ROLE OF ENZYME / INHIBITORS FROM *VIGNA RADIATA* IN HOST – PATHOGEN INTERACTIONS

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Science is a complex stepwise process of wide observation, detailed understanding, logical analysis and precise conclusions. Experiencing this process leads to evolution and maturation. Discovery is where you don't know everything but know only one thing in certainty.

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Aarohi Atul Kulkarni

CERTIFICATE

Certified that the work incorporated in the thesis entitled:

"**Role of enzymes / inhibitors from *Vigna radiata* in host-pathogen interactions**", submitted by Mrs. Aarohi A. Kulkarni, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India. Material obtained from other sources is duly acknowledged in the thesis.

Dr. Mala Rao
(Research Supervisor)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Role of enzymes / inhibitors from *Vigna radiata* in host-pathogen interactions**", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. Mala Rao. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Mrs. Aarohi A. Kulkarni
(Research Scholar)

ABBREVIATIONS

µl	Microliter
Å	Angstrom
ATBI	Alkalo-thermophilic *Bacillus* inhibitor
APS	Ammonium persulphate
CD	Circular dichroism
$CsCl_2$	Cesium Chloride
Da	Dalton
DNSA	Dinitrosalicylic acid
DTNB	5, 5'-dithiobis 2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
FProt	Fungal aspartic protease
g	Gram
Gdn HCl	Guanidine Hydrochloride
$HAuCl_4$	Chloroauric acid
h	hours
IC_{50}	50% inhibitory concentration
KI	Potassium iodide
K_A	Association constant
K_D	Dissociation constant
K_i	Inhibition constant
K_i^*	Total Inhibition constant
K_m	Michaelis Menten Constant

L	liter
LB	Lineweaver Burk
M	Molar
MALDI ToF	Matrix assisted laser desorption / ionization time of flight
µM	Micromolar
min	Minutes
ml	Milliliter
mM	Millimolar
nM	nanomolar
nm	Nanometers
$^{\circ}C$	Degree Celsius
PAGE	Polyacrylamide gel electrophoresis
PHMB	Para hydroxyl mercury benzoate
PMSF	Phenylmethane sulfonyl fluoride
rpHPLC	Reverse phase high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N,N,N'N'- Tetramethyl ethylene diamine
$\mathbf{V_{max}}$	Maximum velocity
WRK	N-ethyl-5-phenylisoxazolium-3'-sulfonate. (Woodward's. reagent K)

ABSTRACT

Every molecule has a predestined course of action. Its journey of life is a chart of tumultuous upheaval, downfalls and peace. The fulfillment of this journey is what each and every object of life strives for attainment.

That is life!

Enzyme molecules are the essence of every life systems, the work horses responsible for maintaining all the life processes of the cell and consequently of the organisms. Their study is essential in understanding not only the fundamental concepts of working of the cell but also in comprehending the interactions in the flora and fauna which are a result of their operation. Complex systems are all the more complicated in their working with a lot of enzyme regulation being involved in the process. The regulatory aspects are critical and mostly performed at the enzyme level itself without involving the genomic level. An elegant system of converting enzymes from their inactive forms to the active molecules is that of proteases, enzymes which cleave other proteins to make them functional and suitable for the cell machinery. The regulation of these specialized enzymes is all the more critical since their overwork can cause havoc in the cell with eventual lysis or cell death due to malfunction of the metabolic processes. Out of all the classes of proteases, aspartic proteases are all the more significant due to their specialized functions in the maintenance and working of the cell. The significance of these enzymes can be outlined by gaining a peek into the life processes of some organisms like pathogenic fungi, HIV, *Plasmodium*, human digestive system, human angiotensin system to name a few wherein this class of enzymes is found to be critical for normal functioning and in gaining nutrition for the cell. Recently these have been implicated

in critical processes like seed germination, storage protein mobilization, programmed cell death and various critical processes in plants also making this class a universally significant one. The regulation of aspartic proteases is critical and therefore the magnitude of their inhibitors is enhanced. This thesis is an attempt to study this class of enzymes with special emphasis on plant aspartic proteases, the enzymes operating in the acidic environment of the plant cell, their regulation and the significance of their inhibitors in the normal regulatory and physiological functioning of the plant. An additional stress has been given on the applications of these enzymes and their inhibitors apart from the insight into the physiological functions. The outline of the thesis is provided below.

ORGANIZATION OF THE THESIS

The thesis is organized into 6 chapters which are as follows:

CHAPTER 1: GENERAL INTRODUCTION

This chapter provides a literature survey on plant proteases with a detailed outline of enzymes and a special emphasis on aspartic proteases and their inhibitors.

CHAPTER 2: PURIFICATION AND PHYSIOLOGICAL ROLE OF ASPARTIC PROTEASE FROM *VIGNA RADIATA*

This chapter describes in detail the purification of an aspartic protease from the seeds of *Vigna radiata*. The prevalent varieties were analysed for the presence of the enzyme. Detailed studies on the varietal and the time based expression of the enzyme are provided. Kopergaon-1 was the variety selected for further purification of the protease since maximum expression was obtained in this variety at 1 hour of germination. The protease was purified using fractional ammonium sulfate precipitation and affinity column chromatography. The enzyme was found to be a

heterodimer identified using SDS-PAGE and NATIVE PAGE. The molecular weight of the enzyme was found to be 67,375 Daltons with subunits of weights, 23, 349 and 44, 024 with SDS-PAGE. Gel filtration using Biogel P 100 was performed to confirm the molecular weight of the native enzyme. It was found to be 67, 406 Daltons. The molecular weight was also confirmed by MALDI mass spectroscopy. The physiological role of the protease was confirmed by suitable germination assays and conducting germination experiments under controlled conditions with Pepstatin-A also.

CHAPTER 3: BIOCHEMICAL STUDIES OF ASPARTIC PROTEASE FROM *VIGNA RADIATA*

This chapter deals with the biochemical studies of the aspartic protease from *Vigna radiata*. It is divided into three sections as:

(a) The biochemical properties: The biochemical characterization of the enzyme revealed that it cleaves the peptide bond between Phe-Tyr residues. The amino acid analysis provides details of the content of the enzyme. The enzyme was found to be pH stable and thermolabile. It was stable under a broad range of pH from 2-10 with the optimum being 3.5 while it was found to have a half life of 20 minutes at 50°C with optimum being 37°C. The chapter also deals with the interactions between VrAP and the universal inhibitor of aspartic proteases, Pepstatin. Kinetic analysis of Pepstatin inhibition of VrAP revealed it to be a competitive reversible inhibitor with a tight mode of binding. The kinetic constants were calculated using Origin 6.1 from Origin Lab.

(b) Active site characterization: The active site of an enzyme is the most conserved domain in the enzyme structure due to its involvement in substrate binding and catalysis. The amino acid residues at the active site can be recognized

by using specific modifiers. In short, chemical modification of the reactive amino acid side chain helps in the identification of those residues which are essential for catalysis. The conditions for modification are so adopted that it results in the quantitative covalent derivatization of the functional group of a single unique amino acid residue in a protein without any demonstratable effect on either any other functional groups or the conformation of the molecule. The presence of aspartic acid and serine in the active site of VrAP was revealed by the use of Woodward's reagent K (WRK) and phenylmethylsulfonyl fluoride (PMSF). The role of aspartic acid and serine in substrate and catalysis was confirmed by substrate protection studies in the presence of the modifiers. The kinetic analysis of the modified enzymes revealed that aspartic acid was more involved than serine in catalysis. The fluorescence studies were based on the tryptophanyl fluorescence and therefore an analysis of tryptophan availability for these studies was done using acrylamide, cesium chloride and potassium iodide. The enzyme being a heterodimer, it was thought that cysteine might be involved in the linkage of the subunits. The modification studies revealed the presence of an essential cysteine residue for complete enzyme activity which is not present in the active site but is involved in the structural aspects of the enzyme which are essential for substrate catalysis.

(c) Structure-function studies: The conformational integrity of an enzyme is essential for its activity. There are few reports of the structural aspects of plant aspartic proteases. The work here presents the folding and unfolding of VrAP in the presence of guanidine hydrochloride. The pH based changes are also discussed. A novel alkali induced molten globule state is seen for VrAP. Detailed analysis of the secondary and tertiary structural changes in VrAP due to guanidine hydrochloride and change in pH is presented.

CHAPTER 4: PURIFICATION AND BIOCHEMICAL STUDIES OF ASPARTIC PROTEASE INHIBITOR FROM *VIGNA RADIATA*

The peptidic/ proteinaceous inhibitors of the aspartic protease from *Vigna radiata* were thought to be present in the seeds since inhibitors are one of the essential elements controlling the action of the enzymes of digestion. The different varieties of *Vigna radiata* were tested for the presence of the inhibitor. The expression of the inhibitor upto 18 hours of germination and in the mature plant was checked. The time-based expression was analysed and it was found that the dormant seeds contained maximum amount of the inhibitor. Accordingly, a peptidic inhibitor of VrAP, viz, VrAPI was purified from the dormant seeds of *Vigna radiata*. The inhibitor was purified by acid precipitation, molecular weight cutoff membrane filtration, gel filtration chromatography and HPLC (High pressure liquid chromatography). The peptidic inhibitor was found to have molecular weight of 820 Daltons. This chapter deals with the purification and the biochemical characterization of VrAPI. The inhibitor was found to be pH and thermostable. The optimum pH for activity was 3.0 while the optimum temperature was 37°C. This chapter also deals with the kinetic analysis of the interactions between the inhibitor and fungal protease from *Aspergillus saitoi*. The fungal protease is inhibited in a competitive reversible manner. This implicates it in interactions with other phytopathogenic fungi. The inhibitor constants were analysed and were found to conform to the slow tight binding model of inhibition. The interactions of the inhibitor with the endogenous protease revealed the competitive reversible nature of inhibition. The kinetic constants calculated indicate a tight binding inhibition of the inhibitor with the endogenous protease.

CHAPTER 5: ASPARTIC PROTEASE INHIBITOR AS A BIOCONTROL AGENT

Biocontrol agents are those which protect the plants from insect and pathogens attack. To date chemical control has been the strategy of choice for protecting the plants from pathogenic attack. The use of pesticides has resulted in severe environmental pollution and harm to the natural flora and fauna. Therefore alternative strategies are being sought to control their spread. This chapter focuses on the use of protease inhibitors as biocontrol agent. The effective management of powdery mildew of mung bean and fusarial wilt of pigeon pea has been demonstrated by laboratory, pot and field experiments. The successful use of the agent indicates that there are simple solutions to complicated problems in nature and we should look for alternatives from nature itself. The successful field trials indicate that without the use of any microorganism and using very simplified approaches plant diseases can be managed effectively. This study lends an altogether different perspective to the research on biocontrol of essential crop plants.

CHAPTER 6: SYNTHESIS OF METAL NANOPARTICLES USING ASPARTIC PROTEASES

The synthesis of nanoparticles using biologically derived molecules has been an area of current interest in the field of nanobiosynthesis. This chapter presents a novel and significant leap in this direction. The chapter is aimed at demonstrating effectively the use of purified aspartic proteases in the synthesis of nanoparticles of gold and silver. Flat structures were obtained with all three aspartic proteases tried and tested with chloroauric solution of 0.0001M. There were some triangles obtained with VrAP and fungal protease. An interesting observation indicated that the nanoparticles coated the surface of glass and plastic. UV-spectroscopy revealed anisotropic particle synthesis. The AFM and TEM analysis provided the details of the shape and size of the nanoparticles formed.

AWARDS

- Krishnamurthy Award for the Best Research Paper given by the Society of Biological Chemists, India in 2003.

PUBLICATIONS

- Bioprospecting the lat gene in soil samples. Aarohi Dharwadkar, Vidya Gupta and Aditi Pant. J. Biosci. 28 (5): 597-604 (2003).
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- Aarohi Kulkarni and Mala Rao (2004) Protease Inhibitors: Strategies in Plant Defence Mechanism: Plant, Microbes and Biotechnology. Pointer Publishers (India) Dr. Sampat Nehra (Editor), p- 301-315, ISBN 81-7132-398-7

CONFERENCES / ABSTRACTS / POSTERS

- Poster presentation in the International conference on Nano Bioscience titled 'Biosynthesis of gold nanoparticles using aspartic proteases' held in Pune in August, 2006.
- Oral presentation in the Asian conference on "Emerging trends in plant-Microbe interactions" titled 'Control of powdery mildew of mung bean using an alkalothermophilic *Bacillus* sp." held in Chennai in December, 2005.
- Poster presentation in the International conference on plant genomics and biotechnology titled 'Biosynthesis of silver nanoparticles using *Vigna radiata* seed extract' held in Raipur in October 2005.
- Poster presentation in the 4^{th} International food legume research conference titled 'Biocontrol of *Fusarium* wilt in pigeonpea using an alkalothermophilic *Bacillus* species' held in New Delhi in 2005.
- Poster presentation in the 10th FAOBMB Congress titled 'Aspartic peptidase inhibitor from mung bean' held at Bangalore in December 2003.
- Attended National Seminar on Crystallography held at Pune in 2003
- Attended workshop on "Macromolecular Crystallography" held at Pune in 2003.

PATENTS

1. A. A. Kulkarni and M.B. Rao (2006) A Process for the preparation of aspartic protease inhibitor using *Vigna radiata* seeds for application in the biocontrol of infections caused due to phytopathogenic fungi (Applied for Indian and US patents)

CHAPTER I

GENERAL INTRODUCTION

“A man with innovative and novel ideas is considered a crank and a failure unless and until the idea succeeds.”

Mark Twain

ABSTRACT

Enzymes are responsible for the proper and normal functioning of all cellular activities. They are the work horses of the cell and need to be studied in great detail in order to have a glimpse of life's machinery. Their studies have slowly evolved into a major branch of science which encompasses basic as well as applied research. One of the major classes of enzymes responsible for normal functioning of the cell is that of the proteases. This chapter provides general knowledge about enzymes, proteases and lays special emphasis on plant aspartic proteases, a fundamental class of regulatory enzymes in plants.

INTRODUCTION

Enzymes serve a wide variety of functions inside living organisms and are the machinery responsible for proper functioning of the cell. They work in specific order creating a cascade of steps inside the cell called as the metabolic pathways of life. They are biological catalysts that accelerate or catalyze chemical reactions. Almost all cellular processes require them in order to occur at significant rates. They are to date known to catalyze about 4000 reactions and are usually named according to the reaction they catalyze. Classically enzymes work by providing an alternative path of lower activation energy for a reaction and dramatically accelerating its rate. The term enzyme was coined by German physiologist Wilhelm Kühne and was derived from the Greek word *ενζυμον*. Various workers like Buchner, Sumner, Northrop, Stanley, Phillips and several others showed that enzymes are specialized proteins. Enzymes being proteins possess properties such as specificity towards the reactions they catalyze and the substrates on which they act upon.

Enzyme Nomenclature

Enzymes are identified by a common nomenclature system based on the description of what function it performs in the cell and ends in the common phrase –ase. The International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry developed a nomenclature system wherein each enzyme is given an **Enzyme Commission Number** called as the EC number. Accordingly the top level classes based on the mechanism of operation of an enzyme are:

1. **Oxidoreductases**: catalyze oxidation/reduction reactions
2. **Transferases**: transfer a functional group
3. **Hydrolases**: catalyse the hydrolysis of various bonds

4. **Lyases**: cleave various bonds by means other than hydrolysis and oxidation
5. **Isomerases**: catalyze isomerisation changes within a single molecule
6. **Ligases**: join to molecules with covalent bonds

The enzymes belonging to the class of hydrolases form the focus of study in the present thesis. In biochemistry, a hydrolase is an enzyme that catalyses the hydrolysis of a chemical bond. They are further systematically classified as "substrate hydrolase" with common names generally being "*substrate*ase". They are classified as EC 3 and are further divided into several subclasses based on the bonds they act upon as:

EC 3.1: Ester bonds (Esterases)

EC 3.2: Sugars (glycosylases)

EC 3.3: Ether bonds

EC 3.4: Peptide bonds (proteases)

EC 3.5: Carbon-nitrogen bonds other than peptide bonds

EC 3.6: Acid anhydrides

EC 3.7: Carbon-Carbon bonds

EC 3.8: Halide bonds

EC 3.9: Phosphorus-Hydrogen bonds

EC 3.10: Sulfur-nitrogen bonds

EC 3.11: Carbon-Phosphorus bonds

EC 3.12: Sulfur-Sulfur Bonds

EC 3.13: Carbon-Sulfur Bonds

Proteases

Proteases (proteases, proteases, or proteolytic enzymes) are enzymes that break peptide bonds between amino acids of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes. They use a molecule of water for this and are thus classified as hydrolases. There are currently six classes of proteases:

- Serine proteases
- Threonine proteases
- Cysteine proteases
- Aspartic acid proteases
- Metalloproteases
- Glutamic acid proteases

The threonine and glutamic acid proteases were not described until 1995 and 2004, respectively and are therefore recent additions. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the character of a polarized peptide bond (serine, cysteine and threonine proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carbonyl group.

Occurrence

Proteases are ubiquitous in occurrence and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting

cascade, the complement system, apoptosis pathways, etc). Proteases can break either specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components, it can be an activation of a function or it can be a signal in a signalling pathway.

Classification

Proteases have previously been classified according to specificity and catalytic type. Classification by specificity depends on the position of the peptide bond cleaved within the peptide or protein chain. Classification by catalytic type depends on the nature of the nucleophile in the hydrolytic reaction. In serine-, threonine- and cysteine-type proteases, the nucleophile is hydroxyl or thiol moiety on named amino acid side-chain; for aspartic-type, metallo-type and the glutamic type proteases the nucleophile is an activated water molecule bound by aspartate residues or metal atoms respectively.

The classification adopted by 'Enzyme Nomenclature' is based on specificity, and endoproteases are subdivided by catalytic type.

- **Exoproteases** - cleave near the N- or C-termini of peptides and proteins
    - EC 3.4.11 **Aminoproteases** - release an N-terminal amino acid
    - EC 3.4.13 **Diproteases** - hydrolyse dipeptides into amino acids
    - EC 3.4.14 **Peptidyl-proteases** - release dipeptides from the N-terminus
    - EC 3.4.14 **Tripeptyl-proteases** - release tripeptides from the N-terminus

  - EC 3.4.15 **Peptidyl-diproteases** - release dipeptides from the C-terminus
  - EC 3.4.19 **Omega proteases** - hydrolyse non-peptide bonds in peptides and proteins

- **Endoproteases** - cleave internal peptide bonds in peptides and proteins
  - EC 3.4.21 Serine-type endoproteases
  - EC 3.4.22 Cysteine-type endoproteases
  - EC 3.4.23 Aspartic-type endoproteases
  - EC 3.4.24 Metallo-endoproteases
  - EC 3.4.99 Endoproteases of unknown catalytic type

Thus proteases form a major part of the enzyme system and are responsible for protein make and break over. Proteases are therefore fittingly called as the enzymes of digestion and have been reported from all strata of life.

Protease inhibitors

The tremendous significance of enzymes makes the control of their activities even more critical and of utmost importance to the cell. There are 4 main ways in which an enzyme activity is controlled in the cell:

1. Enzyme production can be enhanced or diminished by a cell in response to changes in the cells environment. This is generally done at the gene level and is a form of gene regulation.
2. Enzymes may be compartmentalized with different metabolic pathways occurring in different cellular components and with active enzymes being transferred to an inactive compartment after completion of work.
3. Enzymes can be regulated by inhibitors and activators.
4. Enzymes undergo post translational modification which helps to modulate their activity.

Thus, in biology and biochemistry, protease inhibitors are molecules that inhibit the function of peptidases (old name: protease, hence the term protease inhibitor). Many naturally-occurring protease inhibitors are proteins.

Classification of protease inhibitors

Protease inhibitors are divided into 2 two general classes:

1) The low-molecular weight inhibitors

2) Natural protein protease inhibitors

Over 100 naturally occuring protease inhibitors have been identified from a multitude of organisms. They are strictly class-specific except proteins of the alpha-macroglobulin family (e.g alpha-2 macroglobulin) which bind and inhibit most proteases through a molecular trap mechanism.

Classification of protease inhibitors

Protease inhibitors may be classified either by the type of protease they inhibit, or by their mechanism of action.

Proteases inhibitors can be broadly classified based on the enzyme they inhibit as:

- Cysteine protease inhibitors
- Serine protease inhibitors
- Threonine protease inhibitors
- Aspartic protease inhibitors
- Metalloprotease inhibitors

Classes of inhibitor based on their mechanisms of action are:

- Suicide inhibitor
- Transition state inhibitor
- Protein protease inhibitor
- Chelating agents

Serine protease inhibitors have been the most studied protein inhibitors till date and recently a considerable advance has been made in the study of the natural inhibitors of cysteine proteases (cystatins). In contrast, our knowledge of inhibitors of both aspartyl and metallo proteases is very limited.

The present thesis focuses on aspartic proteases and their inhibitors with special emphasis on those of plant origin.

Plant Proteases

Plant proteases play a fundamental role in protein maturation and in protein degradation during normal cell maintenance and restructuring of tissues. Proteins synthesized as precursors undergo selective enzymatic cleavage of peptide bonds which is crucial, for proper 3-dimensional structure, activity, or correct cellular localization. The characterization of specific proteolytic enzymes yields a better understanding of their function in physiological processes. Proteases have been classified according to the amino acids at their active site, the 3-dimentional structures and mechanism of action. Their classification is also based on the sensitivity of the activity to various specific inhibitors. The main classes of proteases include serine-, cysteine-, aspartic- and metalloproteases. Aspartic proteases (EC.3.4.23) are a widely distributed class of proteases present in animals, microbes, viruses and plants (Davies 1990, Rawlings and Barret 1995). All aspartic proteases contain two aspartic acid residues at the active site, are active at acidic pH and

specifically inhibited by pepstatin A (Rawlings and Barret 1995). In most aspartic proteases, the catalytic Asp residues are contained in a common Asp-Thr-Gly motif in both lobes of the enzyme; however plant aspartic proteases contain Asp-Ser-Gly at one of the sites. Based on the information about the functions of aspartic proteases from microbial and animal origins, some of the associated functions include protein degradation by pepsin and chymosin, proprotein processing by cathepsin D, renin and yeast aspartic protease A and polyprotein processing by the human immunodeficiency virus aspartic protease (Davies 1990, Rawlings and Barret 1995). The plant aspartic proteases have been implicated in similar processes, although conclusive evidence is lacking.

Isolation and characterization of the proteins

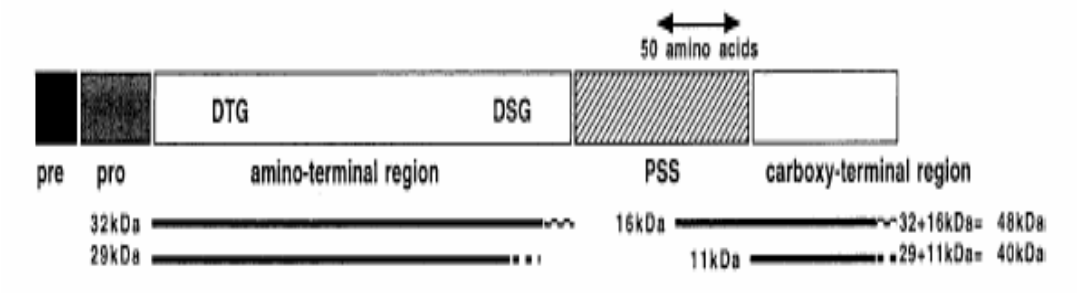
The rapid purification of these enzymes from crude extracts is done using pepstatin-A affinity chromatography due to the selective nature of pepstatin-A for aspartic proteases. Many aspartic proteases from various plant species have been purified using pepstatin A-agarose columns (Sarkkinen et al. 1992, Hiraiwa et al. 1997, Mutlu et al. 1998).

Aspartic proteases have been detected or purified from monocotyledonous (Doi et al. 1980, Belozersky et al. 1989, Sarkkinen et al. 1992, Radlowski et al. 1996) and dicotyledonous plants (Garcý´a-Martinez and Moreno 1986, Mikkonen 1986, Rodrigo et al. 1989, Heimgartner et al. 1990, Rodrigo et al. 1991, Biehl et al. 1993, D'Hondt et al. 1993, Stachowiak et al. 1994, Kuwabara and Suzuki 1995, Verý´ssimo et al. 1996, Hiraiwa et al. 1997, Mutlu et al. 1998) (Table 1). These proteases have been characterized mainly from different parts of resting or germinating seeds, although some have also been isolated from leaves of tobacco, tomato and from flowers of cardoon.

Plant aspartic proteases purified to date are either heterodimeric or monomeric (Table 1). For example, the barley seed aspartic protease contains two isoforms of the enzyme, each with two subunits (Sarkkinen et al. 1992). The larger enzyme has a molecular weight of 48 kDa and is composed of 32 and 16 kDa subunits, while the smaller enzyme has a molecular weight of 40 kDa and is composed of 29 and 11 kDa subunits, likely linked by disulfide bridges. Three different heterodimeric enzymes from dried cardoon flowers and two from fresh flowers have been isolated with similar sized peptides to those found in the barley system (Heimgartner et al. 1990, Verý´ssimo et al. 1996). A heterodimeric aspartic protease with subunits of molecular weight 30 and 11 kDa has been reported from figleaf gourd (Stachowiak et al. 1994). Recently, an aspartic protease from *Arabidopsis* seeds has been purified and characterized (Mutlu et al. 1998). The enzyme contains four subunits (apparent molecular weights 31, 28.5, 15 and 6 kDa), two of which are probably linked by disulfide bonds. Several plant aspartic proteases are monomeric and have a broad range of sizes (Table 1).

The genes for plant aspartic proteases have been isolated predicting that the active heterodimeric enzymes are derived from the processing of a single proprotein. A typical gene sequence for plant aspartic proteases is depicted in figure 1.

Figure 1: A typical gene sequence for plant aspartic proteases



The gene organization typically consists of the pre and the pro regions preceding the actual gene which in turn is interspaced with the plant specific sequence (PSS) region

The genes and predicted proproteins for both monomeric and dimeric plant aspartic proteases are very similar. Thus, it is not clear why apparently similar prepropeptides from different plant species should produce such vastly different proteins. Tissue source does not appear to be the determining factor, because there are monomeric enzymes from rice and brassica seeds, and heterodimeric proteinases from barley and Arabidopsis seeds. The distinction is also not based on the whether the plant is monocotyledonous or dicotyledonous since examples of monomeric and heterodimeric enzymes are found in both types of plants. The differences most probably lie in the presence or absence of protein-processing enzymes, which convert the preproform of the aspartic protease to the mature enzyme. Differences in the sizes of the heterodimeric and monomeric enzymes may be due to slight variations in the processing of a common precursor, or to differences in glycosylation, or other post-translational modification of the mature proteins.

Enzyme localization

Aspartic proteases have been primarily isolated from seed tissues (Table 1). Voigt et al. (1997) analyzed the acid proteinase activity in seeds from ten different plants and found that pepstatin A inhibited 52% 100% aspartic protease activity in beans and

barley seeds respectively. Aspartic proteases have also been detected in many other parts of the plant. In barley different forms of the same enzyme can be found in various locations of a plant giving rise to distinct tissue specific functions (Sarkkinen et al. 1992, Runeberg-Roos et al.1994, Runeberg-Roos and Saarma 1998, To¨rma¨kangas et al. 1994). Aspartic proteases have been isolated from a number of other dry seeds, including *Arabidopsis* and castor bean (Hiraiwa et al. 1997, Mutlu et al. 1998), and have been detected in the germinating kidney bean (Mikkonen 1986). Aspartic proteases are primarily localized in either the vacuolar: protein body or the extracellular space of rice, wheat, buckwheat, sunflower, etc (Table 1). The barley protein was detected in multiple locations (Runeberg-Roos et al. 1994; Marttila etal. 1995, Bethke et al. 1996). The localization of the *Arabidopsis, cardoon* and castor bean aspartic proteases in the protein bodies of seeds, or parts of flowers, has been confirmed using immunohistochemical techniques and subcellular fractionation (Hiraiwa et al. 1997, Ramalho-Santos et al. 1997, Mutlu et al. 1999). The aspartic proteases from tobacco and tomato and *Nepenthes* are found secreted (Juniper et al.1989, Rodrigo et al. 1989, 1991), while the enzyme in maize pollen is believed to be in the cell wall (Rad³owski et al.1996). An aspartic protease from spinach has also been localized to photosystem II membranes of plastids (Kuwabara and Suzuki 1995), an unusual site for an enzyme with an acidic pH optimum. It is not clear why plant aspartic proteases are found in distinct parts of different plants, or whether they have similar functions in different tissues.

Table 1: Summary of the properties of purified plant aspartic proteases

Plant Organ Source	Size (kDa)	M / H[b]	Localization	References
Arabidopsis Dry seeds	31, 28, 5, 15, 6	H	Protein bodies	D'Hondt et al, 1997 Mutlu et al. 1998, 1999
Barley Grains	31+16, 29+11	H	Protein bodies	Runeberg-Roos et al. 1991 Sarkinnen et al, 1992
Brassica napus Seeds	28	M	NK	D'Hondt et al, 1997
Cardoon Flowers	32+16, 33+16, 35+13, 31+15, 34+14	H	Protein bodies	Heimgartner et al, 1990 Cordeiro et al, 1994 a,b Veríssimo et al, 1996 Ramalho-Santos et al, 1997
Castor bean Seeds	32+16, 29	M	Protein bodies	Hiraiwa et al, 1997
Figleaf gourd Seeds	30+11	H	NK	Stachowiak et al, 1994
Maize Pollen	60	M	Cell wall	Radlowski et al, 1996
Rice Seeds	60-65	M	NK	Doi et al, 1980 Asakura et al, 1995b
Spinach Leaves	51	M	Thylakoids	Kuwabara and Suzuki, 1995
Tobacco Leaves	36-40	M	Extracellular	Rodrigo et al, 1991
Tomato Leaves	37	M	Extracellular	Rodrigo et al, 1989 Schaller and Ryan, 1996
Wheat Seeds	58	M	NK	Belozersky et al, 1989

Primary structure of plant aspartic proteases

A number of aspartic protease cDNAs have been isolated from different plants including Arabidopsis, barley, brassica, cardoon, pumpkin, rice and tomato (Runeberg-Roos et al. 1991, Cordeiro et al. 1994b, Asakura et al. 1995a, Schaller and Ryan 1996, Chen and Foolad 1997, D'Hondt et al. 1997, Hiraiwa et al. 1997). In addition, other enzymes have been identified in Arabidopsis and cauliflower from partial clones, expressed sequence tags and genomic DNA databases (Fujikara and Karssen 1995, S. Gal, unpublished observations). These sequences correspond well with the partial protein sequences available for some of the isolated aspartic proteases from Arabidopsis, barley, Brassica, castor bean and figleaf gourd (Sarkkinen et al. 1992, Stachowiak et al. 1994, D'Hondt et al. 1997, Hiraiwa et al. 1997, Mutlu et al. 1998).

The overall sequence of plant aspartic proteases is similar to their mammalian and microbial counterparts (Runeberg-Roos et al. 1991, D'Hondt et al. 1997, Hiraiwa et al. 1997). Following a likely signal sequence and a propeptide, the sequence of plant aspartic proteases can be divided into three regions; an amino-terminal region, a plant specific sequence (PSS) and a carboxy-terminal region (Fig. 1). The amino- and carboxy-terminal domains of plant aspartic proteases are regions of high similarity with each other and also to the enzymes from mammalian and microbial sources. The amino-terminal region contains the two active site aspartic acids in the sequence, Asp-Thr-Gly and Asp-Ser-Gly, a conserved catalytic triad.

The PSS contains a 100 amino acid insert, which is present in most plant enzymes. Although the positions of six cysteine residues are conserved, the PSS of plant aspartic proteases from different species are less homologous with each other than are the amino- and carboxy- terminal regions, suggesting a greater diversion of this

sequence throughout evolution. There is a conserved glycosylation site within the PSS that has been shown to be modified in some of the enzymes (Costa et al. 1997).

The plant aspartic proteases show sequence homology to the mammalian or microbial enzymes, however, the gene organization of the plant proteinases appears to have diverged in terms of the intron positions.

Expression of genes and their regulation

Many aspartic proteases isolated from dry seeds are also expressed in other tissues of the plant. For example, the barley aspartic protease mRNA, in addition to its detection in seeds, is also present in flower, shoot and root tissues (To¨rma¨kangas et al. 1994). Thus, it appears the aspartic proteases play a role although undefined beyond that in the seeds of plants.

Function of plant aspartic proteases

The mammalian, microbial and viral aspartic proteases have been shown to be involved in specific protein processing events, as well as in protein degradation (Davies 1990, Rawlings and Barret 1995). Plant aspartic proteases have been demonstrated to process and degrade proteins in vitro, and in many cases, the localization of the protein or expressed gene would be consistent with such a role in vivo. However, work assigning functions to these enzymes in plant systems lags behind information gained from animals and fungi. During seed development, seed-storage proteins are expressed in specific storage tissues (e.g. endosperm or cotyledon mesophyll), and are generally synthesized as precursor proteins, which are processed prior to deposition in the protein bodies (Higgins 1984). Aspartic proteases have been reported to be involved in storage protein breakdown especially 2S albumins (Krebbers et al. 1988; D'Hondt et al. 1993; Hiraiwa et al. 1997; Runeberg-

Roos et al. 1994, Mutlu etal. 1998; De Clercq et al. 1990, Mutlu et al. 1999; V. Kovaleva and N.V. Raikhel unpublished observations; Higgins 1984).

Aspartic proteases are often dominant in the resting seeds whereas during germination the level of cysteine proteinases increases (Marttila et al. 1995, Voigt et al. 1997). It has been suggested that the seed aspartic proteases may initiate the hydrolysis of seed-storage proteins before the massive de novo synthesis of cysteine proteinases (Belozersky et al. 1989). Several endogenous seed-storage proteins have been tested in vitro, as substrates for plant aspartic proteases. Wheat aspartic protease hydrolyzes the storage protein gliadin in vitro (Belozersky et al. 1989). The degradation of gliadin and gluten proteins in the germinating seeds is probably caused by de novo synthesized cysteine proteinases. However, preincubation of wheat gliadin with the aspartic protease and a carboxyprotease in vitro, increases the susceptibility of gliadin to degradation by cysteine proteinases (Belozersky et al. 1989, Dunaevsky et al. 1989). This suggests the involvement of aspartic protease in the degradation of seed-storage proteins during germination. The localization of aspartic proteases in the protein storage vacuoles of a number of seeds, suggests these enzymes could be involved in storage protein breakdown, or in the activation of proteinase zymogens during seed germination (Runeberg-Roos et al. 1994, Bethke et al. 1996, Hiraiwa et al. 1997, Mutlu et al. 1999). The evidence that the plant aspartic proteases play a role in storage protein degradation is still indirect and requires more vigorous proof. The aspartic proteases have also been associated with protein degradation in other plant tissues. In carnivorous plants, the organs specialized for trapping and digesting insects and other small invertebrates secrete aspartic proteases, as well as other hydrolyzing enzymes (Juniper et al. 1989). Extracellular aspartic proteases from the leaves of infected tobacco and tomato degrade secreted pathogenesis-related proteins in vitro (Rodrigo et al. 1989, 1991).

These aspartic proteases are present in healthy tobacco and tomato leaf cells at similar levels, but they have no activity towards intracellular proteins and their function in uninfected tissue is unknown (Rodrigo et al. 1989, 1991).

Possible roles for aspartic proteases in protein degradation during organ senescence and cell death have been suggested wherein the possible function of this enzyme to autolyse during formation of those tissues undergoing programmed cell death is observed. This would be consistent with the work of Chen and Foolad(1997), who found an aspartic protease-like gene expressed in the degenerating cells of the barley nucellar cells of the embryo. However, more work will be needed to confirm that this class of enzymes functions in autolysis of tissues in plants.

Two major avenues of research are likely to dominate the future work on the plant aspartic proteases: (1) research on the structure of these enzymes and (2) work to understand the functions of these enzymes in the plant.

The general structure of the plant aspartic proteases is very similar to many other enzymes of this class, but there are two regions whose function we have yet to understand. These include the plant specific sequence (PSS) and the pro-region lying between the signal peptide and the amino terminus of the mature protein.

The other major work with these enzymes is an attempt to understand their function in the plant. Work to elucidate these functions has concentrated on a few major substrates and in vitro assays, but the future lies in trying to identify a function for these enzymes in vivo. A logical search for their substrates comes from information collected on the cell type expression of the aspartic protease gene and the subcellular localization of the enzyme.

It is clear that understanding of the plant aspartic proteases has improved over the past several years. A variety of molecular, genetic and biochemical approaches should address a number of questions on the structure of these enzymes in the future and shed light on the function of these important enzymes in plants.

This thesis work is an attempt to throw more light on the enzyme systems of this plant, understand their physiological significance in addition to their importance in nutritional values. A part of the study is aimed at the regulatory ways used by the plant to protect the seeds from pathogen attack and study the interactions of these inhibitory molecules on the enzyme systems of the host as well as those of the pathogen. Apart from the physiological roles of these proteases and their inhibitors, their applications in other significant areas are delved into in some parts of the thesis.

CHAPTER II

PURIFICATION AND PHYSIOLOGICAL ROLE OF ASPARTIC PROTEASE FROM *VIGNA RADIATA*

Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under your observation in life.

Marcus Aurelius

ABSTRACT

Although thorough studies on microbial and mammalian aspartic proteases have been conducted the class of enzymes is of recent emergence in plants. Reports of their physiological importance in plants are emerging. This chapter describes in detail the purification of an aspartic protease from the seeds of *Vigna radiata*. The prevalent varieties were analysed for the presence of the enzyme. Detailed studies on the varietal and the time based expression of the enzyme are provided. Kopergaon-1 was the variety selected for further purification of the protease since maximum expression was obtained in this variety at 1 hour of germination. The protease was purified using fractional ammonium sulfate precipitation and affinity column chromatography. The enzyme was found to be a heterodimer identified using SDS-PAGE and NATIVE PAGE. The molecular weight of the enzyme was found to be 67,375 Daltons with subunits of weights, 23, 349 and 44, 024 with SDS-PAGE. Gel filtration using Biogel P 100 was performed to confirm the molecular weight of the native enzyme. It was found to be 67, 406 Daltons. The molecular weight was also confirmed by MALDI mass spectroscopy. The physiological role of the protease was confirmed by suitable germination assays and conducting germination experiments under controlled conditions with Pepstatin-A also.

INTRODUCTION

Proteases play an important role in biotechnology since proteolysis modifies the chemical, physical, biological and immunological properties of proteins. Some plant proteases are used in the food industry, in manufacturing cheeses and drinks, meat tenderizing, cookie baking and the production of protein hydrolysates [Uhlig H, 1998].

Aspartic proteases (EC 3.4.23) are ubiquitous in distribution and are present in animals, microbes, viruses and plants [Davies DR, 1990; Rawlings N.D and Barret A.J; 1995]. All aspartic proteases contain two aspartic acid residues at their active site, are active at acidic pH and are specifically inhibited by pepstatin A [Rawlings N.D and Barret A.J; 1995]. In most of the reported enzymes the catalytic Asp residues are contained in a common Asp-Thr-Gly motif in both lobes of the enzyme, but plant aspartic proteases contain Asp-Ser-Gly at one of the sites.

Two plant aspartic proteases have been completely characterized [Frazão C, et al 1999; Kervinen J, et al 1999]. The structure of most of the plant aspartic proteases identified so far contain a prepro-domain and subsequently converted to mature enzymes. A characteristic feature of the majority of plant aspartic proteases precursors is the presence of an extra protein domain of about 100 amino acids known as the plant-specific insert [Simões I, Faro C, 2004; Mutlu A and Gal S, 1999].

Plant aspartic proteases have been detected and purified from a number of plants. Although the mammalian, microbial or viral enzymes perform diverse and different functions, biological functions have not yet been assigned definitively to the plant

aspartic proteases. They have been implicated in protein processing and/or degradation in different plant organs, as well as in plant death, stress responses, programmed cell death and reproduction [Simões I, Faro C, 2004; Mutlu A and Gal S, 1999].

Proteolysis is an essential step in many biological processes like protein degradation and turnover, protein processing and pathogen attack. In addition to these essential roles in the cells, in plants proteolysis is a critical step during the mobilization of seed storage proteins in the process of germination and early growth of plants. The reserve protein is cleaved by class specific enzymes giving rise to intermediates, which are susceptible to digestion. The early germination events are therefore subject to limited proteolysis by class specific proteases. Leguminous seeds contain large amounts of storage proteins. The characteristics of these storage proteins, their synthesis during seed development, and their metabolism during seed germination have been studied extensively. The storage proteins consist of a 7S component, vicilin, and an 11S component, legumin, both localized in special organelles called protein bodies. These protein bodies also contain some proteolytic enzymes as well as other hydrolases. Germination is accomplished by an increase in the proteolytic activity in the cotyledons of peas and beans but the nature of enzymes responsible for this increase (endo-or exopeptidases), and their location is not conclusive (in the protein bodies or in the cytoplasm) [Chrispeels M.J and Boulter D, 1975].

In *Vigna radiata* (L. Wilczek), the only investigated variety, vignain and carboxypeptidase III have been reported from the dormant seeds with an aim to understand and implicate them in the germination process [Chrispeels M.J and Boulter D, 1975; Chrispeels M.J, Baumgartner B and Harris N, 1976; Harris N and

Chrispeels M.J, 1975]. All these reported enzyme activities have been noticed after 48 hours of initiation of germination. However, the events initiating the mobilization of the reserve protein in seeds remain uncharacterized. In this paper we report for the first time the purification, characterization of an aspartic protease and suggest its role in the early germination events in seeds of *Vigna radiata*. The roles of cysteine and serine proteases in germination are also discussed.

MATERIALS AND METHODS

Plant material

Vigna radiata (Mung bean) seeds of Kopergaon-1, Tata, Vaibhav and Yellow mung were purchased from the market. They were washed and sterilized in 0.01% of $HgCl_2$ before use. All experiments were performed with Kopergaon-1 variety unless and otherwise mentioned.

Assay for cysteine, serine and aspartic protease activity

For all the activities, the different varieties were analyzed and aspartic protease activity was specially checked. Seeds of Kopergaon-1 were used for all subsequent purification and analysis. Protease activity was measured using Hammersten casein as a substrate. The standard reaction mixture contained 0.5ml of Hammersten casein (1%) dissolved in 0.05 M Tris-HCl buffer, pH 7.5, in the presence of 20 μl 1M dithiothreitol and 200μl of buffer extract for cysteine protease activity. The standard reaction mixture contained 0.5ml of Hammersten casein (1%) dissolved in 0.05 M carbonate-bicarbonate buffer, pH 10.0 and 200μl of buffer extract for serine protease activity. After incubation at 37°C for 20 minutes, 1ml of 1.7mM PCA was added to stop the reaction. The standard reaction mixture contained 1ml of 5% (w/v) hemoglobin in glycine-HCl buffer, 0.05 M, pH 3.0 and 200μl of enzyme solution for

aspartic protease activity. After incubation at 37°C for 30 minutes, 1ml of 1.7mM PCA was added to stop the reaction. After the mixture was left for 30 minutes at room temperature, undigested material was removed by filtration (Whatman number 1). Proteolytic activity was measured as the increase in absorbance at 280nm of the PCA soluble fraction. Adding PCA before the enzyme made appropriate blanks. One unit of proteolytic activity was defined as the amount of enzyme necessary to increase absorbance by 0.001 under the conditions previously described.

Expression profile

100g of seeds of the locally prevalent Kopargaon variety were soaked in 300 ml of deionised water. The samples were removed at hourly intervals and ground in 400ml of glycine-HCl buffer, 0.05 M; pH 3.0 at ambient temperature till a homogenous mixture was obtained. The samples were then analyzed for aspartic protease activity using hemoglobin as the substrate at pH 3.0 and by confirming the results by activity gel for the aspartic protease.

Germination Assay

30 seeds of mung bean Kopergaon-1 variety were soaked in 10ml of water and 0.1mM pepstatin-A solution for germination and kept at 30°C for 1 day. The seeds soaked in pepstatin-A solution were transferred to water on the second day and further incubated for one day at 30°C. The effect of soaking in the respective solutions was monitored by observing the length of the germination tube and was recorded with photographs.

Activity gel

The seminative gels (10% acrylamide) were copolymerized with 0.5% hemoglobin. The hourly samples were treated in 25mM Tris, pH 6.8/0.08%SDS, without β-

mercaptoethanol. After electrophoresis, the gels were washed first with 1% Triton X-100 in 25mM Tris-HCl, pH 6.8, and then in glycine-HCl buffer, 0.05 M, pH 3.0. After incubation in this buffer for 18h at 37°C, proteolytic activity appeared as negative bands upon staining with Coomassie Brilliant Blue. Reduction in the activity of the enzyme on treatment with pepstatin was visualized by the activity gel staining. The expression profile of the enzyme was confirmed by activity gel for the hourly samples of imbibition (Timotijevic, 1999).

Purification of enzyme

The seeds of mung bean Kopargaon variety (100g) were imbibed in 300ml water at 28°C for 1 hour. After imbibition the seeds were homogenized in 400ml glycine-HCl buffer, 0.05 M, pH 3.0 at ambient temperature to obtain a homogenous mixture. The extract was allowed to remain at room temperature for 3 hours with slow stirring and then centrifuged at 10000rpm for 20 minutes. The supernatent was subjected to 80% ammonium sulfate precipitation at 4°C. The pellet was dissolved in 100ml of glycine-HCl buffer, 0.05 M, pH 3.0 and extensively dialysed against deionised water using a 10kDa cut off membrane at 4°C. The enzyme solution was concentrated by lyopohilisation and subjected to affinity column chromatography on pepstatin A-agarose column (Sigma-Aldrich, USA). The column was equilibriated in glycine-HCl buffer, 0.05 M, pH 3.0. 5mg of protein was loaded onto the column and it was washed with glycine-HCl buffer, 0.05 M, pH 3.0. The enzyme was eluted with Tris-HCl buffer; 0.05 M, pH 9.2 and the active fractions were collected.

Molecular weight analysis

The molecular weight of the enzyme was determined by gel filtration chromatography using Biogel P100 previously equilibrated with 50 mM Tris-HCL, pH 7, which was calibrated using the following marker proteins: albumin (67,000),

ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). The elution volume of blue dextran determined the void volume of the column. The presence of subunits in the protein was determined by 12% SDS–PAGE and MALDI-TOF.

Protein measurement

Protein was measured by the Bradford method using bovine serum albumin as the standard (Bradford, 1976). The protein elution patterns of purification steps were measured by absorbance at 280nm.

RESULTS

The proteolytic activity was analyzed in dormant and in germinating seeds of *Vigna radiata* varieties Kopergaon-1, Tata, Vaibhav and Yellow mung bean. As seen in Table 1 the activity of aspartic protease was found to be predominant in the initial stages of germination especially in the dormant seeds of Kopergaon-1 variety. Therefore further efforts on purification and characterization of the enzyme were performed using this variety. The highest activity was recorded in the extract of the seeds imbibed in water for 1 hour after which it declined steadily till 18 hours of germination after which no activity was detected. The expression of the aspartic protease from the total protein extract of germinating seeds is depicted in Figure 1.

Table 1: Activities of aspartic protease from various varieties of mung bean

Variety	Enzyme (Units / g of seed)
Kopergaon	1518
Tata	738
Vaibhav	50
Yellow	14

Figure 1: Time bound expression of the enzyme

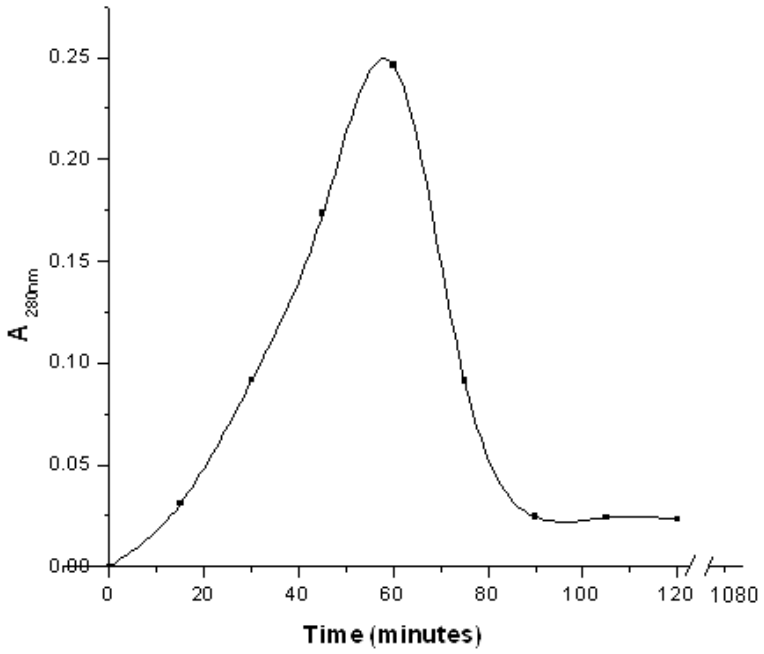
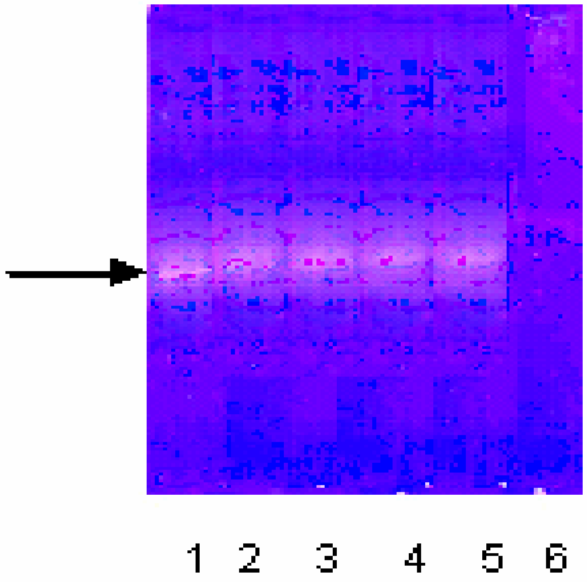


Figure 1 shows the time dependent expression of the aspartic protease in the germinating seeds of *Vigna radiata*. Maximum expression was observed in the first hour of germination which steadily declined such that there was minimum expression after 1 ½ hour of initiation of germination.

The protein extract was subjected to an activity gel with incorporated hemoglobin as the substrate for confirming and following the time bound expression of the enzyme (Figure 2). A band represented by a clearance zone was detected to have proteolytic activity (Figure 2 Lane number 1-4). No such clearance was visualized in the lane 5 of Figure 2 inset containing sample from 18 hours of germination indicating the absence of protease activity. In the presence of pepstatin A there was no visible band, indicating the absence of aspartic protease activity (Figure 2 Lane number 6).

The results were also confirmed by performing the assay for aspartic protease in the presence of pepstatin-A throughout the time period of expression for aspartic protease (data not shown). *De novo* synthesis of cysteine protease activity was observed after 24 hours of imbibition of water after which it continued to rise steadily while serine protease activity was observed after 72 hours (Table 2).

Figure 2: The expression pattern of the enzyme analyzed by activity gel



In the above figure lanes 1-4 show the expression of the enzyme at 1,4,6,12,18 hours of imbibition of water as indicated by the arrowhead while lane 6 shows the effect of pepstatin-A on the expressed enzyme as visualized by the absence of the clearance zone.

Table 2: Time bound activities of proteases from *Vigna radiata*

Class of Protease	Time of expression	Stage of germination	Activity (Units / mg protein)
Aspartic Protease	1-18 hours	Initiation of germination	1.182
Cysteine Protease	24 hours onwards	Later stages of germination	0.476
Serine Protease	72 hours onwards	Later stages of germination	0.793

In a comparative experiment the role of aspartic protease in germination was further confirmed by the germination assay in which the seeds of mung bean were soaked in water and pepstatin-A solution concurrently. The seeds soaked in water for 1 day were swollen and soft with loose seed coats indicating the initiation of germination with an average germ tube length of 8-10 mm (Figure 3A). The seeds soaked in pepstatin-A solution on the other hand did not swell and remained hard with no loosening of the seed coat (Figure 3B). This indicates that the aspartic protease is involved in the germination process. The seeds when transferred from pepstatin-A solution to water showed the initiation of germination. They were swollen, soft and had loosened seed coats with an average length of 4 mm of the germination tube. This indicated that the effect of pepstatin-A was reversible and confirmed the role of aspartic protease in the early stages of germination (Figure 3C).

Figure 3: Effect of aspartic protease on germination of mung bean seeds

3A

3B **3C**

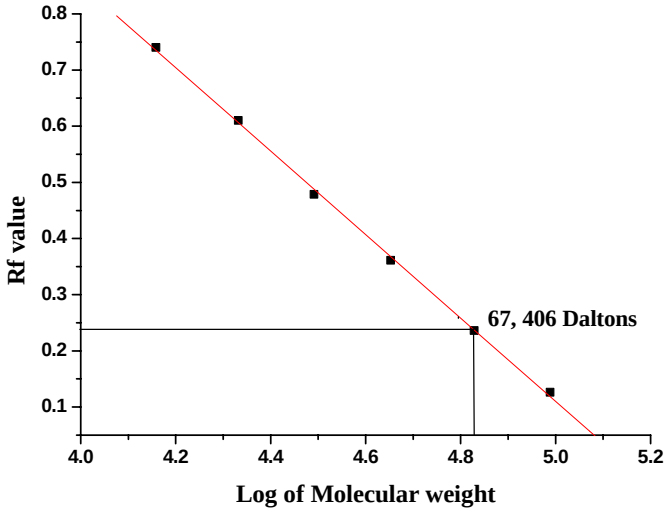
In the above figure 3A represents the seeds soaked in water for 1 day, 3B the seeds soaked in pepstatin-A for 1 day while 3C the seeds transferred from pepstatin-A solution to water after 1 day and further incubated for 1 day.

The purification of aspartic protease from *Vigna radiata* variety Kopergaon-1 is summarized in Table 3. The enzyme was purified by fractional ammonium sulfate precipitation (0-80%) followed by affinity column chromatography on pepstatin-A agarose. The purity of the enzyme was checked by gel filtration and native PAGE followed by silver staining.

Table 3: Purification of aspartic protease

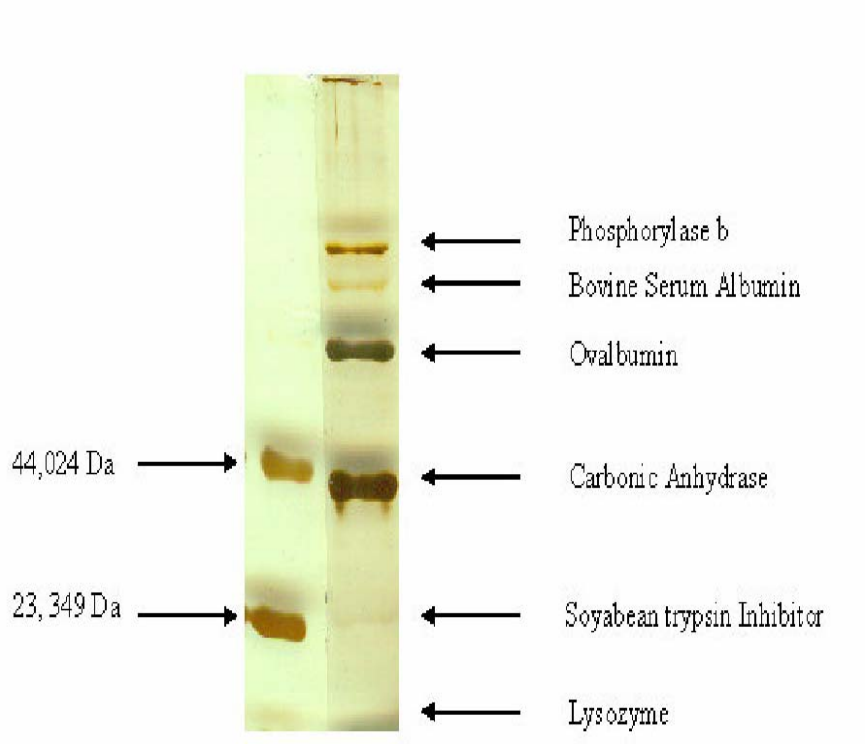
Sample	Activity (units/ml)	Protein (mg/ ml)	Total Activity (Units)	Total Protein (mg)	Specific Activity (units/mg)	Fold purification
Extra cellular filtrate	14.18	12	709	600	1.18	1
$(NH_4)_2SO_4$ Saturation	17	11	850	550	1.45	1.23
Affinity Column Fraction	51.18	0.897	2590	44.85	57.75	48.85

Figure 4: Gel filtration chromatography of pure aspartic protease on Biogel P100



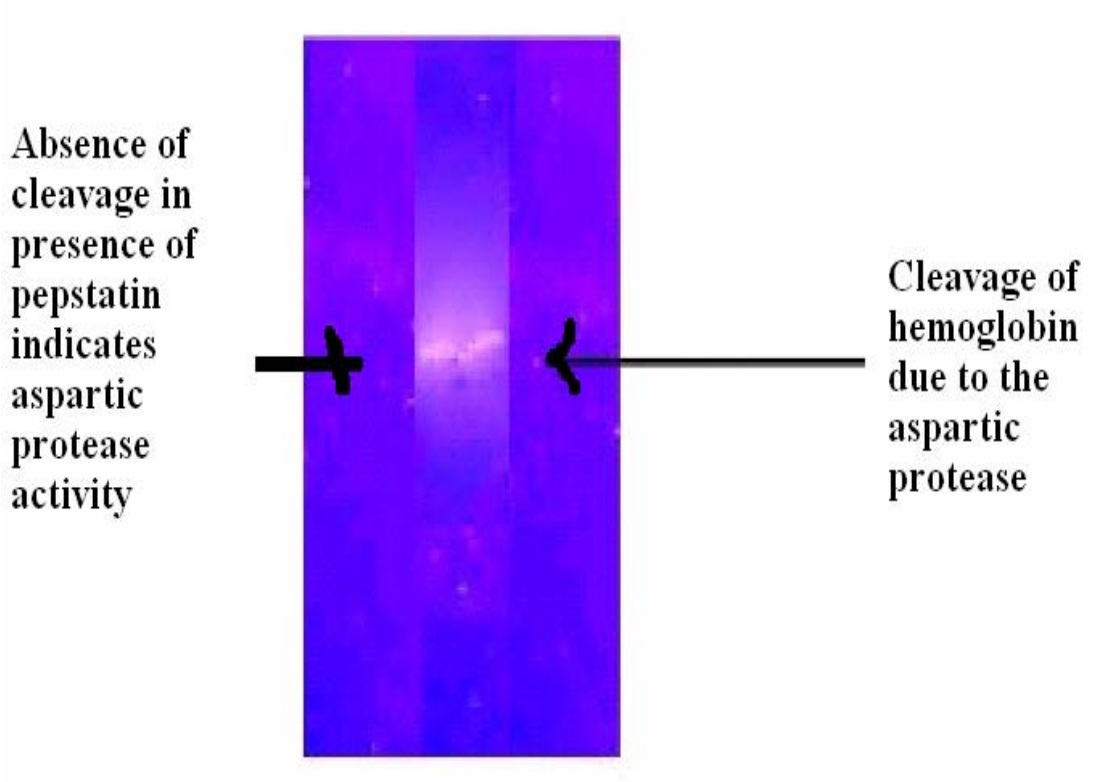
The enzyme showed a Mr of 67, 406 daltons on gel filtration with Biogel P100 (Figure 4). A single band was obtained on native PAGE suggesting the molecular integrity of the enzyme after affinity chromatography. SDS-PAGE analysis however revealed the presence of two bands suggesting that the enzyme is a heterodimer with distinctly different subunits (Figure 5). The molecular weights of the subunits are 23,349 and 44,024 Daltons as deduced from SDS-PAGE.

Figure 5: SDS-PAGE analysis of purified enzyme



The catalytic activity of the enzyme was verified by using hemoglobin as the substrate in an activity gel. The cleavage of hemoglobin in the presence of the purified enzyme was visually observed as bands of clearance. No such clearance was observed in the sample treated with pepstatin A, the universal inhibitor of aspartic proteases. It was also noticed that majority of the substrate cleavage activity could be associated with the larger subunit of the heterodimer (Figure 6).

Figure 6: Substrate cleavage activity of the individual dimers



Majority of the cleavage activity was associated with the larger subunit implicating the smaller subunit in the stabilization of the enzyme.

DISCUSSION

Plant aspartic proteases have been purified from dicotyledonous and monocotyledonous species as well as from gymnosperms [Rodrigo I, Vera P, Conejero V, 1989; Timotijevic GS, Radovic SR, Maksimovic V, 2003; Dunaevsky YE, Sarbakanova ST, Belozersky MA, 1989; Park H et al., 2000; Doi E et al., 1980; Zhang N and Jones B, 1999]. They are heterodimers or monomers ranging in molecular weight from 6 to 65 kDa. The specificities of some of them have been worked out using synthetic substrates. There are reports emerging about the roles of these enzymes in plants. In *Nepenthes* these enzymes have been implicated in the digestion of insects due to their presence in the pitchers of these plants. In tomato and tobacco, an Asp protease activity is implicated in the hydrolysis of pathogenesis related proteins [Rodrigo I, Vera P, Conejero V., 1989]. They are associated with the storage proteins in hemp and buckwheat seeds [Timotijevic GS, Radovic SR, Maksimovic VR, 2003]. They are associated with the breakdown of storage protein in wheat [Dunaevsky YE, Sarbakanova ST, Belozersky MA, 1989], sunflower [Park H, et al, 2000], rice [Doi et al, 1980], barley [Zhang N and Jones B, 1999; Kervinen J, Kontturi M, Mikola J, 1990; Tõrmäkangas K et al, 1994; Runeberg-Roos P, 2004], *Vigna* [Carvalho M et al, 2001] and recently in *Centaurea* [Salvador SM, Novo C, 2006] and *Cynara* [Faro C et al, 1999]. In Arabidopsis, they are involved in the processing of the 2S albumin precursors in vitro [Chen X, Pfiel J and Gal S, 2002; Mutlu A, Reddy X.C.S.M and Gal S, 1999; D' Hondt et al, 1993]. The studies on the biological functions of aspartic proteases are of recent origin and till to date represent an uncharacterized area.

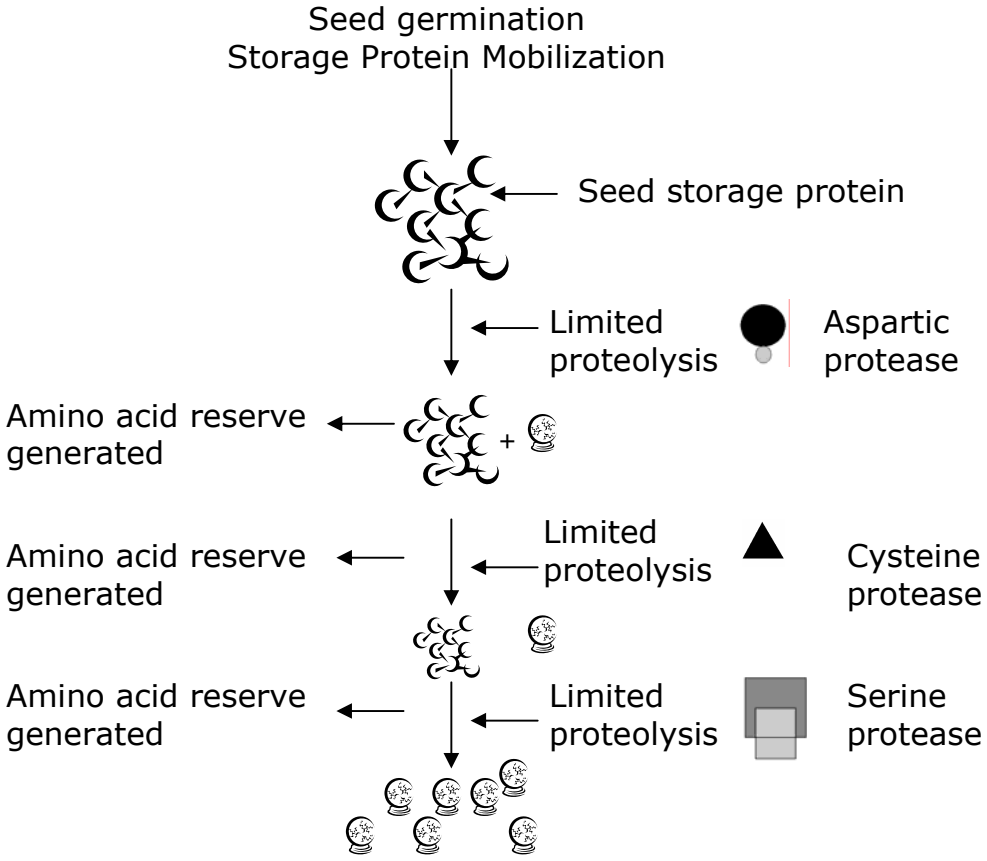
The activities of cysteine and serine proteases have been studied in *Vigna radiata* L. Wilczek variety. The cysteine protease activity was detected after 48 hours while

serine protease activity was found after 72 hours of initiation of germination [Frazão C et al, 1999; Kervinen J, et al,]. However the events before that have been uncharacterized. This is the first report of proteolytic activities from *Vigna radiata* variety Kopergaon-1. We demonstrate here for the first time the role of aspartic protease in mung bean (*Vigna radiata*) seed germination. An aspartic protease activity was detected for the first time in the germinating seeds of *Vigna radiata*. Maximum activity of aspartic protease was obtained after 1 hour of imbibition of water. This indicates its significance in the first few hours of germination. The cysteine protease activity in Kopergaon-1 seeds was detected after 24 hours while serine protease was observed after 72-hours of initiation of germination. The presence of aspartic protease in the first few hours of commencement of germination provides a new dimension to the events in seed germination and ends the speculation of the enzyme mobilizing the reserve protein. The inhibition of germination in the presence of pepstatin-A and its reversible action suggests unambiguously the role of aspartic proteases in the initiation of germination

It has been observed that the germination of seeds requires the action of a number of proteolytic enzymes, which hydrolyse the seed storage proteins to provide amino acids to the growing plants. The schematic representation of the events occurring during seed germination is provided in Figure 7. Our experimental results suggest that the cascade of seed germination is initiated by aspartic protease. The enzyme mobilizes the reserve protein by limited proteolysis generating the amino acid reserve required for building cellular constituents. The reserve seed protein is mobilized during the initiation of germination by limited proteolysis by an enzyme belonging to the class of aspartic protease. During further seed development, the amino acid reserve is depleted. Our experimental results demonstrated that further proteolytic activity, which is necessary for continuation of germination, is provided by

the arrival of other proteases. The limited proteolysis is continued in the absence of aspartic proteases by the action of cysteine proteases, which catalyse the limited proteolysis generating the amino acid reserve to continue the events. For further germination events to occur cysteine and serine proteases may be involved. This hypothesis corroborates well with the experimental results obtained and therefore could be taken as a base model to explicate the role of proteases especially aspartic protease in seed germination.

Figure 7: Schematic representation of the events occurring during germination of mung bean seed.



Seed germination in *Vigna radiata* is initiated by an aspartic protease. The first amino acid reserves are generated by limited proteolysis of the storage proteins. This activity is later

taken over by the *de novo* synthesized cysteine proteases and continued by serine proteases establishing the start of germination in *Vigna radiata*.

The aspartic protease purified in this report is a heterodimer with the active site on the larger subunit of the dimer as visualized in the activity gel patterns. The presence of aspartic protease ensures that the initial stages of storage protein mobilization are being mediated by aspartic protease whose role is later taken over by cysteine proteases after 24 hours of imbibition of water. This is the first report of such a high molecular weight enzyme active in the initial stages of germination of seeds. In mung bean, this report is significant with the understanding of the germination process whose initiation events were not understood. It sheds light on the importance of this class of enzymes in the mobilization of storage protein. This also ends the speculation of the protease involved in the early events governing germination. The presence of aspartic protease in the seeds leads to a new insight into the events governing the germination cycle of *Vigna radiata.*

CHAPTER III

BIOCHEMICAL STUDIES OF ASPARTIC PROTEASE FROM *VIGNA RADIATA*

Enzymes are things invented by biologists that explain things which otherwise require harder thinking.

Lettvin, Jerome

ABSTRACT

Biochemical characterization and studies on their structure function relationships are fundamental studies in understanding the normal functioning of enzymes. This chapter deals with the biochemical studies of the aspartic protease from *Vigna radiata.* The biochemical characterization of the enzyme revealed that it cleaves the peptide bond between Phe-Tyr residues. The enzyme was found to be pH stable and thermolabile. The interactions between VrAP and the universal inhibitor of aspartic proteases, pepstatin were analyzed revealing it to be a competitive reversible inhibitor with a tight mode of binding. The active site of an enzyme is the most conserved domain in the enzyme structure due to its involvement in substrate binding and catalysis. Chemical modification of the reactive amino acid side chain helps in the identification of those residues which are essential for catalysis. The presence of aspartic acid and serine in the active site of VrAP was revealed by the use of Woodward's reagent K (WRK) and phenylmethylsulfonyl fluoride (PMSF). The modification studies revealed the presence of an essential cysteine residue for enzyme activity. The fluorescence studies were based on the tryptophanyl fluorescence and therefore an analysis of tryptophan availability for these studies was done using acrylamide, cesium chloride and potassium iodide.

The conformational integrity of an enzyme is essential for its activity. There are few reports of the structural aspects of plant aspartic proteases. The work here presents the folding and unfolding of VrAP in the presence of guanidine hydrochloride. The pH based changes are also discussed. Detailed analysis of the secondary and tertiary structural changes in VrAP due to guanidine hydrochloride and change in pH is presented along with evidence for an alkali induced molten globule state.

INTRODUCTION

Enzymes, one of the most remarkable and important class of biomolecules, have astounded and fascinated both chemists and biologists for over a century by virtue of their extraordinary specificity and enormous catalytic power. The beginning of the study of mechanism of action of enzymes can be traced back to the end of the nineteenth century when Emil Fischer (1894) developed his famous 'lock and key' hypothesis to explain the substrate specificity of enzymes. He considered the enzyme as a rigid template of well defined structure and a correctly shaped substrate molecule would bind to the surface of the enzyme to form an enzyme-substrate complex, like a key fitting into a lock. Considering relative sizes of substrate and the presumed big size of enzymes, it appeared that only a small portion of the enzyme is essential for catalytic action and thus the concept of 'active site' originated. Koshland (1960) described the active site as being composed of a group of "contact" amino acids which remain within $2A^{O}$ of the substrate and some "auxiliary" amino acids which participates in the catalytic process even if they may not be in contact with the substrate. The contact and auxiliary amino acid residues are connected to 'contributing' amino acids, which serve as a stable framework for the active site and have no dynamic role in the catalytic action.

The immediate goal for the biochemists was the identification of the amino acids responsible for the formation of enzyme-substrate complex, determination of their approximate arrangement in space and development of enzymatic reactions. The role of specific amino acid residues in the catalytic process and the nature of the active site became clearer when various inhibitors were developed for enzyme study. The idea of using chemical inhibitors for studying the mechanism and pathway of enzyme catalysis was developed in 1930 from the observation that addition of

lodoacetate to fermenting yeast extract caused an accumulation of fructose 1, 6-diphosphate. Among the amino acids commonly occurring in proteins, sulphydryl group of cysteine first drew attention of the biochemists because of its distinctive properties and Hellerman (1937) introduced *p*chloromercuribenzoate for modifying this amino acid. Since then a variety of reagents including some new reagents for sulphydryl group were developed for the study of enzyme active site. Modification of amino acids in enzyme active site by chemical compounds is a powerful tool in the study of structure function relationship of enzyme and has been reviewed by several authors (Cohen LA, 1970; Means GE and Feeney, RE, 1971; Hirs CHW, 1967; Hirs CHW and Timasheff SN, 1972). The most commonly used reagents for chemical modification studies are known as group specific reagents. The group specific reagents react with the side-chains of specific amino acid residues. The reactivity of a particular side chain in a protein towards different reagents may vary with the size, shape and charge of the reagents depending on the protein environment around the reactive group and its accessibility.

The first step in these studies is to select a specific reagent suitable for use under conditions where the enzyme will not be denatured. If the enzyme loses activity on treatment with the reagent, the amino acid modified by it could be considered to be involved in the actual catalytic process or substrate binding process or maintenance of the active site conformation. The situations are distinguished by carrying out the same modification in presence of saturating amount of substrate or competitive inhibitors which would protect the active site. If the amino acid is part of the active site itself and is involved in the binding of the substrate, then there will be no loss of activity on treatment with the modifying reagent in presence of these ligands. Modification of amino acid residues involved in substrate binding process may not result in complete elimination of activity because the substrate might still be able to

approach the catalytic site by random motion. This could result in V_{max} remaining unchanged but as the substrate concentration required achieving it would be very high; the K_m would be increased greatly. Modification of amino acid residues in the catalytic site inactivates the enzyme completely. However chemical modification studies do not provide information about the three dimensional structure of the enzyme, its folding pathway as also the correlation between the enzyme structure in space and its function. One of the ways of inactivating the enzyme in the cell is to change its folding pattern. The folding and unfolding of a protein can be studied by using denaturants such as guanidine hydrochloride or urea. The changes can also be monitored by altering the environment of the enzyme by changes in temperature and pH. Studies wherein the environment is altered provide direct clues to the active conformation of the enzyme.

In the absence of any knowledge regarding the chemical nature, an indirect but powerful method utilized for the study of enzyme catalysis was the study of enzyme kinetics. By applying kinetic methods it was possible to gain information about the conditions optimum for the action of different enzymes, the effect of various factors on enzymatic rate and also the mechanism of enzyme action. The classical hyperbolic relationship between the initial substrate concentration and initial velocity of an enzyme reaction was demonstrated first by Brown (1902) when working with the enzyme invertase. The phenomenon was subsequently found to be of general application for all single substrate enzyme catalysed reactions and for multisubstrate reactions where the concentrations of all the substrates but one were kept constant. The assumption of the formation of enzyme-substrate complex was central to all subsequent thinking on the mechanism of enzyme action. In 1913, Michaelis and Menten (1913) proposed a kinetic model for the enzyme action and a kinetics which was based on the assumption that an equilibrium between enzyme, substrate and

enzyme-substrate complex almost instantly set-up and maintained, the breakdown of enzyme-substrate complex to products being too slow to disturb this equilibrium. Subsequent analysis of the Michaelis-Menten equation by Briggs and Haldane (1925) showed that the assumption of a steady-state for the enzyme-substrate complex is central to this derivation and as a necessary corollary to this, an important relationship connecting the thermodynamic equilibrium constant with the Michaelis constants and the maximal velocities of catalysis for both the substrate and the product emerges for any enzyme catalyzed reaction. The Michaelis-Menten equation has found general application in all aspects of enzymology and much of the later development of enzyme kinetics is in many ways, a refinement and extension of this fundamental equation of enzyme catalysis. The basic assumptions of Michaelis-Menten equation and its general method of treatment of kinetic parameters were found to be adequate for dealing with more complicated enzymological reactions. As more and more enzymes were being studied it became evident that simple idealized one substrate reaction involving only one enzyme-substrate complex which was assumed in the development of Michaelis-Menten kinetics are vary rare indeed. Many one-substrate reactions may involve two or more intermediate complexes and most enzymes have two or more substrates and may have two or more products. Kinetic behaviour of such reactions are rather complex. Development of the principles and derivation of the basic rate laws for these reactions were done primarily by Alberty (1956, 1962), Dalziel (1957), Wong and Hanes (1962) and Cleland (1963, 1970). However, the starting point for kinetic analysis of all enzymatic reactions is still the Michaelis Menten relationship. Cleland proposed a variety of mechanism for the multisubstrate reactions depending on the relative order of binding and release of the substrates and products of the enzymes (1967, 1970).

While enzyme kinetics was advancing in its understanding of enzyme catalysis, inhibitors assumed a central role in understanding the interactions between substrate and its respective enzyme. Studies of the structural requirements of competitive inhibitors also provided useful information about the structure and geometry of the enzyme active site. The kinetic study of enzyme inhibition for both competitive and noncompetitive inhibitors was greatly facilitated by the work of Lineweaver and Burke (1934). The minimal structural requirements for the structural and ionic geometry of the active site for quite a few enzymes were worked out from such studies.

Enzyme inhibitors are often used to further the understanding of enzyme mechanisms. Inhibitors serve as probes for kinetic and chemical processes during catalysis. Alternative applications for inhibitors are the detection of short-lived enzyme-bound reaction intermediates, or the identification of amino acid residues at the active site that are necessary for the catalytic activity of the enzyme. Inhibitors are also used for in vivo studies to localize and quantify enzymes in organs or to mimic certain genetic diseases that involve the absence of an enzyme in a given biosynthetic pathway. In pharmacological research, enzyme inhibitors are used to inactivate specific enzymes or groups of enzymes, leading to the treatment of many diseases.

The enzyme inhibitors may be classified as follows:

Non-covalent inhibitors	**Covalent inhibitors**
Rapid reversible inhibitors	Mechanism-based inhibitors
Tight, slow, slow-tight binding inhibitors	Affinity labels
Transition-state analogues	Pseudo-irreversible inhibitors
Multi-substrate analogues	

All of these enzyme inhibitors are active site-directed. They bind to the substrate binding site where the catalysis specific for that particular enzyme takes place.

Rapidly Reversible Inhibitors

Some inhibitors can be described solely by their kinetic behaviour. Rapidly reversible inhibitors, which are classified by their Michaelis-Menten kinetics specifically, are so named because they rapidly and reversibly establish their binding equilibria with enzymes. They bind to enzymes noncovalently, obtaining binding energy from thermodynamic sources such as the hydrophobic effect, van der Waals contacts (induced dipole interactions), hydrogen-bond formation, and charge-charge interactions. The strength of enzyme-inhibitor binding is measured by the K_i value, defined as the inhibition constant or dissociation constant of the inhibitor from the enzyme-inhibitor complex. A low *Ki* value represents a tightly bound enzyme-inhibitor complex. An other measurement of inhibition is the $IC_{50'}$ an approximation of the $K_{i,}$ which is the inhibitor concentration that produces 50% enzyme inhibition.

The rapidly reversible class of inhibitors is comprised of competitive, noncompetitive, and uncompetitive inhibitors. These types of inhibitors are distinguished by conducting competition experiments, which determine how an inhibitor interacts with the substrate at the active site of the enzyme.

Tight, Slow, and Slow, Tight-Binding Inhibitors

These inhibitors can be distinguished from the classical, rapidly reversible inhibitors because they do not display Michaelis-Menten kinetics. Each of the different inhibitors can be identified by its concentration relative to that of the enzyme and the rate of equilibrium formation during the reaction (Table 1). Tight-binding inhibitors inhibit reactions at concentrations comparable to that of the enzyme and under

conditions where the equilibrium of complex formation sets up rapidly. Tight-binding non-covalent inhibitors may have such high affinities for their target enzymes that they become functionally equivalent to covalent or irreversible inhibitors. These compounds may have half-lives on the order of hours, days, or even months. They have very low Ki values (eg, picomolar [pM]), and their off-rates are very slow. In the cases where one equivalent of inhibitor binds irreversibly to one equivalent of enzyme, the inhibitors have been called stoichiometric. Such inhibitors usually show noncompetitive kinetics even though they compete with the substrate for the active site.

Table 1: Reversible Enzyme Inhibitors

Inhibitor class	**I/E ratio**	**Attainment of equilibrium (E + I = EI)**
Classical	I > > E	Fast
Tight binding	I = E	Fast
Slow binding	I > > E	Slow
Slow, tight-binding	I = E	Slow

Slow-binding inhibitors operate by one of two mechanisms. Either the inhibitor binds slowly in an initial binding step, or the initial binding step occurs quickly, followed by a slow rearrangement of the EI complex. Most examples in the literature follow the slow rearrangement route. Slow binding can result when the form of the inhibitor or enzyme required for binding is present in low concentrations in solution, or if barriers to binding are encountered at the binding site (slow initial binding). Slow binding has also been correlated to the extrusion of an enzyme-bound water molecule from the active site after the formation of an initial enzyme-inhibitor complex (slow rearrangement). Slow binding may arise from conformational changes in the enzyme-inhibitor complex for the formation of the activated complex at the transition-state (slow rearrangement).

Slow, tight-binding inhibition occurs when slow-binding inhibition takes place at inhibitor concentrations comparable to that of the enzyme, in which case the previous two mechanisms can still apply. Comprehensive review articles on the subject of tight, slow, and slow, tight-binding inhibitors are available in the literature.

The present chapter is divided into three sections as biochemical properties of the enzyme, active site characterization of the enzyme and structure function studies.

The biochemical properties of the enzyme

Protein inhibitors occupy a central position in the elucidation of the functioning of enzymes. Aspartic proteases are a significant class of enzymes controlling key mechanisms and pathways in the cells. Protein inhibitors of aspartic proteases are relatively uncommon and are found in specialized locations. Few examples include proteins from the plants such as potato, squash, a pleuripotent inhibitor from sea anemone and an 8 kDa polypeptide inhibitor from yeast inhibiting saccharopepsin (Lenarcic, B. and Turk, V. 1999; Christeller JT, et al 1998; Kreft S, et al 1997). Pepstatin, a low molecular weight inhibitor isolated from various species of streptomyces is a universal inhibitor of this class of enzymes (Umezawa H, et al, 1970). The potency of pepstatin toward aspartic proteases has been widely exploited as a research tool for unraveling the mechanism of this group of enzymes, its biological functions and in affinity chromatography (Devaux, C, et al, 1976). To our knowledge there are no reports on the kinetics of inhibition of plant aspartic proteases.

A number of enzymatic reactions respond immediately to the presence of competitive inhibitors and thereby display a mechanism of tight binding inhibition. Thus, the formation of the enzyme-inhibitor complex takes place in a very short time. There is no clear distinction between the classical type of inhibition and that caused by tight binding inhibitors. Classical inhibition is observed only at inhibitor concentrations considerably higher than the enzyme concentrations whereas tight binding inhibitors are those which inhibit the enzyme-catalyzed reactions at concentrations comparable to that of the enzyme and under conditions where the equilibrium is set up rapidly (Szedlacsek S and Duggleby RG, 1995). Although a limited number of studies have been made on the kinetics of tight binding inhibitors interest in the subject is increasing constantly mainly because of their significance as therapeutic agents. The kinetic analysis is complex since the Michaelis-Menten approach is not valid and the double reciprocal plots are non linear in the presence of such inhibitors. Morrison (1982) gave a general initial steady state rate equation for any enzyme catalysed reaction in the presence of a tight binding reversible inhibitor. Henderson derived the linear form of the Morrison equation thus allowing graphical representation of the inhibition constants wherein secondary plots provided the constants in case of competitive inhibition (Williams, JW and Morrison JF, 1979).

In this section the use of pepstatin in understanding the mechanism of plant aspartic proteases is outlined. There have been no reports on the kinetics of inhibition of plant aspartic proteases with pepstatin. The substrate progress curve analysis provides the rate constants for the hydrolysis of hemoglobin under the assay conditions. The progress curve analysis demonstrates the time and concentration dependent inhibition of the inhibitor and indicates a tight binding mechanism of enzyme inhibition.

MATERIALS AND METHODS

Enzyme assay

Proteolytic activity of the purified aspartic protease was measured by assaying the enzyme activity using hemoglobin as the substrate and performing the reaction as given in Chapter II.

Protein measurement

Protein was measured by the Bradford method using bovine serum albumin as the standard (Bradford MM, 1976).

Determination of optimum pH, temperature, and stability of purified aspartic protease

Estimation of aspartic protease activity at different pH (2–10) and temperature (28°–60°C) values was carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 10U of the enzyme at 37°C in buffer of desired pH for 1 h. The temperature stability was determined by incubating 10U of the enzyme at different temperatures. The samples were removed at regular intervals and the residual activity was estimated under standard assay conditions.

Activity gel

The seminative gels (10% acrylamide) were copolymerized with 0.5% hemoglobin. The hourly samples were treated in 25mM Tris, pH 6.8, 0.08% SDS, without β-mercaptoethanol as explained by Timotijevic et al., 2003. After electrophoresis, the gels were washed first with 1% Triton X-100 in 25mM Tris-HCl, pH 6.8, and then in glycine-HCl buffer, 0.05 M, pH 3.0. After incubation in this buffer for 18h at 37°C,

proteolytic activity appeared as negative bands upon staining with Coomassie Brilliant Blue. Reduction in the activity of the enzyme on treatment with pepstatin was visualized by the activity gel staining.

Amino acid analysis

The amino acid analysis was done by hydrolyzing the 100 pM with 6N HCl for 24 hours. The hydrolyzed amino acids were derivatized with AccQ Fluor Reagent (6-amino quinolyl-Nhydroxysuccinimide carbamate) and run on a prepacked RP-HPLC 3.9×150mm column AccQ.Tag.

Substrate cleavage sites

VrAP was assayed in the presence of the synthetic substrate N-acetyl-L-phenylalanyl-L-3, 5-diiodotyrosine (APD). To 0.25ml of the enzyme solution (50 nM) at 37°C is added 0.125ml of 0.05N HCl. After 10 minutes 0.125 ml of APD solution is added to the test reaction and 0.5 ml of ninhydrin reagent is added to the blanks (final enzyme conc. 12.5 nM). After 30 minutes more, 0.5 ml ninhydrin reagent is added to the test reactions and at any time 0.125 ml of APD solution is added to the blanks. All the tubes are placed in a boiling water bath for exactly 10 minutes and are then cooled. The contents of tubes are diluted with 2.5 ml of 60% (v/v) ethanol and the tubes are then shaken to mix the solutions. The absorbance of the solutions at 570 nm is read against water. The activity can be expressed in APD units. One APD unit is the quantity of enzyme which librates 1 micromole of diiodotyrosine per minute under the above conditions.

Initial kinetic analysis for determination of K_m and K_i

For initial kinetic analysis the kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The IC_{50} values were

determined by nonlinear regression of percent inhibition data by using the four parameter logistic equation 1 where p is percent inhibition and is the relative decrease in enzymatic activity due to the inhibitor concentration $[I]_0$. The regression analysis was performed by Microcal Origin 6.1.

$$p=p_{min}+(p_{max}-p_{min})/\ 1+\ ([I]_0/I_{50})^n \qquad (1)$$

The inhibition constant K_i was determined by Dixon method (Dixon M, 1953) and also by the Lineweaver Burk double reciprocal plot (Lineweaver H and Burke D, 1934). For the Lineweaver Burk analysis VrAP (20μM) was incubated with the inhibitor at 2 and 6μM and assayed at increasing concentration of hemoglobin (78-546μM) at 37°C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. In Dixon's method, hydrolytic activity of VrAP (20μM) was measured in the presence of 234 and 462 μM of hemoglobin at concentrations of inhibitor ranging from 1-10μM at 37°C for 30 min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the K_i was determined by fitting the data using Microcal Origin 6.1.

Initial apparent inhibition constants

Stock solution of the inhibitor was prepared as mentioned above. Inhibition studies were performed by adding 100μl of the enzyme (0.02μM) to 300 μl of 234 μM hemoglobin solution in standard buffer containing varying concentrations of the inhibitor (1-10μM) at 37°C for 30 min. The product was estimated as mentioned above. Relative enzymatic activity R was computed from the ratio of product amounts obtained in the presence and absence of inhibitors as $R=1-[P]/\ [P]_{0.}$ The relative inhibition was fit by non-linear least squares regression to equation

mentioned below where $[I]_0$ is the total concentration of inhibitor and K_{app} the fitting parameter is the apparent inhibition constant.

Substrate progress curve analysis

The percentage of product formed, p, versus reaction time, t, was fitted to the recursive integral rate equation which is a variant of a known method for the estimation of Km and Vm from the reaction progress. The instrumental offset parameter p_0 accounts for the possible systematic error of the detection method. Other optimized parameters in the least squares regression were the specific molar instrumental response of the reaction product rp, the Michaelis constant Km, and the maximum velocity Vm. The fixed parameters were the mixing delay time (t=0) and the initial substrate concentration $[S]_0$. Auxiliary variables are defined as mentioned below:

$$Y^{(a+1)}=y^a+r_p(1-[P]/[S]_0{}^{-b})/(1/[S]_0{}^{-b/Km}) \quad (2)$$

$$b=([P]-tVm)/Km \quad (3)$$

$$[P]=(y^a - p_0)/r_p \quad (4)$$

Inhibitor progress curve analysis

The reaction time course in the presence of inhibitor was fitted to the equation given below, which is a modification of the standard kinetic model.

$$p= p_0+ Vst + (V_0-V_s)[1-\exp(-k_{app}t)]/k_{app} \quad (5)$$

The instrumental offset p0 is treated as an adjustable parameter to account for the possibility of systematic errors in measuring the product conversion degree. Each individual progress curve was fitted separately. The local fitting parameters were the initial velocity V_0, the steady state velocity V_s, the apparent first order rate constant k_{app}, and the instrumental offset p_0. These fitting parameters were analysed to extract approximate inhibition constants and is described in detail later. Several

progress curves obtained in the presence and absence of the inhibitor at various concentrations were combined and fitted as a whole. The equation used as theoretical model is mentioned below and is applicable to a pure tight binding inhibitor.

$$v=v_0([E]_0-[I]_0-K_{app}+\{([E]_0-[I]_0-K_{app})^2 + 4[E]_0K_{app}\}^{-1/2} \quad (6)$$

In order to fit data to this equation, the modified Marquardt-Levenberg least square fitting equation was used. The rate constants were obtained by the regression analysis of this data using the software Origin 6.1.

Another approach used to calculate the apparent Ki* value for Pepstatin applied the following equation:

$$IC_{50} = Et/2 + K_i^* \quad (7)$$

Et is the total enzyme concentration and K_i^* is the apparent enzyme-inhibitor dissociation constant value.

For competitive inhibition, the true K_i was obtained by dividing K_i^* by $(1+S/K_m)$.

RESULTS

pH and temperature stability and optima

The enzyme was active in a wide range of pH from 2 to 6 with optimum activity at pH 3.5. It was stable in an expansive range of pH 2-10 with more than 70% activity (Table 2). The optimum temperature wherein the purified enzyme had maximum activity was 37°C. The enzyme had a half life of 6 minutes at 50°C (Table 2).

Substrate cleavage sites

Use of the synthetic substrate revealed that the enzyme recognizes the peptide bond between the amino acids Phe-Tyr with subsequent release of diiodotyrosine residues. This indicates that the enzyme is similar in nature to pepsin with similar recognition site.

Table 2: Biochemical properties of the enzyme

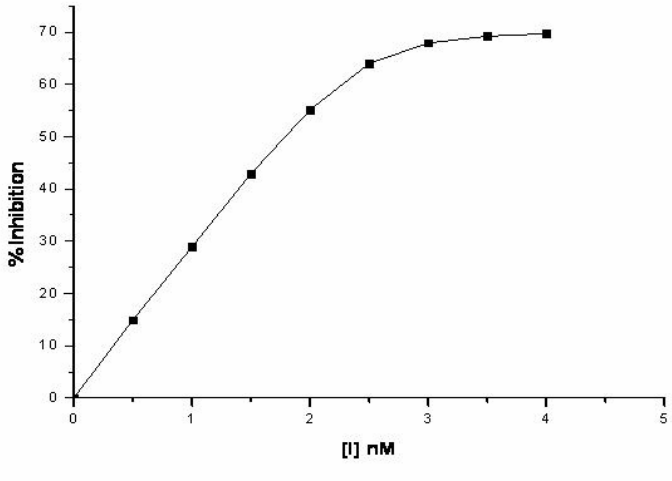
Property of Enzyme	
Temperature Stability	Half life of 5 minutes at 50°C
Temperature Optima	37°C
pH Stability	Stable in pH range 2-10
pH Optima	3.5
Substrate Cleavage Site	Phe-Tyr bond

Determination of IC_{50} value of pepstatin against VrAP

The inhibition of VrAP followed a hyperbolic pattern with increasing concentrations of the inhibitor. The non linear regression analysis of the best fit data yielded an IC_{50} value of 1.6 µM (Fig. 1). However, the secondary plot (the slope of inhibition graph

versus pepstatin concentration) was not linear, suggesting that the application of Michaelis-Menten inhibition kinetics was not appropriate in this study.

Figure 1: Determination of IC50 value of pepstatin against VrAP

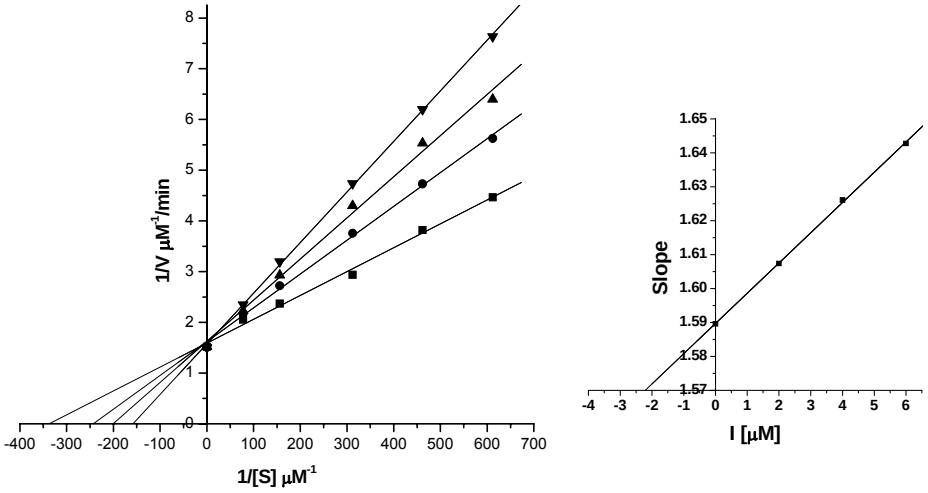


Estimation of IC50 values based on the application of equation of Cha to the sigmoidal curve. The concentrations of pepstatin used were 0.5 – 4 µM.

Determination of Vm, Km and Ki of VrAP

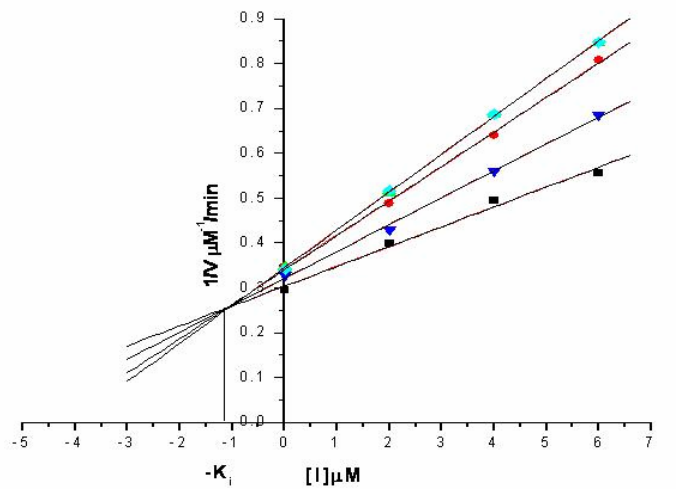
Pepstatin showed a linear inhibitory response in the dose range of 1-10µM. During this course of inhibition classical competitive experiments can be performed to determine the K_i values. Initial kinetic assessments by the Lineweaver Burk analysis revealed that the aspartic protease was competitively inhibited by pepstatin (Figure 2). The K_m value of the enzyme was 179.7 µM. The secondary plot of the slope versus inhibitor concentration gives the K_i value of 2.70768 µM (Figure 2 inset 1) while with the Dixon plot a K_i value of 1.1388 µM was obtained (Figure 3).

Figure 2: Determination of Vm, Km and Ki by Lineweaver Burk analysis.



The enzyme activity was assayed at increasing concentrations of the substrate using inhibitor concentrations of 0 (■), 2 (▲), 4 (●) and 6 (▼) μM. The reciprocal of the rate of the substrate hydrolysis for each inhibitor concentration were plotted against reciprocals of the substrate concentrations. Ki was determined from the formula for competitive type of inhibition.

Figure 3: Determination of Ki by Dixon plot



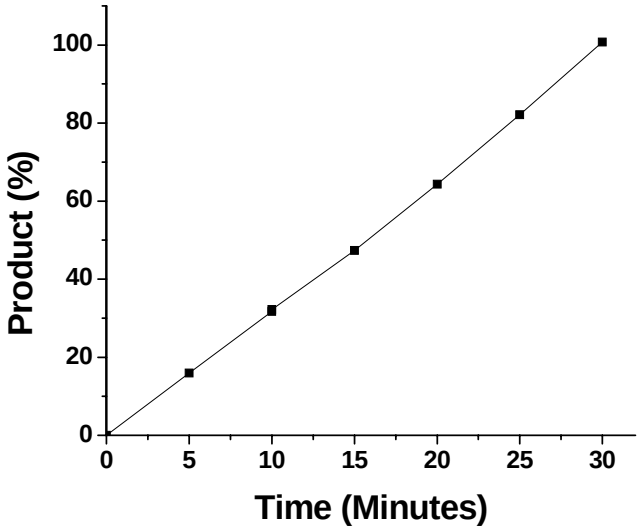
The substrate concentrations used were 78 (◆), 234 (●), 462 (▼) and 546 (■) μM. The straight lines indicate the best fit of the data obtained. The inhibition constant Ki was calculated from the point of the intersection of the plots.

Substrate kinetics

Although the K_m and V_m values are obtained from the double reciprocal plot a more comprehensive and correct understanding of these comes from the analysis of the substrate hydrolysis progress curve. Figure 4 provides the non linear least squares fit to the equations mentioned in the text previously. The initial substrate concentration was fixed at 525 µM while the best fit values of the adjustable parameters were Vm = 0.548 µM per minute, Km = 165 µM, rp = 0.177 au µM and p0= 0.25 au.

The arbitrary unit (au) of molar response is defined as percent of product formed in the reaction. From the results of fit, the maximum velocity expressed in the arbitrary units is .177 × 0.548 = 0.097% of product per second, and, for control, the maximum conversion is 0.177 × 525 + 0.25 =93.18 % of product.

Figure 4: Analysis of time dependent substrate cleavage by VrAP



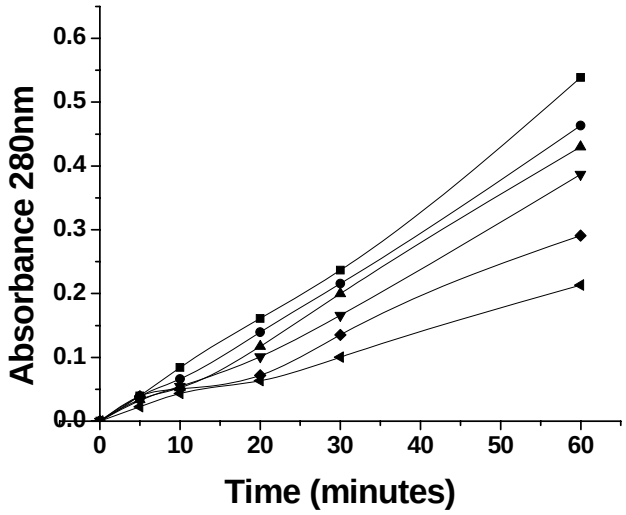
20 µM of VrAP was reacted with 3mg/ ml of hemoglobin for different time intervals. Aliquots removed at specified time intervals of 5, 7.5, 10, 15, 20, 25 and 30 minutes were analyzed for the amount of product formed. The least squares fit of substrate progress curve to the

modified integral Michaelis-Menten equation provided accurate estimates of the kinetic constants Vm and Km. The best fit values were obtained are mentioned in the text.

Progress Curve analysis

The reaction progress was analyzed by two different methods. The results of the analysis based on the assumption of rapid equilibrium are shown in Figure 5.

Figure 5: Progress curve of time dependent inhibition of VrAP by Pepstatin.



The concentration of the substrate used for monitoring the progress of the reaction in the presence of varying inhibitor concentration was 500µM. The inhibitor concentrations were 0 (■), 2 (●), 4 (▲), 6 (▼), 8 (◆) and 10 (◀) µM. The experimental conditions and the best fit values are mentioned in the text. Least square fit of progress curve data of the enzyme inhibition with pepstatin to equation mentioned in the text.

The progress curves obtained at 2, 4, 6, 8 and 10 µM inhibitor were fitted individually to the equation mentioned below. The best fit values of the individual parameters obtained are listed in Table 2. Based on these results it is obvious that the results corroborate with the one-step inhibition mechanism wherein the enzyme inhibitor complex is rapidly formed. The complex thus formed is stable and does not

revert to another form as is the case with the slow-tight binding mechanism. The onset of inhibition is rapid and the binding indicates a tight binding mechanism.

Table 2: Kinetic rate constants obtained by various methods

Method of analysis	IC_{50} (µM)	V_m (µM per minute)	K_m (µM)	K_i (µM)	K_{i*} (µM)	K_A (per second)	K_D (per second)
Inhibitor curve analysis	1.6						
Lineweaver Burk		0.630	179.7	2.70768			
Dixon Plot				1.1388			
Substrate kinetics		0.548	165				
Inhibitor progress curve		0.650		0.87	0.7271	6.85×10^{-3}	11.91×10^{-3}
Dissociation constant analysis					1.5183		
Cha analysis				1.1245	1.075		

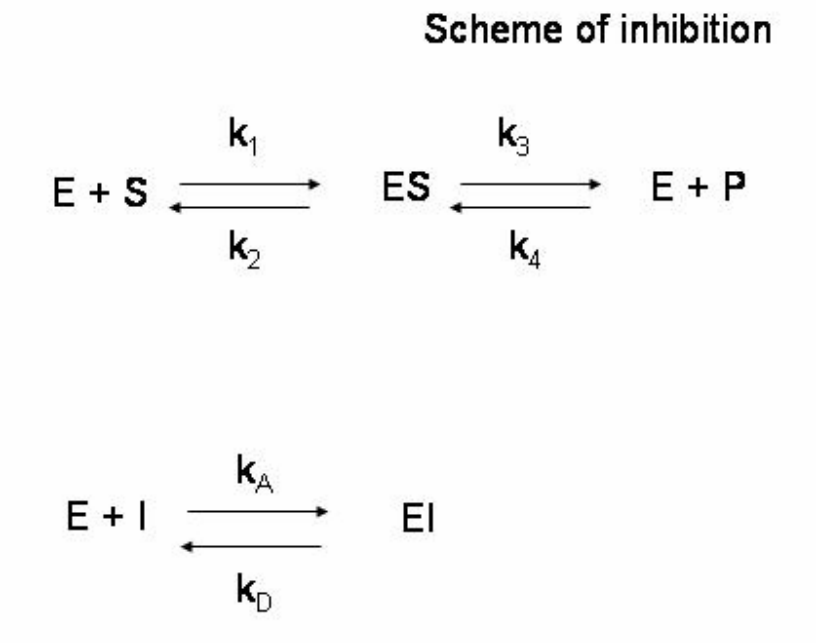
The rate constants are in agreement with the model scheme outlined in the figure10 and rule out the possibility of a slow onset of inhibition. The initial velocity V_0 obtained is constant and the apparent rate constant k_{app} increases linearly with the inhibitor concentration. The rate constants were obtained by fitting the data to the equations mentioned below.

$$V_0 = V_m[S]_0/([S]_0 + K_m) \quad (8)$$

$$V_s = V_m[S]_0/([S]_0 + K_m\,(1 + [I]_0/Ki)) \quad (9)$$

$$Kapp = kD + kA[I]0/(1 + [S]0/KM) \quad (10)$$

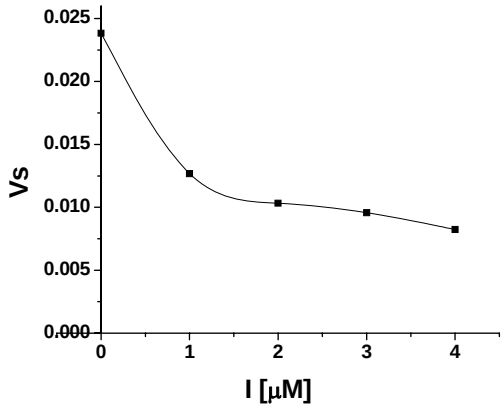
The parameters listed in Table 3 favor the one-step mechanism, because the initial velocity does not decrease with the concentration of the inhibitor, as predicted by eq 8. Also, the increase of the apparent rate constant with $[I]_0$ is linear.



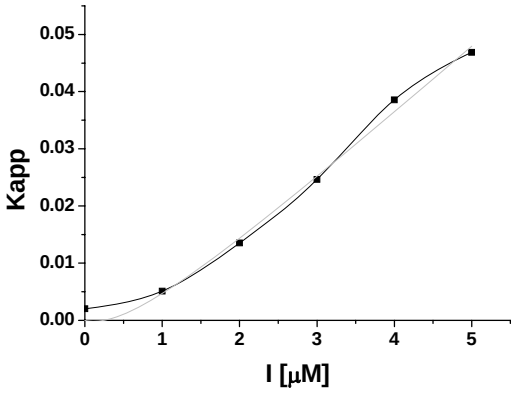
The nonlinear least squares fit of V0, V_S, and *K*app to eq. 8a-c is shown in Fig. 6 and Fig. 6. From fitting of V_S to eq. 9, the overall dissociation constant *Ki** is 0.7271 ± 0.005 μM; the fitted value of *V*max is 0.650 ±0.001 au s-1 in this case. From fitting of *K*app to eq. 8c (fig. 6), the initial inhibition constant *Ki* was 0.87 ± 0.12 μM. The isomerization rate constants were k_A = 6.85 ± 0.1 × 10^{-3} s-1 and *k*D = 11.91 ± 0.06 ×10^{-3} s-1, from which the total dissociation constant $Ki^* = Kik_D/k_A$ is 1.5183 μM.

The parameters obtained with the mechanism as explained by Cha, Williams et al, corroborate well with the above mentioned mechanism yielding a K_i^* of 1.075 μM and a K_i of 1.1245 μM.

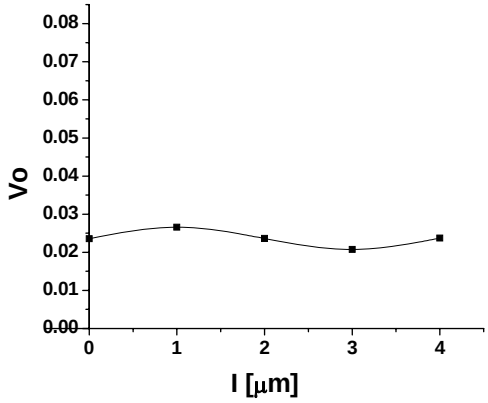
Figure 6: Least square fit of the steady state velocities V_0, V_s and the apparent first order rate constant K_{app}.



A



B



C

A: From fitting of V_S to eq. 9, the overall dissociation constant *Ki** is 0.7271 ± 0.005 μM; the fitted value of *V*max is 0.650 ±0.001 au s-1 in this case. B: From fitting of *K*app, the initial inhibition constant *Ki* was 0.87 ± 0.12 μM. The isomerization rate constants were k_A = 6.85 ± 0.1 × 10^{-3} s-1 and *k*D = 11.91 ± 0.06 ×10^{-3} s-1, from which the total dissociation constant $Ki^* = Kik_D/k_A$ is 1.5183 μM. The change in Vo as per the inhibitor concentration was found to be almost constant fitting into the theory of tight binding of the inhibitor to the enzyme.

DISCUSSION

Pepstatin is a universal inhibitor of aspartic proteases but has not been studied with plant aspartic proteases. The interactions of pepstatin with aspartic proteases of fungal origin and viral origin as also of human origin are well known. The kinetics of the enzyme with the inhibitor leads to a better understanding of the relationship of the enzyme-inhibitor interactions. Pepstatin has been shown to exhibit a tight binding effect on cathepsin-D and pepsin by many researchers (Rich D and Sun E, 1980; Knight CG et al, 1980; Knight CG and Barrett AJ, 1976).

A parallel finding here implicates that it exhibits the same mode of action for plant enzymes as that for animal enzymes. The data reported in this study showed that it is a tight binding inhibitor of the plant aspartic protease, VrAP. During initial kinetic analysis the inhibitor showed competitive inhibition against the enzyme in vitro. The 1:1 molar ration of the interaction of the inhibitor with the target enzymes classified it under the "tight-binding inhibitor" group [53]. The inhibitor demonstrated an IC_{50} value of 1.6 µM and the inhibition constant *Ki*, determined by the different methods was found between 1-3 nM (Table 4), which are almost equal to the IC50 value of the inhibitor. Characteristically the concentration of a tight binding inhibitor required to give 50% inhibition depends on the concentration of the enzyme. Therefore IC_{50} values for tight binding inhibitors become meaningless in the absence of information on the concentration of the enzyme used. A reversible tight-binding inhibitor is one that exerts its effect on an enzyme catalyzed reaction at a concentration comparable to that of the enzyme. Also several authors have pointed out the discrepancy in calculating the K_i values. As a rule when comparing K_i values for tight binding inhibitors, it is essential to examine the mathematical methods used for their

estimation since different methods even when applied to the same data can yield K_i estimates differing by several orders of magnitude (Szedlacsek SE, Duggleby RG and Vlad MO, 1991; Reich JG, 1992). The Lineweaver-Burk reciprocal plot shows that pepstatin is a competitive inhibitor of VrAP. For the inhibition kinetic studies, the enzyme activity was monitored in the presence of different concentrations of inhibitor and substrate as a function of time. In the region of K_i, the k_A value is high while the k_D value is low. This signifies that pepstatin binds rapidly to the enzyme but the rate of dissociation is slow. This indicates a fast inactivation of the enzyme in the presence of the inhibitor. The tight binding inhibitors combine at the active site and rapidly cause the enzyme to loose activity. This is the same as can be observed between VrAP and Pepstatin.

Active site characterization of the enzyme

VrAP is a member of the plant aspartic protease group of enzymes. It is an important component of the germination cascade in the seeds of *Vigna radiata* and is primarily responsible for the mobilization of the reserve seed storage protein. The physiological significance of aspartic proteases in plants is recently emerging. The structural studies of this class of enzymes are recently being pursued with only two crystal structures being solved to date. Despite their distribution in the living world, occurring from retrovirus to mammals, aspartic proteinases share significant similarities in primary and tertiary structures. Members of this class display Asp-Thr/Ser-Gly motifs within their sequences and are specifically inhibited by pepstatin, a peptide produced by *Streptomyces*. The technique of chemical modification has been used on other enzymes to understand the composition of the active site of the enzyme. It is used here to confirm the presence of the catalytic triad and prove the existence of an additional cysteine in maintaining the active conformation. Additional information about the accessibility of the tryptophan residues provides a strong basis for the fluorimetric analysis of the structure of the enzyme. Quenching studies of tryptophan indicate a homogenous distribution of the tryptophans thus adding a new dimension to the structure of the enzyme.

MATERIALS AND METHODS

Materials

VrAP used in these experiments was purified from the seeds of *Vigna radiata*. The purification involved fractional ammonium sulfate precipitation and affinity column chromatography. Hemoglobin, phenylmethylsulfonyl fluoride, p-hydroxy mercuribenzoate, Woodward's reagent K, acrylamide, cesium chloride, potassium iodide and DTNB were procured from Sigma.

Methods

Purification of VrAP

VrAP was purified as mentioned in chapter II. The protease activity and protein were checked using hemoglobin as the substrate and Bradford respectively. VrAP (1.5 µM) was prepared in glycine-HCl buffer, 0.05 M, pH 3.0 for estimating the optimum activity. Enzyme activity was estimated as mentioned in Chapter II.

Preparation and kinetics of enzyme

VrAP (1.5 µM) was diluted in glycine-HCl buffer, 0.05 M, pH 3.0 for estimating the optimum activity. The same procedure was followed for other pH values using acetate buffer at pH 6.0, Tris-Cl buffer at pH 8.0 and Glycine NaOH buffer at pH 10.0. The substrate hemoglobin was prepared as a 1mM stock in the buffers of respective pH. For the Lineweaver Burk analysis of enzyme kinetics at different pH, VrAP (20µM) was incubated at the pH values of 2, 4, 6, 8 and 10 and assayed with increasing concentration of hemoglobin (78-546µM) at 37°C for 30 min. The reciprocals of substrate hydrolysis (1/v) at each pH value were plotted against the reciprocals of the substrate concentrations. The corresponding Vm and km values

were obtained for each pH value. A graph of log Vm/ km against pH was plotted to obtain the pKa values. The approximate pK values of ionizing groups were determined by fitting straight lines with slopes of one or two to plots of the log Vm/kM versus pH as described by Tipton and Dixon. This method is a well established, common procedure for developing pK values from pH data.

Modification of VrAP with WRK (Woodward's Reagent K)

Enzyme sample (20µg) was incubated with varying concentrations of WRK (0.1-0.5µM) in 50mM Gly-HCl buffer, pH 3.0, at 25°C and assayed for aspartic protease activity at different time intervals. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions. Substrate protection studies were performed by incubating the enzyme with the substrate hemoglobin along with the modifier.

Modification of VrAP with PMSF (Phenylmethylsulfonylfluoride)

Enzyme sample (20µg) was incubated with varying concentrations of PMSF (0.1-0.5mM) in 50mM phosphate buffer, pH 7, at 25 °C. The modified enzyme was assayed for aspartic protease activity at different time intervals using the standard assay protocol mentioned in Chapter II. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions. Substrate protection studies were performed by incubating the enzyme with the substrate hemoglobin along with the modifier under similar experimental set up and using suitable controls.

Reaction with DTNB

VrAP (0.1 mg/mL) in 50mM phosphate buffer, pH 7, was incubated with 0.2mM DTNB at 25 °C for 25 min. The reaction of DTNB with sulfhydryl groups of the native

enzyme was followed spectrophotometrically at 412 nm. Control tubes having only enzyme or only inhibitor or inhibitor and substrate were incubated under identical conditions.

Modification of VrAP with PHMB (Para-hydroxymercuribenzoate)

Enzyme sample (20µg) was incubated with varying concentrations of PHMB (0.1-0.5mM) in 50mM phosphate buffer, pH 7,at 25°C and assayed for aspartic protease activity at different time intervals.

Kinetics of modified enzyme

Enzyme sample (20µg) modified with 0.3mM of PMSF, PHMB and 0.3µM of WRK in 50mM Gly-HCl buffer, pH 3.0, at 25°C was assayed for aspartic protease activity with different substrate concentrations from 0.1-1 mg/ml. The kinetic constants were determined from the Lineweaver-Burk double reciprocal plots. One unit of protease activity is defined by an increase of 0.001 at 280 nm/min at pH 3.0 at 37 °C measured as PCA-soluble products using hemoglobin as the substrate.

Kinetics of inactivation

The apparent first-order constant of inactivation depends on the concentration of the modifier and can be expressed as

$Kapp = K (M)n$

Where, Kapp is the apparent first order rate constant, M is the concentration of the modifier and n is the number equal to the average order of reaction with respect to the concentration of modifier

$\log Kapp = \log K + n \log (M)$

Kapp can be calculated as a slope from a semi-logarithmic plot of residual enzyme activity as a function of time. The second order rate constant for inactivation was

determined from the slope of the plot of pseudo-first order rate constant against inhibitor concentration. The order of the reaction (n) can be experimentally estimated by determining the Kapp at different concentrations of the modifier. A plot of log Kapp against log (M) gives a straight line with a slope equal to n, where n is the number of the molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme inhibitor complex (Levy *et al*., 1963).

Fluorescence quenching experiments

The tryptophan environment of the native enzyme was analyzed with solute quenching technique. Quenching titrations with either acrylamide or cesium chloride or KI were performed at 30ºC by sequentially adding aliquots of the concentrated quencher stock solutions (5M) to the enzyme solution. Sodium thiosulfate (0.1mM) was added to the KI stock solution to prevent I_3^- formation. The excitation wavelength was set at 295 nm and the fluorescence emission spectra were scanned from 300-400 nm. The fluorescence quenching data in the presence of acrylamide, CsCl or KI were analysed by the Stern-Volmer equation (Eftink and Ghiron., 1981)

$F_0/F = 1+ K_{sv} X [Q]$

In which F_0 and F are the fluorescence intensities in the absence or the presence of the quencher, respectively. K_{sv} is the Stern-Volmer quenching constant and [Q] is the quencher concentration (Stern- Volmer, 1919).

For proteins containing more than one fluorescing tryptophan residues differing in their accessibility to the quencher, the Stern- Volmer plots will be non linear and hence a modified Stern-Volmer equation has been applied

$F_0/ \Delta F = 1/f_a + 1/f_a K_{sv} [Q]$

$\Delta F = F_0-F$

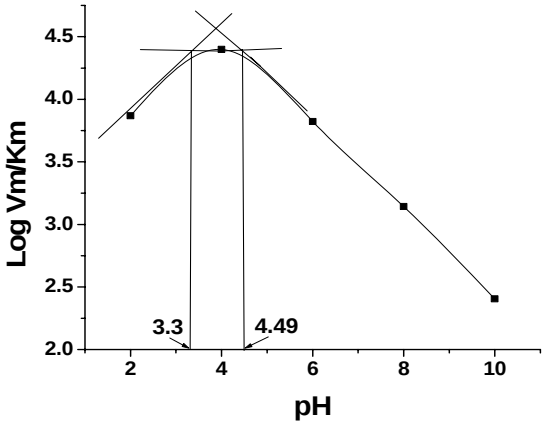
f_a is fraction of the quenchable fluorescence obtained from the ordinate intercept of the linear portion of the $F_0/\Delta F$ vs 1/Q plot (Lehrer, 1971).

RESULTS

pH dependent activity

The enzyme was found to be stable in a wide range of pH from 2-12. The double reciprocal plots of enzyme activity at different pH against various substrate concentrations gave the Vm and km values at the respective pH values. The secondary plot of the log Vm/km versus pH gave two pKa values of 3.3 and 4.29 which are representative of acidic amino acids at the active site of the enzyme (Figure 7).

Figure 7: Identification of the pKa values of ionizable side chains of active site amino acids

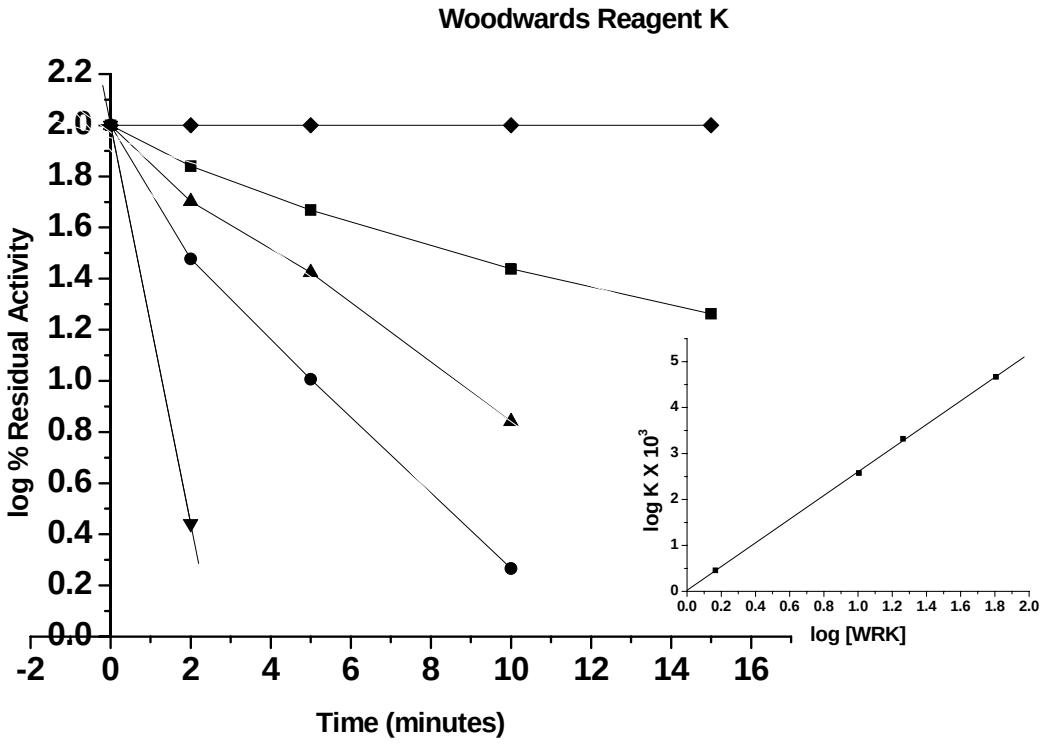


The secondary plot of the log Vm and km versus the pH provided tha pKa of the active ionizable groups at the active site of the enzyme. The tangential fitting of the curve and the subsequent extrapolation of their intersection point onto the X axis provided the pKa values of 3.3 and 4.49, which correspond to the carboxylic acid residues in the active site of the enzyme.

Modification of carboxyl groups

Inactivation of VrAP by WRK led to fast inactivation of the enzyme with increase in the concentration of the modifier and the exposure time. The reaction followed pseudo first order kinetics (Figure 8). The double logarithmic plot of slope against concentration of the reagent showed a straight line with the value of the slope to be two. Thus two carboxylate residues are proposed to be present at the active site.

Figure 8: Kinetics of inactivation of VrAP by WRK. Pseudo first order plots for the inactivation of VrAP by WRK



VrAP (20µg) was incubated with varying concentrations of WRK (0.1-0.5µM) in 50mM phosphate buffer, pH 3.0 and control (s) at 25 °C. Aliquots were removed at indicated time

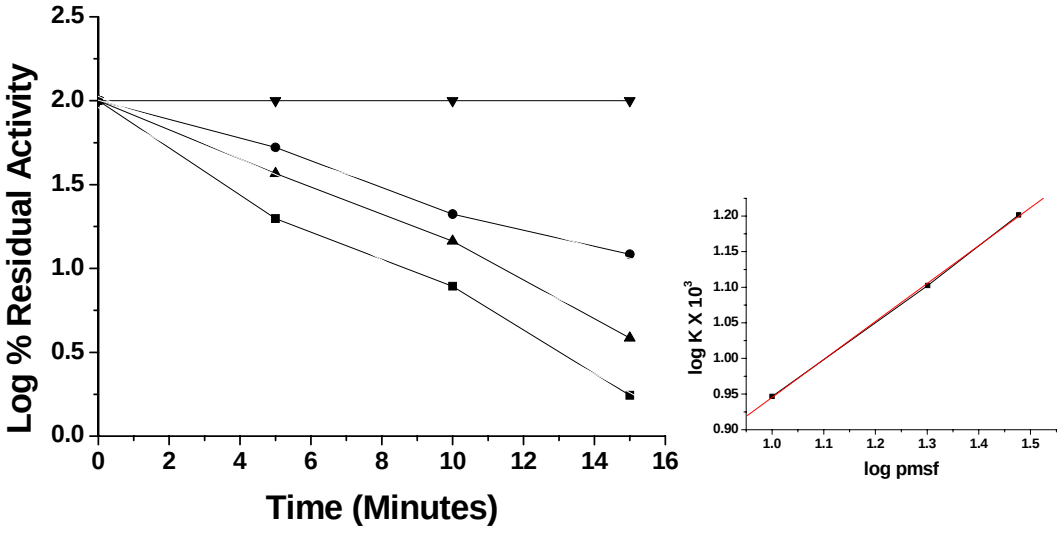
intervals and the reaction terminated by adjusting the pH to 3.0. Inset: Double logarithmic plots of pseudo first order rate constants as a function of WRK concentration.

The Vm of the enzyme treated with WRK remained unchanged while km had increased indicating decreased affinity towards the substrate (Table 1). The enzyme was fully protected from inactivation by 200 µM of the substrate (Table 2).

Modification of serine

Treatment of VrAP with PMSF was found to be time and concentration dependent. The reaction followed pseudo first order kinetics and double logarithmic plot of slope against concentration of the reagent showed a straight line with the value of the slope to be one (Figure 9).

Figure 9: Kinetics of inactivation of VrAP by PMSF. Pseudo first order plots for the inactivation of VrAP by PMSF



Enzyme sample (20µg) was incubated with varying concentrations of PMSF (0.1-0.5mM) in 50mM phosphate buffer, pH 7 and control (s) at 25 °C. Aliquots were removed at indicated

time intervals and the reaction terminated by adjusting the pH to 3.0. Inset: Double logarithmic plots of pseudo first order rate constants as a function of WRK concentration.

The single serine residue present at the active site seems to be involved in substrate binding as the km of the modified enzyme increased while the Vm remained unchanged (Table 1). Substrate protection studies confirmed the presence of serine at the active site (Table 2).

Table 1: Kinetics of modified enzyme

	Vm	**Km**
Native Enzyme	0.06	15.24
WRK modified enzyme	0.0602	36.28
PMSF modified enzyme	0.0597	22.13

Table 2: Substrate protection studies of chemically modified VrAP

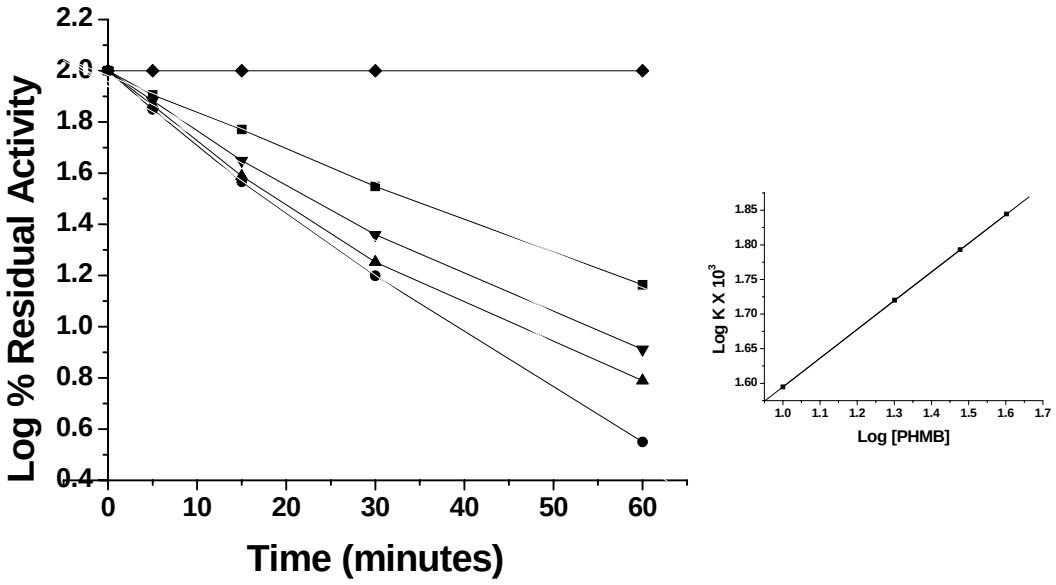
Incubation mixture	**Residue Modified**	**Residual activity (%)**
Enzyme	None	100
WRK	Aspartic acid	0
WRK+ Hemoglobin (50µM)		34.8
WRK+ Hemoglobin (100µM)		67.3
WRK+ Hemoglobin (200µM)		100
PMSF	Serine	30
PMSF + Hemoglobin (100µM)		48.6
PMSF + Hemoglobin (200µM)		66.9
PMSF + Hemoglobin (300µM)		85
PHMB	Cysteine	70

PHMB + Hemoglobin (300μM)		70

Modification of cysteine

The number of free cysteine residues in native VrAP as determined by modification of enzyme by DTNB was found to be 1. Treatment of the enzyme with PHMB slowly inactivated the enzyme (Figure 10). The enzyme lost 80% of its activity with 0.5mM reagent after 60 minutes. However, substrate protection from inactivation was not observed in case of enzyme inactivated by PHMB.

Figure 10: Kinetics of inactivation of VrAP by PHMB. Pseudo first order plots for the inactivation of VrAP by PHMB



Enzyme sample (20µg) was incubated with varying concentrations of PHMB (0.1-0.5mM) in 50mM phosphate buffer, pH 7 and control (s) at 25 °C. Aliquots were removed at indicated time intervals and the reaction terminated by adjusting the pH to 3.0. Inset: Double logarithmic plots of pseudo first order rate constants as a function of WRK concentration.

Assessment of the microenvironment of Trp residues of VrAP

The fluorescence spectrum of the native enzyme had an emission maximum of 341 nm. The fluorescence intensity of the enzyme was as high as 800 fluorescence units.

A drastic decrease in the intensity was noted in the presence of the quenchers especially acrylamide. The fluorescence characteristics of Trp residues depend strongly on the microenvironment and thus provide a sensitive probe of the conformational state of the protein. The intrinsic fluorescence measurement in presence of acrylamide, KI and CsCl were measured and the quenching was calculated by Stern Volmer and modified Stern Volmer's plots. The percent Q value for KI was calculated to be 93.12 which were higher as compared to that of CsCl (52.45). Quenching by acrylamide was estimated to be 85% (Table 1).

Table 1: Tryptophan environment as detected by quenchers

	K_{SV}	**%Q**	**Fa**	**KQ**
KI (5M)	5.75	93.12	0.9312	6.452
CsCl (5M)	12.45	52.45	0.5245	0.57
Acrylamide (5M)	30.65	85.51	0.8551	6.785

DISCUSSION

Plant aspartic proteases have been in recent focus due to the critical steps especially in seed germination being under their control. Studies on the DNA and the enzymes have indicated the presence of two aspartic acid residues in the active site of the enzymes along with Threonine, Serine and Glycine in the form of the catalytic triad Asp-Thr-Gly on one lobe and Asp-Ser-Gly on the other lobe (Uhlig H, 1998). The crystal structures of two of the proteases have been reported confirming the presence of the catalytic triad (Kervinen J, et al, 1999; Frazão C, 1999).

Although the functioning of these enzymes in acidic conditions is well known, there have been no studies on the correlation of the structure of the enzymes with their functions. The LB plots and the subsequent secondary plot of log Vm/ Km against pH indicated two pK values in the acidic range for the ionizable groups. This correlates to the presence of the carboxylic groups of aspartic acid determined by the modification studies.

Chemical modification is a useful tool to understand the significance of the amino acids at the active site. Among the various modifiers used the following reagents showed effect on the enzyme. WRK, PMSF and PHMB were seen to reduce the activity of the enzyme substantially, while the others did not indicating the presence of carboxylate group of aspartic acid, serine and cysteine to be essential for the activity of the enzyme. The time and concentration dependent effect obtained with micromolar quantities of WRK indicated the presence of highly active carboxylic acid residues in the active site of the enzyme which were extremely sensitive to modification due to their exposure. Further to confirm their role in substrate binding

or catalysis simultaneous incubation of the enzyme with the substrate and modifier was performed. The complete substrate protection offered during the simultaneous incubation confirmed the role of the carboxylic groups in substrate cleavage. These results corroborate the crystal data. Similar experiments with PMSF, PHMB and DTNB which reduces the activity of the enzyme in micromolar amounts indicated serine to be involved in substrate binding while cysteine was found to be significant in maintaining an active conformation of the enzyme. The kinetics of the modified enzyme in the presence of PMSF and WRK indicated that the modifiers reduced the affinity of the enzyme towards the substrate hemoglobin. This was reflected in the increased Km and constant Vm values obtained with WRK and PMSF.

In addition to these amino acid residues the environment of tryptophan was studied as a basis for all subsequent fluorimetric work. Fluorescence quenching studies have been widely used for studying the degree of exposure and electronic environment of aromatic amino acid residues. Acrylamide is an efficient neutral quencher of tryptophan fluorescence and can distinguish between buried and exposed side chains (Lehrer, 1971) while KI and CsCI are charged, highly hydrated molecules and their quenching ability is limited to surface exposed Trp and also depends upon the neighbouring charged groups (Lehrer, 1971; Eftink and Ghiron, 1981). The quenching of VrAP with increased concentration of acrylamide resulted in a Stern-Volmer plot with an upward curvature, whereas those for CsCI and KI were linear. The $K_{(Q)}$ and fa values were extrapolated from a replot of quenching data according to modified Stern- Volmer equation (Fig. 15). The Ksv value for acrylamide was much higher than that of KI and CsCI since it is a neutral molecule and can penetrate into the interior and is an efficient quencher. Acrylamide is a useful neutral quenching probe for topological studies with proteins. It can penetrate a protein matrix and its ability to collisionally quench the emitting groups depends only on the

ease with which it is able to diffuse to meet the excited groups (Eftink & Ghiron, 1976). KI and CsCI have been suggested as companion probes since these bear opposite charges and their relative quenching efficiencies should be related not only to the accessibility of the protein fluorophore but also to the net charge in the vicinity of the fluorophore. In the presence of acrylamide, VrAP showed a slightly curving Stern- Volmer plot, indicating that the tryptophans are partially exposed as a heterogenous population (Eftink & Ghiron, 1976). This indicates the presence of both static and collisional populations of this amino acid. A linear Stern-Volmer plot with KI and CsCI indicates that no static quenching was observed, as is usually the case. The larger steric value obtained with KI- ion compared to Cs+ as quencher suggests that the tryptophans are located in a positively charged environment.

From the results obtained we can conclude that the two aspartates are critical for the functioning of the enzyme whereas serine in involved more so in substrate binding. Also the presence of an essential cysteine was detected which could be responsible for the maintenance of the active conformation of the enzyme. The quenching studies indicate that tryptophan can be used as an effective probe for further fluorimetric analysis for the elucidation of structural details.

Structure-function studies

Protein folding is characterized by the presence of kinetic intermediates, which can accumulate in the folding process. It has become increasingly evident that a polypeptide chain can adopt conformations different from the functional, native conformation of the protein. In order to understand the principles governing protein folding it is important to study these partly folded conformations (Baldwin, 1991, Dill and Shortle, 1991; Dobson, 1992). The development of wide range of sensitive techniques has led to the identification and characterization of stable intermediates in several proteins (Kuwajima, 1989). The classic examples of such conformations are the molten globule states adopted by several proteins at extremes of pH (typically acid pH) and ionic strength (Goto et al., 1990). It has been widely accepted that the molten globule state is general intermediate populated during the early stage of protein folding (Kuwajima, 1989; Matthews, 1993; Baldwin, 1995). Molten globule is as compact as the native conformation with pronounced secondary structure but has no rigid tertiary structure with exposed hydrophobic surface that binds a hydrophobic dye or induces extensive aggregation (Ptitsyn, 1987). Though many proteins in their molten globule and partially folded states have been characterized, it has not yet been possible to generalize the concept of the molten globule as the universal equilibrium protein folding intermediate. Interest in the molten globules has intensified because the properties of the molten globule support the argument that it may be one of the first conformations embraced by the polypeptide chain in folding from the unfolded state (Kim and Baldwin, 1990). In this work, we have shown the presence of a partially structured intermediate at high pH that binds to the hydrophobic dye 1-anilinonaphthalene-8-sulfonic acid and resembles a 'molten globule'-like state in the equilibrium-unfolding pathway of plant aspartic protease, VrAP.

MATERIALS AND METHODS

Glycine, acetic acid, and sodium acetate were from Sisco Research Laboratories Pvt. Ltd. Either glass double-distilled or Milli-Q water from Millipore set-up was used to make the buffers. The pH of the buffer solutions was adjusted on a Control Dynamics pH meter model APX 175 by adding hydrochloric acid or sodium hydroxide solutions.

METHODS

Enzyme unfolding

In the chemical unfolding experiments, the enzyme was incubated with various concentrations of Gdn HCl in Gly-HCl (0.05 M, pH 3.0) at 30ºC for 4 hours. The process of enzyme unfolding was monitored by assaying the initial rate of the residual enzyme activity, the fluorescence spectrum, and the circular dichroism (CD) change. In the enzyme activity assays, control experiments were performed simultaneously with the same amount of denaturant added into the control tube where the enzyme was not preincubated with the denaturant; eg., if the enzyme was denatured in 5M Gdn HCL then the enzyme activity was assayed in the presence of 5M Gdn HCl. Residual enzyme activity (E_t/E_0) was plotted versus denaturant concentration in which E_0 and E_t are the enzyme activity in the control and experimental tubes respectively.

For monitoring the refolding process, the enzyme denatured with 5M Gdn HCl was diluted with Gly-HCl buffer (0.05M, pH 3.0) to lower Gdn HCl concentrations and the fluorescence spectrum taken.

Analysis of the unfolding curves of the unfolding process followed the procedure of Pace (1986) assuming a two-state unfolding mechanism.

Spectrofluorimetric analysis

Fluorescence spectra of the enzyme were monitored with a Perkin Elmer LS 50B spectrofluorimeter.at 30ºC and all the spectra were corrected for buffer absorption. The Raman spectrum of water was also corrected. The excitation wavelength was set at 280 nm and the fluorescence spectra were scanned from 300-400 nm. The maximal peak of the fluorescence spectrum and the change of fluorescence intensity at 360nm were used in monitoring the unfolding processes of the enzyme. Slit widths of 5 and 5 nm were used on the excitation and emission monochromators, respectively.

Spectropolarimetric analysis

The secondary structure of the enzyme was analysed by monitoring the CD spectrum of the protein at 25ºC in a Jasco J-700 spectropolarimeter in the near and far UV ranges under constant N_2 flush. In general, an average of 6 scans was used, and the data was presented as molar ellipticity. All the spectra were obtained at an interval of 1 nm with a scanning speed of 200nm/min at 30 °C. Nitrogen gas was continually flushed at the rate of 5 liters min_1 through the instrument. 0.1 and 1 cm path length cuvettes were used in the far and near-UV regions, respectively. The protein concentration for the far-UV and the near-UV spectra acquisition was 300 µg/ml and 1.0 mg/ml, respectively.

ANS Binding measurement

ANS fluorescence studies were carried out to characterize the pH-induced transitions. Fluorescence measurements were carried out on a Perkin Elmer LS 50B

spectrofluorimeter. 1-cm path length cuvettes were used to record the spectra of the samples. A protein concentration of 20µg/ml for the intrinsic fluorescence and 20µg/ml for the ANS fluorescence was used. For intrinsic fluorescence, an excitation wavelength of 280 nm was used, with an excitation slit width of 7 nm and an emission slit width of 7 nm. The emission was recorded from 300 to 400 nm. For ANS fluorescence, an excitation wavelength of 375 nm was used, and the emission was measured from 500 to 600 nm with slit widths of 5 nm for excitation and 5 nm for emission. All the spectra and the transition curves were recorded at 30°C. Following this, ANS concentration of 50 µM was used for the emission scans as a function of pH.

Structural analysis

The structural analysis after Gdn HCl unfolding was carried out by using the K2D programme. The secondary structure of the native enzyme was estimated by CONTINL, SELCON and CDSSTR methods with an expanded reference set (Sreerama and Woody, 2000).

RESULTS

Secondary structure analysis

The secondary structure of VrAP was analyzed by monitoring the changes in circular dichroism spectra of the enzyme from 190-250nm. In this far-UV region, the CD spectra of the native VrAP show characteristic double minima at 208 and 222 nm (Figure 11). The enzyme was found to belong to an α/β class of proteins and the secondary structure content determined from the K2D analysis indicated it to have 16% α helices and 32% β sheets. The detailed structural analysis using CDPRO software is provided in Table 1 which indicates pronounced β structures than α helices. The calculated α helical and β sheets contents as a function of pH using the K2D program for analysis, are listed in Table 2.

Table 1: Secondary structure analysis by CDPRO

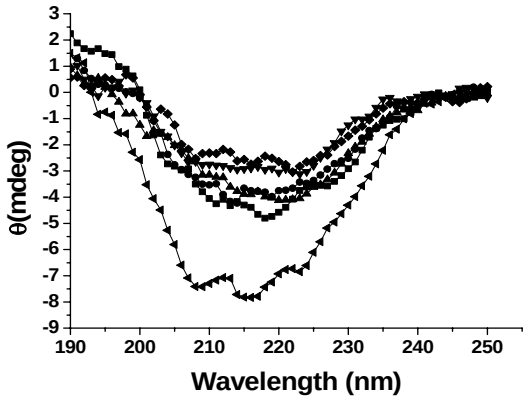
CDPRO Analysis	Value (%)
α helix regular	14
α helix distorted	11
β sheet regular	18
β sheet distorted	9
β turns	21
Unordered	28

Table 2: Alterations in secondary structure as determined by K2D

pH	α Helix content (%)	β sheet content (%)	Random Coil structure (%)
2	17	32	51
4	16	32	51
6	11	41	48
8	9	43	48
10	24	20	56

The native secondary structure is preserved up to pH 6.0 in the acidic conditions (Figure 11). However, at pH 8.0, the protein loses 44% of its helical nature. At pH 10.0, there is an increase in the helical nature from 16 to 24%. The β conformation reduces from 32 to 20% while the random unorganized structure accounts as much as 56%. In the alkali conditions, VrAP loses tertiary structure rapidly at pH 10.0 (Figure 11). The compact secondary structure and the collapsed tertiary structure are indicative of a molten globule state for the enzyme.

Figure 11: Secondary structure analysis of VrAP by circular dichroism

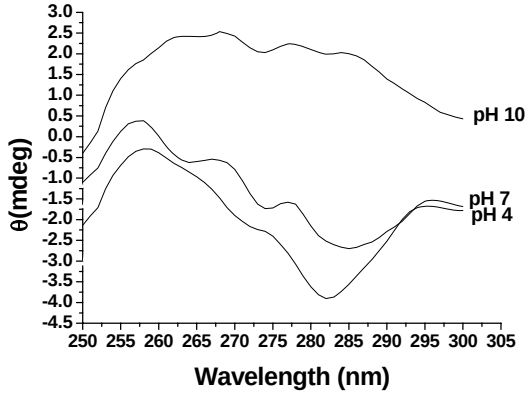


Far-UV CD spectra were recorded for native VrAP (■) and for VrAP at pH 2 (●), pH 4 (▲), pH 6 (▼), pH 8 (◆) and pH 10 (◀) from 260-190 nm at 25 °C. The spectrum represents the average of six scans.

Tertiary structure analysis

The near UV circular dichroism spectrum of VrAP at different pH values indicated its tertiary structure spectrum of native VrAP at pH 10.0 revealed a single negative ellipticity peak at 270nm while most of the structure is unfolded with no prominent peaks (Figure 12). Under neutral conditions at pH 7.0 the negative ellipticity peaks at 263, 273, 286 and 291nm and positive ellipticity peaks at 256, 268, 276 and 295nm were observed for VrAP. The peaks at 276, 289 and 295 become more enhanced than in the native structure suggesting that more tryptophan and phenylalanine residues are exposed in the neutral range than the acidic range. Under alkaline conditions (Figure 12), the ellipticity values at these wavelengths respond differently to changes in the pH. The ordered tertiary structure of the native enzyme is significantly altered at pH 10.0 suggesting that maximum tryptophan and phenylalanine residues are rearranged in the alkaline range than the acidic range.

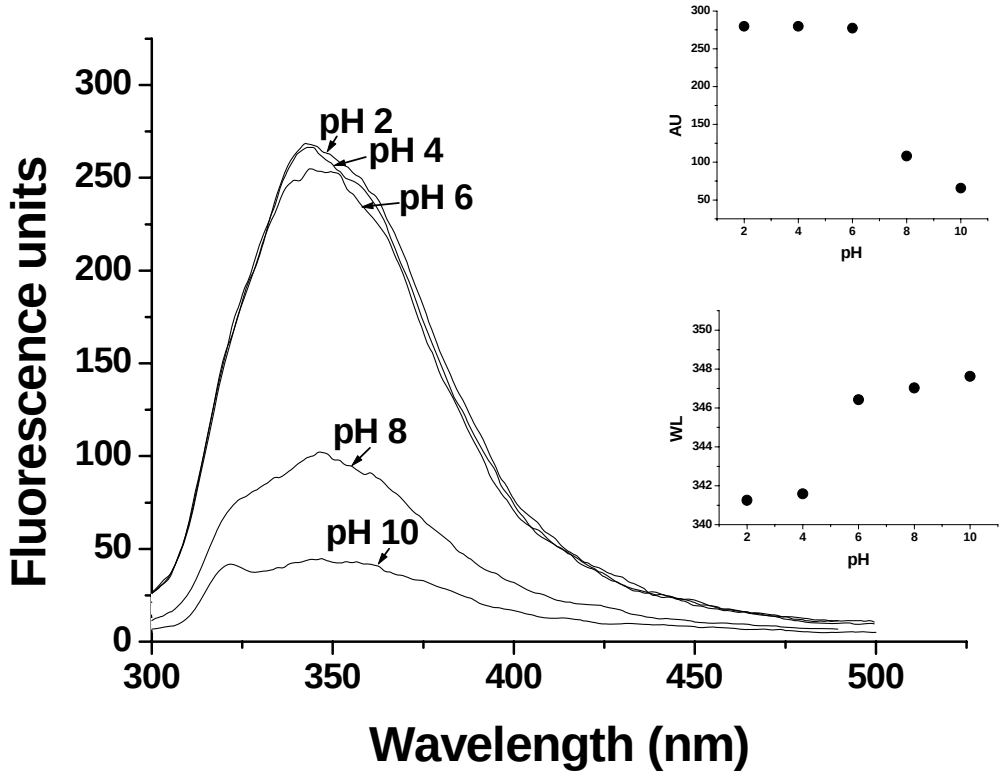
Figure 12: Tertiary structure analysis of VrAP by circular dichroism



Far-UV CD spectra were recorded for VrAP at pH 4, 7 and 10 from 250-300 nm at 25 °C. The spectrum represents the average of six scans.

The fluorescence emission was measured upon excitation at 280 nm, in which case the emission arises both from Tyr and Trp residues as well as due to the result of energy transfer from Tyr to Trp residues is accounted for. Thus, the excitation wavelength of 280 nm links the Tyr probes distributed throughout the protein with fluorescence emission. Emission spectra of the native state indicate maxima at 341 nm, suggesting that the tryptophan residues although buried in a non polar environment are partially exposed to the solvent. The emission maximum of the protein at the extreme acidic pH is red-shifted to 343 nm, and that of the alkali-denatured protein is shifted rapidly to 348 nm (Figure 13), indicating that the solvent exposure of tryptophans in the acid and the alkali-denatured protein takes place to varying extents. These data are in accordance with the near-UV CD data, which show loss of tertiary structure for the alkali denatured protein as compared with the acid denatured protein.

Figure 13: Tertiary structure analysis of VrAP by fluorescence spectroscopy



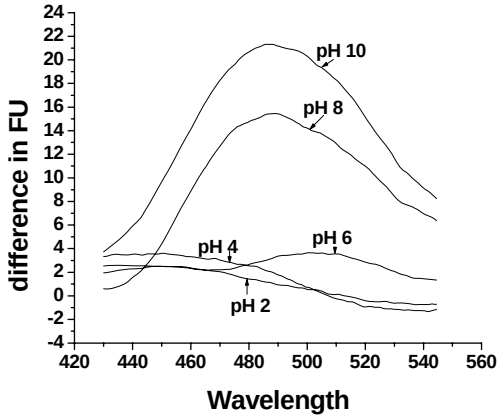
VrAP sample (20µg/ml) was incubated at pH of 2, 4, 6, 8, 10 for 4 hours at 25 °C. The fluorescence of pH modified VrAP was monitored at λex 280. The inset above gives the decrease in the fluorescence intensity as a function of pH while the inset below provides an insight into the shift in peak maxima as a function of time.

The fluorescence intensities are seen to decrease from 270 to 50 AU (almost 5 times) with subsequent increase in pH (Figure 13 inset above). A subsequent shift in optima from 341 for native enzyme to 349 for the enzyme under extreme alkaline conditions is seen (Figure 13 inset below). This can be correlated to the gradual loss in the tertiary structure.

ANS Fluorescence

ANS has been shown to bind to hydrophobic regions of partially unfolded proteins that become exposed to solvent. The results of ANS binding to VrAP are shown in Figure 14. Treatment of the protein with ANS as a function of pH ranging from 2.0 to 10.0 suggested enhanced fluorescence (almost 5 times) in the alkaline region and no binding in the acidic range. ANS binding in the alkaline region led to an increase in the emission intensity with a concomitant blue shift in the emission maximum from 521nm to 491nm, more so at pH 10 indicating rapid exposure of hydrophobic patches (Figure 14). These studies support the near-UV CD absorption data at pH 10.0 and show loss of tertiary structure without any loss in secondary structure. The reduced binding of ANS to the protein at pH 8.0 suggests partial loss of tertiary structure of the enzyme wherein the secondary structure is retained as evidenced by far-UV CD and by wavelength of emission maximum at pH 8.0.

Figure 14: ANS Fluorescence measurements



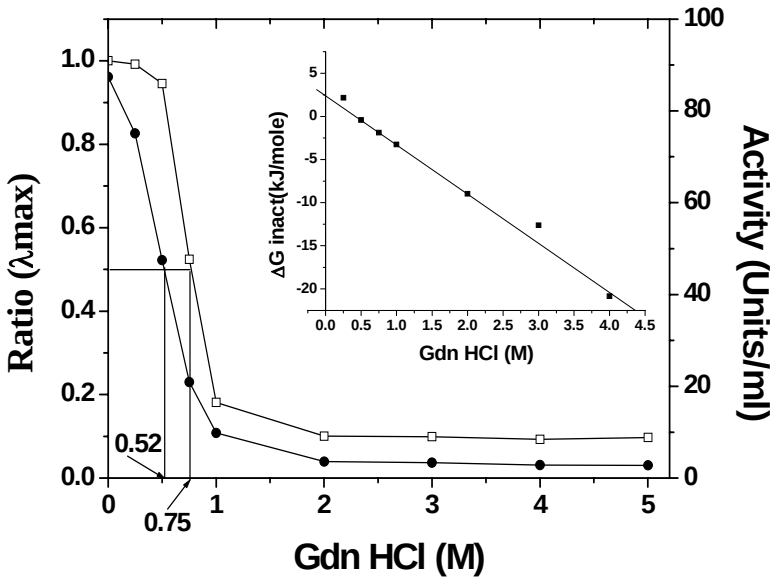
VrAP (20μg/ml) was incubated at pH 2, 4, 6, 8 and 10 for 4 hours at 25°C. The binding of ANS was analysed by exciting the modified protein at 375nm and monitoring the fluorescence from 400-600nm. Each reading was an average of ten scans. The difference in fluorescence intensity was calculated based on the peak intensities.

The readjustment of pH indicated a reversal in ANS binding and consequent regain of the tertiary structure. The tertiary structural changes induced by pH were found to be reversible.

Denaturant induced changes

The activity of VrAP declined rapidly at low concentrations of guanidine hydrochloride. A complete loss of activity was noted after 0.75M of Gdn HCl. The loss in activity could be correlated to the structural changes observed in the fluorescence and CD analysis. The energy changes associated with the loss of activity were calculated which indicated that only 2.5 kJ / mole energy is required to inactivate the enzyme (Figure 15). This proves the delicate nature of the enzyme in its active conformation. Thus the D ½ required for inactivation is 2.5 kJ/ mole which is much less than 4kJ/ mole required for unfolding.

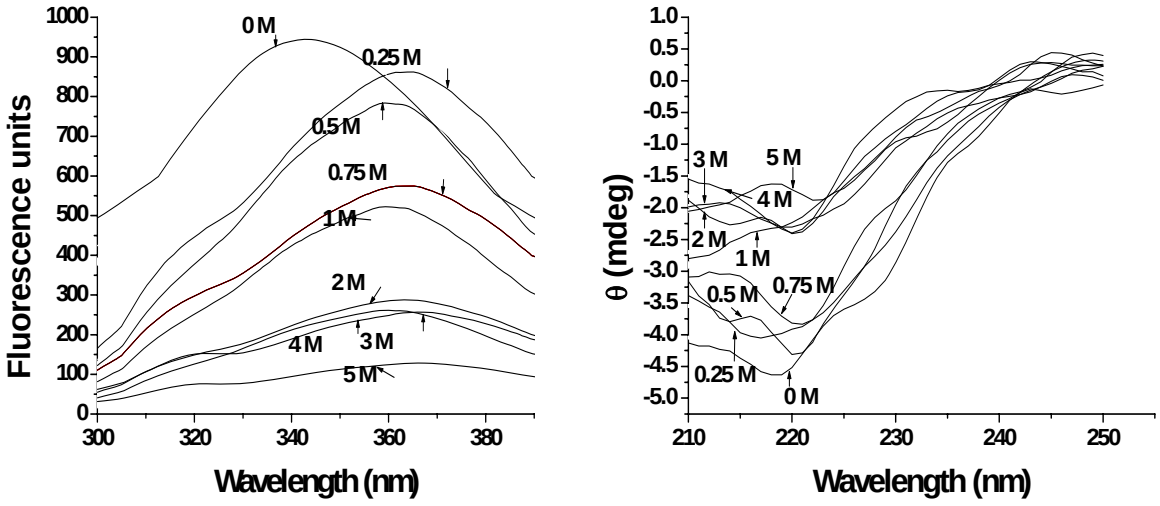
Figure 15: Effect of guanidine hydrochloride on enzyme activity and structure.



The changes in the secondary and tertiary structure were monitored in the presence of guanidine hydrochloride. There was substantial loss of secondary structure and folded conformation above 0.75M as indicated in the CD and fluorescence studies. The ratio of the peak intensities at different concentrations of Gdn HCl amd the activity profile demonstrated the effect of Gdn HCl on these. The inset indicates the free energy changes associated with the denaturation of the enzyme.

VrAP lost structure above 0.75M Gdn HCL with a corresponding loss in activity. There was a concomitant shift in the maxima from 341 to 360nm from 0-1M Gdn HCl which was maintained at all higher molarities of the denaturant (Figure 16).

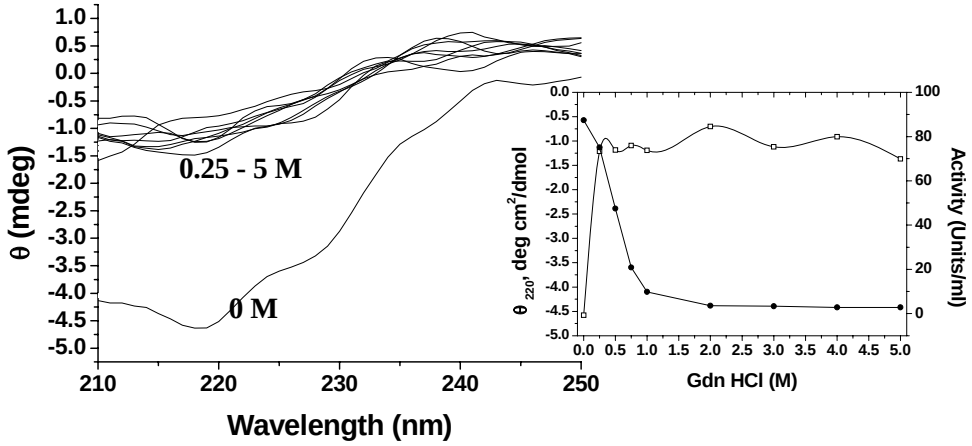
Figure 16: Effect of Gdn HCl on the structure of VrAP



VrAP was exposed to different concentrations of the denaturant from 0.25-5M. The immediate shift in the fluorescence peak indicated the drastic effect on the tertiary structure of the enzyme and demonstrating a two state model N to U. This pattern is seen even in the secondary structure data from circular dichroism studies.

The renaturation studies with guanidine hydrochloride indicated that on retrieval of the denaturant partial renaturation of the enzyme was possible (Figure 17). There was partial recovery of the tertiary structure while the secondary structure did not recover (Figure 17). This explains the loss of activity which is not recovered on withdrawal of Gdn HCl.

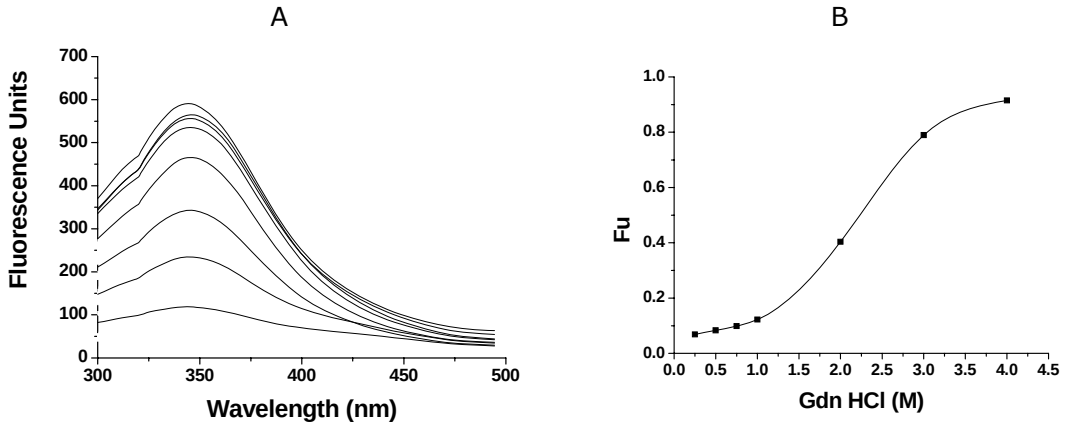
Figure 17: Effect of guanidine hydrochloride on renaturation of VrAP



The effect of guanidine hydrochloride on the renaturation of VrAP was studied by diluting the denaturant 10 folds. There was no recovery of the secondary structure of the enzyme indicating the permanent nature of the loss of structure. The activity also could not be regained as visualized in the inset of the figure.

The fluorescence studies indicated that the fraction unfolded increased drastically after 1M of the denaturant where there was complete loss of activity and structure (Figure 18).

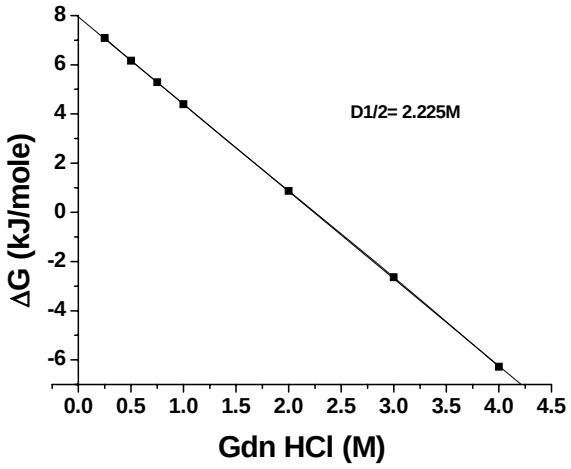
Figure 18: Effect of Gdn HCl on the tertiary structure of VrAP



The renaturation of VrAP was studied after diluting out the denaturant Gdn HCl 10 fold times. There was partial recovery of the tertiary structure of the enzyme as indicated in A. Although the peak maxima was recovered the intensity of the enzyme had reduced drastically suggesting the partial recovery of the structure of VrAP. B indicates the fraction unfolded as the concentration fo Gdn HCl increases. It is clear that from 1M onwards the enzyme unfolds exponentially with concomitant loss in activity.

The free energy changes associated with the loss of structure were calculated by the Gibbs free energy equation, $\Delta G = -RT \ln k$ ($k=Fu/[1-Fu]$), wherein ΔG is the free energy in kJ / mole, R is the Arrhenius constant, T is temperature in °kelvin, Fu is the fraction unfolded. The energy associated with the unfolded structure was 8 kJ / mole. The D ½ value i.e. the amount of denaturant required to half denature the enzyme was found to be 2.225M (Figure 19).

Figure 19: Free energy changes in VrAP during folding-unfolding



The delta G associated with VrAP at different concentrations of Gdn HCl were plotted against the concentration of the denaturant to obtain the D1/2 value of 2.225M.

DISCUSSION

The detailed mechanism that directs the primary structure of a polypeptide chain to fold into the correct biologically active enzyme has been a subject of endless speculation and discussion (Aurora et al., 1997; Beasley and Hecht, 1997; Brockwell et al, 2000; Dill, 1997, 1999; Federov and Baldwin, 1997; Ruddon and Bedows, 1997; Tsai et al, 1999). Different models proposing the mechanism of protein folding have been outlined for small as well as the larger complex proteins. Protein folding is studied by inducing denaturant dependent conformational changes or environmental based changes such as different pH or temperatures. During protein folding the formation of a molten globule state as an intermediate is observed. The molten globule (MG) state is classically defined as a compact and largely mobile molecular state containing native like secondary structure and hydrodynamic radius but without rigid tertiary structure. It is a thermodynamically distinct state assuming significance in the protein folding mechanism due to its occurrence as a late folding intermediate. The MG state is important for protein function in the living cell especially in protein-protein interactions (Kumar R, et al, 2006). The impact of pH in the formation of the MG like states has been demonstrated for numerous enzymes wherein such states have occurred under acidic conditions of pH. The study of the MG state has therefore always been an area of significant research interest. Although there are numerous examples of acid induced molten globules, the alkali induced molten globule state has been a matter of discussion for few enzymes.

The overall structural dynamics of the enzyme imposing functional constraints were scrutinized by pH and denaturant induced changes. Thus using various techniques like CD and fluorescence analysis in addition to activity measurements helped to correlate structural and functional data. Also it is known that the classical triad of all

aspartic proteases contains two aspartates in the native conformation of the enzyme. The circular dichroism analysis indicates a secondary structure with more of β sheets than α helices in the native conformation. The secondary structure however changes into a conformation wherein the α helices increase and the β sheets decrease such that at the extreme alkaline pH of 10 the structure contains more of the previous and less of the later. The randomness of the structure is also enhanced. At the extreme pH of 10.0 the enzyme falls rapidly into a highly compact structure which has lost its activity. The far UV CD spectra thus indicated clearly the compactness of the structure. The near UV CD showed that the tertiary structure was significantly changed.

The fluorescence studies indicate that at pH 10.0 there is a complete loss of the tertiary structure and a compact secondary structure indicating a molten globule like state of the enzyme. The red shift in fluorescence analysis indicates the exposure of tryptophans to the solvents. This correlates well with the quenching wherein more of tryptophans are on the surface of the enzyme. Monitoring the pH dependent changes in structure signified hydrophilic moieties to be more exposed for the functionality of the enzyme. In addition to the alkali-induced conformational changes responsible for the effect of ANS, repulsive electrostatic interactions at acidic pH between ANS and the exposed protein surface could also affect ANS from having complete binding effect. At pH 10.0 there is substantial ANS binding observed, indicating an exposure of all the hydrophobic residues. The strong signals suggest altered tertiary structure for the enzyme with retained compact secondary structure. The fluorescence results corroborate well with the CD spectra observed. The compact secondary structure, disorganized tertiary structure and the exposure of hydrophobic patches together are indicative of the formation of molten globule like structure of VrAP at pH 10.0.

The finding of an alkali induced molten globule is in contrast with most of the enzymes wherein acid induced molten globule state is more predominant (). However in most of the cases the enzymes are active at neutral or alkaline pH whereas they are inactive in the acidic conditions. However aspartic proteases being active at acidic conditions are postulated to fit into the criteria of alkali induced molten globule state. The only other enzymes known to exist in such states are ferrocytochrome c reductase, (Kumar R, et al, 2005) ferricytochrome c reductase (Kumar R, et al 2006) and Bar Star Viral protein. There are no reports of molten globule states for aspartic proteases especially plant aspartic proteases with structural studies being of recent origin. This is the first report on alkali induced molten globule state for a plant aspartic protease.

The use of denaturants is an elegant way of understanding the dynamics of folding and unfolding of the enzyme due to changes in environment. Chemical denaturation is one of the most extensively used methods for the quantitative characterization of protein stability because this technique provides access to conformational states that are generally undetectable under native solution conditions. There have been several models proposed for the mechanism of protein folding. These studies shed more light on the relationship between structural and functional aspects of the enzyme. Inactivation of the enzyme at lower concentrations of Gdn HCl is indicative of the presence of acidic amino acids at the active site. This again correlates with the presence of two aspartates essential for activity. The calculation of free energy changes suggest that the native protease is highly fragile and the associated energy is very less whereas the unfolded enzyme has more energy associated with it. The results also indicate that there is partial reversion of the tertiary structure whereas the changes in the secondary structure are irreversible. These conformational

changes are responsible for the irreversible loss in enzyme activity by guanidine hydrochloride.

CHAPTER IV

PURIFICATION AND BIOCHEMICAL STUDIES OF ASPARTIC PROTEASE INHIBITOR FROM *VIGNA RADIATA*

"Every great advance in science has issued from a new audacity of the imagination."

John Dewey

ABSTRACT

Protease inhibitors contribute significantly to the regulatory framework of the cell by way of controlling the activities of proteases. Seeds are by far the richest source of PIs and it is probable that all seeds contain one or more inhibitors. There are a multitude of reports on the occurrence of serine, cysteine and metalloprotease inhibitors in seeds whereas there is a distinct lacuna of literature on aspartic protease inhibitors from plants. The different varieties of *Vigna radiata* were tested for the presence of the inhibitor. The expression of the inhibitor upto 18 hours of germination and in the mature plant was checked. The time-based expression was analysed and it was found that the dormant seeds contained maximum amount of the inhibitor. Accordingly, a peptidic inhibitor of VrAP, viz, VrAPI was purified from the dormant seeds of *Vigna radiata*. The inhibitor was purified by acid precipitation, molecular weight cutoff membrane filtration, gel filtration chromatography and HPLC (High pressure liquid chromatography). The peptidic inhibitor was found to have molecular weight of 820 Daltons. This chapter deals with the purification and the biochemical characterization of VrAPI. The inhibitor was found to be pH and thermostable. The optimum pH for activity was 3.0 while the optimum temperature was 37°C. This chapter also deals with the kinetic analysis of the interactions between the inhibitor and fungal protease from *Aspergillus saitoi*. The inhibitor constants were analysed and were found to conform to the competitive reversible slow tight binding model of inhibition. The interactions of the inhibitor with the endogenous protease revealed the competitive reversible nature of inhibition. The kinetic constants calculated indicate a slow tight binding inhibition of the inhibitor with the endogenous protease, signifying its physiological role.

INTRODUCTION

Protease Inhibitors

Protease inhibitors contribute significantly to the regulatory framework of the cell by way of controlling the activities of the enzymes of digestion i.e. proteases. Proteases are responsible either directly or indirectly for all systemic functions and therefore require effective counterchecks for their activity. The ubiquitous occurrence of protease inhibitors and extensive research on them has brought out their multifaceted roles in prokaryotic as well as eukaryotic systems. Protease inhibitors are classified into two types:

i) Proteinaceous inhibitors

ii) Low Molecular Weight Inhibitors

Protease inhibitors in plants

Protease inhibitors (PIs) are of ubiquitous occurrence in all kingdoms. Reports on their physiological significance and applications as drugs have made them a topic of recent research focus. They have been characterized from many microbial and mammalian systems. Protease inhibitors have been found in plant species belonging to diverse taxa (Richardson M, 1977). These proteins are abundant in the seeds of monocot and dicot angiosperms and in gymnosperms and comprise up to 5-10% of the total content of water-soluble proteins (Mutlu A and Gal S, 1999). They form a major part of wound induced defense components of plants especially in leaf tissue. Almost all the PIs known till date belong to serine and cysteine classes with recent reports on aspartic and metalloproteases emerging in the past few decades. PIs were first isolated from seeds in the 1940s, with the characterization of Kunitz and Bowman-Birk type protease inhibitors (Kunitz M, 1945; Bowman, 1946) from soybean and of an α-amylase inhibitor from cereal grains (Kneen and Sandstedt,

1943). Seeds are by far the richest source of PIs and it is probable that all seeds contain one or more inhibitors, although the levels vary greatly and their detection is dependent on the selection of the appropriate target enzymes and assay conditions. The vast majority of the inhibitors characterized to date are active against endoproteases, with a smaller number of amylase inhibitors, some of which are bifunctional (i.e. inhibit both amylases and proteases. There are a multitude of reports on the occurrence of serine, cysteine and metalloprotease inhibitors in seeds whereas there is a distinct lacuna of literature on aspartic protease inhibitors from plants.

Physiological role of PIs

Protein protease inhibitors amount to a considerable part of seed protein: in legumes, this amount is about 6% (Weder, 1981), whereas, in cereal crops, it is as high as 10% of the total water-soluble protein (Pusztai, 1972). Because of their high content in seeds, Pusztai [5] assumed that they play the role of storage proteins. By their amino acid composition, some of protease inhibitors constitute an important source of amino acids for developing plants.

PIs in legume seeds irreversibly inhibit the action of the animal digestive enzymes and hence they are considered antinutritional (Ryan, 1990; Ryan, 1981; Pearce, et al, 1982; Ryan, 1990; Jouanin, et al, 1998).

PIs regulate endogenous protease levels before and during seed germination for storage protein digestion and to control protein turnover (Hilder, et al, 1987; Baumgartner and Chrispeels, 1976).

The involvement of PIs in the protection of seed reserves from premature hydrolysis has been established (Collins and Sanders, 1976; McGurl, et al, 1995). The

concentration of inhibitors is reduced during germination, facilitating the hydrolysis of protein for utilization in the germination process.

PI gene expression has been detected in leaves of several species following wounding, suggesting their role in protecting plants from insect attack and microbial infection. After the identification of PI as a valuable trait suitable for developing insect-resistant transgenic plants, there was intense interest to identify the *PI* gene from different plant species. *PI* gene has been identified and cloned from a wide array of plant sources, including alfalfa, tomato, potato, maize, mustard, poplar, tobacco, rice, sweet potato, soybean, amaranthus, cowpea and barley (Ussuf, 2001).

During seed development, PIs accumulate relatively later, and rapidly increase in the desiccation phase, implying a role in protein stabilization.

Dehydration-related stresses such as drought, salinity and abscisic acid induce the expression of trypsin inhibitors (TIs) in developing seeds of moong bean and lettuce (Ussuf, 2001). Accumulation of TIs closely resembles that of late embryogenesis abundant protein, which has a specific function in stress dehydration.

A new role for PI in the modulation of apoptosis or programmed cell death has been identified in soybean. Cysteine protease plays an important role in the regulation of programmed cell death leading to hypersensitive (HR) reaction, following pathogen attack. It has been shown that the ectopic expression of cystatin inhibits the induced cysteine protease activity, which in turn blocks programmed cell death (Ussuf, 2001). It is suggested that in plants balancing between the cysteine protease and

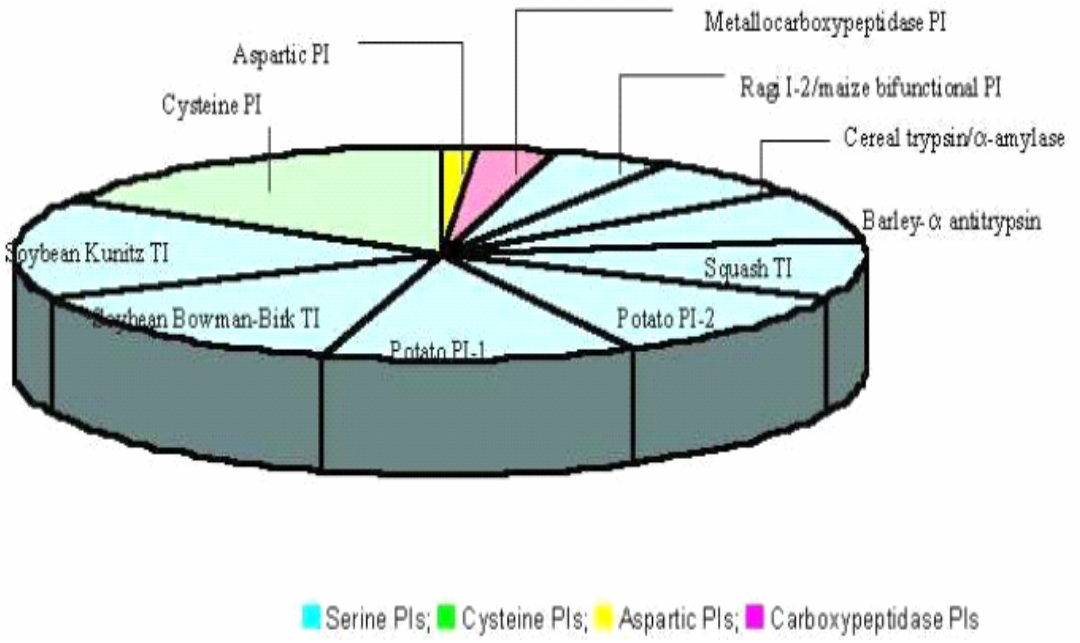
cysteine protease inhibitor activity regulates the programmed cell death. Thus a new role for PI in modulating the programmed cell death in plants has been identified.

Factors involved in expression of PIs

Plants react to wounding by activating a set of genes, most of them playing a role in wound healing and prevention of subsequent pathogen invasion. The gene activation takes place in both wounded and non-wounded plant parts. This systemic accumulation of defense-related products then serves a preventive function, checking the spread of the damaging agent to the healthy part of the plant. The tomato and potato PI families are the best-studied examples of genes, systemically expressed upon wounding. The detailed analysis of *PI* gene expression in different plant species has shown that it is regulated by a variety of developmental and environment factors.

Protease inhibitor families in plant tissues

The overall distribution of plant protease inhibitor families is provided in the figure below.



As observed majority of the inhibitors belong to the serine protease inhibitor families. Recent developments into the PIs belonging to the cysteine, aspartic and metalloproteases families have brought into focus their significance and roles in plants. The knowledge of aspartic protease inhibitors in plant protection is very scanty. There have been reports of aspartic proteases in insect guts, but conclusive evidence showing the role of the inhibitors in protection has not been reported. Pepstatin, a powerful and specific inhibitor strongly inhibited the gut enzymes of the Colorado potato beetle (Wolfson and Murdock, 1987). Potato tubers have shown the presence of a cathepsin D inhibitor that exhibits considerable amino acid homology with the soyabean trypsin inhibitor (Mares et al., 1989). In fact, many aspartic protease inhibitors from potato tubers have now been reported. The physiological significance of the inhibitors in addition to their role in defense is under investigation.

The current chapter reports the purification and biochemical characterization of an aspartic protease inhibitor from the seeds of *Vigna radiata* variety Kopergaon-1. The physiological role of the inhibitor, its kinetic interactions with the fungal protease from *Aspergillus saitoi* and the endogenous protease is described.

MATERIALS AND METHODS

Vigna radiata seeds were purchased from the local market. Fungal protease from *Aspergillus saitoi* and hemoglobin were purchased from Sigma Limited. All other reagents were of analytical grade.

Varietal screening for aspartic protease inhibitor

Seeds of different varieties of *Vigna radiata* were purchased from the local market. 10g seeds of each variety were blendorised in 50ml Gly HCl buffer (0.05M, pH 3.0). The mixture was centrifuged at 10000rpm for 20 minutes. The corresponding supernatants were analyzed for the aspartic protease inhibitory activity.

Inhibition of aspartic proteases

The proteolytic activity of the purified aspartic protease from *Aspergillus saitoi* (F-Prot) and of the endogenous aspartic protease VrAP was measured initially by assaying residual enzyme activity. The effect of VrAPI was analysed by checking the activity in the absence or presence of inhibitor. In a classical assay, F-Prot (50 µl; 1 mg/ml) or VrAP (25µl; 1 mg/ml) in glycine-HCl buffer (0.05 M; pH 3.0) was incubated with the inhibitor (25 µg/ml) for 5 min. The reaction was initiated by the addition of 0.5ml of hemoglobin (5 mg/ml) and was allowed to proceed for 30 min at 37°C. The enzymatic activity was quenched by the addition of 1 ml of perchloric acid (PCA) (1.7 M) followed by 30 min of incubation at 37°C. The precipitate formed was removed by filtration. The optical absorbance of the PCA-soluble products in the filtrate was read at 280 nm. One unit of aspartic protease activity is defined by an increase of 0.001 at 280 nm per min at pH 3.0 and 37°C, measured as PCA-soluble products, with hemoglobin as the substrate. One unit of the inhibitor is defined as

the amount of inhibitor required to inhibit one aspartic protease unit under standard assay conditions.

Purification of VrAPI

The seeds were blendorised in a mixer using sterile Milli Q water in the proportion 1:4 weight by volume. The blendorised seeds were incubated on a shaker at room temperature for 2 hours. The insoluble fraction was removed by centrifugation at 10000 rpm for 10 minutes. The supernatant was subjected to acid precipitation using 100% TCA (trichloroacetic acid) to a final concentration of 5%. The mixture was incubated at 4°C for 3 hours. The precipitated proteins were removed by centrifugation at 10000rpm for 15 minutes. The pH of the resulting supernatant was adjusted to neutral using sodium hydroxide. This supernatant was subjected to treatment with activated charcoal to remove the coloring impurities which adhered to the charcoal. The resulting filtrate was subjected to ultrafiltration using membrane cutoffs of 10000 and 3000 Daltons successively. The resulting filtrate of 3000 dalton cutoff was then desalted using Biogel P2 column. The active fractions were loaded onto a prepacked waters RP-HPLC C-18 Microbondapak column for purification. The peaks were collected and assayed for activity. Peak 1 was found to be highly active with 75% inhibitory activity towards fungal protease from *Aspergillus saitoi*.

Determination of optimum pH, temperature, and stability of purified aspartic protease inhibitor

Estimation of aspartic protease inhibitory activity at different pH (2–10) and temperature (28°–100°C) values was carried out under standard assay conditions to determine optimum pH and temperature for enzyme inhibitory activity. The pH stability of the inhibitor was measured by incubating 2mg of the inhibitor at 37°C in

buffer of desired pH for 1 h. The temperature stability was determined by incubating 5mg at different temperatures. The samples were removed at regular intervals and the residual activity was estimated under standard assay conditions.

Amino acid analysis

The amino acid analysis was done by hydrolyzing 5mg of inhibitor with 6N HCl for 24 hours. The hydrolyzed amino acids were derivatized with AccQ Fluor Reagent (6-amino quinolyl-Nhydroxysuccinimide carbamate) and run on a prepacked RP-HPLC 3.9×150mm column AccQ.Tag.

Protein measurement

Protein was measured by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Molecular Weight

The molecular weight of the inhibitor was estimated by Maldi ToF mass spectroscopy.

Kinetics of inhibition of VrAP, an endogenous aspartic protease and F Prot, a fungal aspartic protease by VrAPI

For initial kinetic analysis of F Prot and VrAP, the kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant *K*i was determined as described by Dixon (*29*) and by the Lineweaver- Burk equation, and the *K*m values were calculated from the double reciprocal plots. In Dixon's method, proteolytic activity of the enzymes was measured at two different concentrations of substrate as a function of inhibitor concentration. The kinetic constants were determined by incubating the enzymes in the absence and presence of VRAPI with increasing concentrations of the substrate.

The inhibition of the initial rate of enzyme activity was analyzed by the double reciprocal plot. One unit of protease activity is defined by an increase of 0.001 at 280 nm/min at pH 3.0 at 37 °C measured as PCA-soluble products using hemoglobin as the substrate.

For the progress curve analysis, assays were carried out with 2 mL solutions containing the enzyme, substrate, and inhibitor at various concentrations. Five to six assays were performed in each slow-binding inhibition experiment: one without inhibitor and others with different inhibitor concentrations. At different time intervals, aliquots were removed, and the residual proteolytic activity was measured. Further details of the experiments are given in the respective figure legends. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicate, and the average value was considered throughout this work.

Evaluation of Kinetic Parameters

Initial rate studies that resulted in reversible, competitive inhibition were analyzed according to the equation

$$v = \frac{V_{max} S}{K_m(1 + I/K_i) + S}$$

where *K*m is the Michaelis constant, *V*max is the maximal catalytic rate at saturating substrate concentration [S], *K*i is the dissociation constant for the enzyme-inhibitor complex, and *I* is the inhibitor concentration (Cleland, 1979).

The progress curves for the interactions between VRAPI and aspartic protease as per Scheme 1b were analyzed using the equation (Beith, 1995; Morrison and Stone 1985).

$$[P] = v_s t + \frac{v_0 - v_s}{k}(1 - e^{-kt})$$

where [P] is the product concentration at any time *t*, V0 and Vs are the initial and final steady-state rates, and *k* is the apparent first-order rate constant for the establishment of the final steady-state equilibrium.

For Scheme 1b, the relationship between *k*, the rate, and the kinetic constants is given by the equation

$$k = k_6 + k_5\left[\frac{I/K_i}{1 + S/K_m + I/K_i}\right]$$

The progress curves were fitted to eqs 2 and 3 using nonlinear least-squares parameter estimation to determine the best fit values.

The overall inhibition constant for Scheme 1b is defined as

$$K_i^* = \frac{[E][I]}{[EI] + [EI^*]} = K_i\left[\frac{k_6}{k_5 + k_6}\right]$$

where *K*i) *k*4/*k*3.

For the time-dependent inhibition, there exists a time range in the progress curves in which formation of EI* is small (e.g., see Figure 3). Within this time range, it is possible to directly measure the effect of the inhibitor on V_0, i.e., to measure *K*i directly. Values for *K*i were obtained from Dixon analysis at a constant substrate concentration (eq 5).

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}(1 + I/K_i)$$

The rate of enzyme-inhibitor dissociation, *k*6, was measured directly for the time-dependent inhibition. Small volumes of concentrated enzyme and inhibitor were incubated to reach equilibrium, followed by large dilutions in assay mixtures containing near-saturating substrate. The rate of enzymatic activity regain was measured by the rate of product formation.

Fluorescence Analysis

Fluorescence measurements were performed on a Perkin-Elmer LS50 luminescence spectrometer connected to a Julabo F20 water bath. Protein fluorescence was excited at 295 nm, and the emission was recorded from 300 to 500 nm at 25 °C. The slit widths on both the excitation and emission were set at 5 nm, and the spectra were obtained at 500 nm/min. Fluorescence data were corrected by running control samples of buffer and smoothened.

For inhibitor binding studies, VrAP and F-prot were dissolved in the respective buffer systems. Titration of the enzyme with VRAPI was performed by the addition of different concentrations of the inhibitor to the enzyme solution. For each inhibitor concentration on the titration curve a new enzyme solution was used. All of the data on the titration curve were corrected for dilutions. The magnitude of the rapid fluorescence decrease (F_0 - F) occurring at each VRAPI concentration was computer fitted to the equation (F_0 - F)= ΔFmax/{1 + (Ki/[I])} to determine the calculated value of Ki and ΔFmax. The first-order rate constants for the slow loss of fluorescence, *k*obs, at each inhibitor concentration [I] were computer fitted to the equation *k*obs) *k*5[I]/{Ki + [I]} for the determination of *k*5 under the assumption that, for a tight-binding inhibitor, *k*6 can be considered negligible at the onset of the slow loss of fluorescence.

Time courses of the protein fluorescence following the addition of inhibitor were measured for up to 10 min with excitation and emission wavelengths fixed at 295 and 340 nm, respectively. Data points were collected at 0.5 s intervals during time courses. Corrections for the inner filter effect were performed as described by the formula (Lakowicz, 1983)

$$F_c = F \,\text{antilog}[(A_{ex} + A_{em})/2]$$

where *F*c and *F* are the corrected and measured fluorescence intensities, respectively, and *A*ex and *A*em are the solution absorbances at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering.

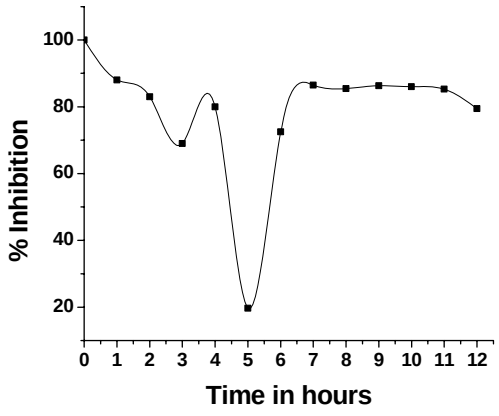
RESULTS

The inhibition of protease activity was analyzed in dormant seeds of *Vigna radiata* varieties Kopergaon-1, Tata, Vaibhav and Yellow mung bean. As seen in Table 1 the activity of aspartic protease inhibitor was found to be primarily present in maximum amounts in the dormant seeds of Kopergaon-1 variety. Therefore further efforts on purification and characterization of the inhibitor were performed using this variety. The highest activity was recorded in the extract of the seeds which were hard and not imbibed in water at all. The expression of the aspartic protease inhibitor, VrAPI from the total protein extract of hard seeds is depicted in Figure 1.

Table 1: Activities of aspartic protease from various varieties of mung bean

Variety	Enzyme Inhibitory Units per g of seed
Kopergaon	83
Tata	70
Vaibhav	56
Yellow	34

Figure 1: Time bound expression of the inhibitor



The time bound expression of the inhibitor indicated that maximum expression was in the hard seeds. The activity fluctuated till 12 hours of germination which could be related to the levels of the endogenous protease.

Figure 1 shows the time dependent expression of the aspartic protease inhibitor, VrAPI in the hard seeds of *Vigna radiata*. Maximum expression was observed at the dormancy stage which declined in the initial stages of germination. The expression increased later from 5 hours onwards and the level was maintained at 85% expression. A marked absence of the inhibitor in the mature plant tissues was noted.

Purification of VrAPI

The purification of VrAPI was done using rp-HPLC and the single peak was analyzed for the molecular weight using Maldi ToF. VrAPI was purified to 250 folds by the protocol followed as indicated in Table 2.

Table 2: Purification chart of VrAPI

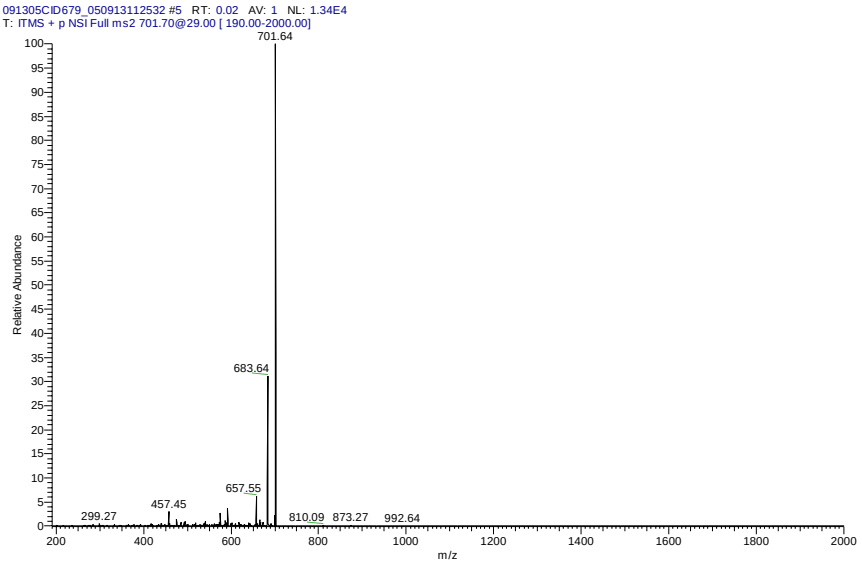
Purification Step	Volume	Total Protein	Total Activity	Specific Activity	Fold Purification
Water Extract	1000	940	9267	9.858	1
Acid precipitation	900	576	6300	10.937	1.109
Charcoal Treatment	800	315	4097	13.00	1.318
Ultrafiltration 10 KDa cutoff	775	248	3784	15.26	1.548
Ultrafiltration 3 KDa cutoff	750	173	3511	20.29	2.058

Biogel P2	50	3.4	2746	807.64	81.93
RP-HPLC	10	1	2516	2516	255.22

Molecular weight of VrAPI

The molecular weight of VrAPI was estimated to be 710 Daltons as deduced by MALDI ToF (Figure 2).

Figure 2: MALDI ToF spectroscopy of VrAPI



The figure shows the profile of the MALDI ToF spectrum of VrAPI. The molecular weight of the inhibitor was estimated to be 710 Daltons.

Biochemical properties of VrAPI

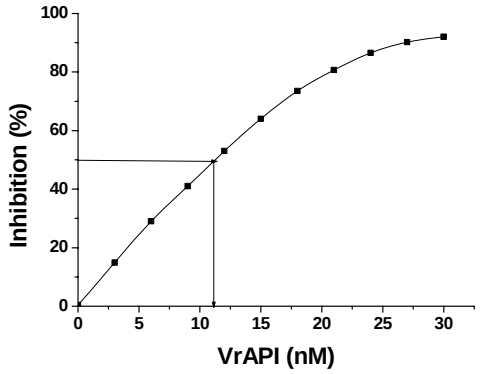
The inhibitor was found to be stable in a broad range of pH from 2-10. The optimum was found to be 3.0. The half life of VrAPI at 100°C was 30 minutes whereas the maximum activity was observed at 37°C. The amino acid analysis indicated the presence of the following eight amino acids:

Aspartic acid, Glutamine, Glycine, Alanine, Valine, Lysine, Isoleucine, Leucine.

Determination of IC_{50} of VrAPI

The initial kinetic analysis of VrAPI against the endogenous protease showed an IC_{50} value of 11nM (Figure 1).

Figure 1: Determination of IC_{50} of VrAPI

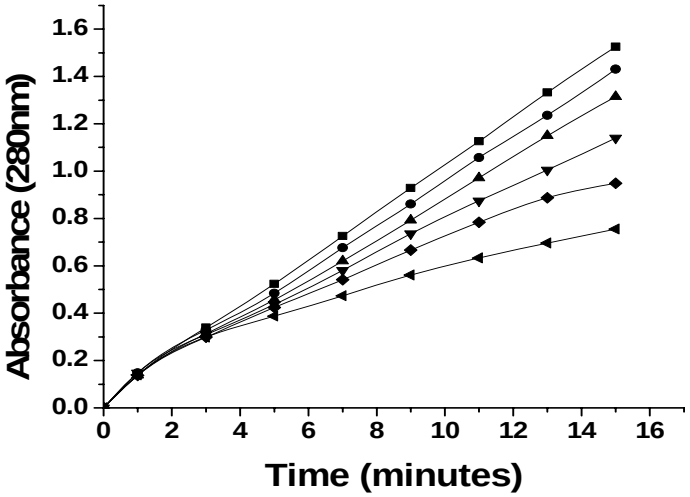


The IC_{50} value for inhibition of VrAP was determined in the presence of 0-30nM VrAPI. The non linear regression analysis of the best fit of the data gave a value of 11nM for IC_{50}.

Progress curve analysis

Assessment of the progress curves suggested that, in the absence of VRAPI, the steady-state rate of proteolytic activities was reached rapidly, whereas a time dependent decline in the rate was observed in its presence (Figure 2).

Figure 2: Time dependent inhibition of VrAP by VrAPI



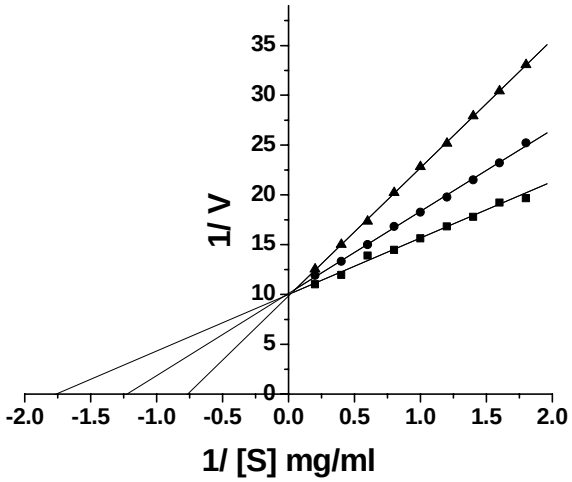
The reaction solution for progress curve analysis contained VrAP (50nM) in 0.05M Gly HCl pH 3.0 at increasing concentrations of VrAPI using hemoglobin (5mg/ ml). Reactions were initiated by the addition of the enzyme at 37°C. The points represent the hydrolysis of substrate as a function of time. The lines indicate the best fits of data obtained from equations 2 and 3. The concentrations of VrAPI were 0nM (■), 20nM (•), 40nM (▲), 60nM (▼), 80nM (◆) and 100nM (◄).

The progress curves also revealed a time range where the conversion of EI to EI* was minimal. For a low concentration of VRAPI, the range was 0-3 for VrAP (Figure 2), within which classical competitive inhibition experiments can be used to determine the *K*i values (eq 3).

Determination of K_i by Lineweaver Burk and Dixon plots

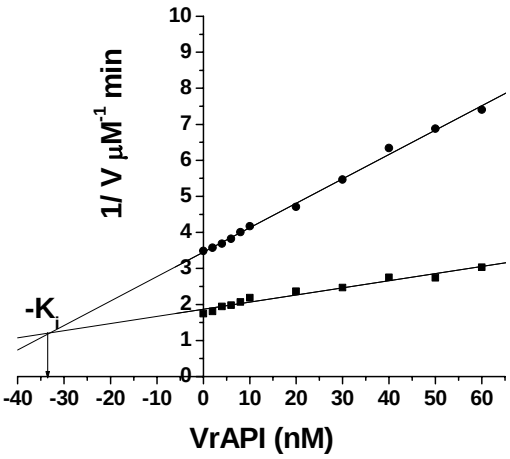
The value of the inhibition rate constant *Ki*, associated with the formation of the reversible enzyme-inhibitor complex (*EI*) determined from the fits of data to the reciprocal equation was 34 x 10^{-9} M (Figure 3), which was corroborated by the Dixon method (Figure 4).

Figure 3: Determination of K_i by double reciprocal plots



Initial rate of reaction in the presence of VrAPI was analysed by the double reciprocal plot. Initial rate of proteolysis by VrAP were estimated using hemoglobin (5 mg/ml). The enzyme was incubated without the inhibitor (▪) or with the inhibitor at 25nM (•) and 40nM (▲) and assayed at increasing substrate concentrations. The reciprocals of the substrate hydrolysis for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. The straight lines obtained indicated the best fit for the data obtained by nonlinear regression as analyzed by the Lineweaver-Burk reciprocal equation and the Km and Ki values were determined from the graphs. Velocities are in micromoles per minute per milligram.

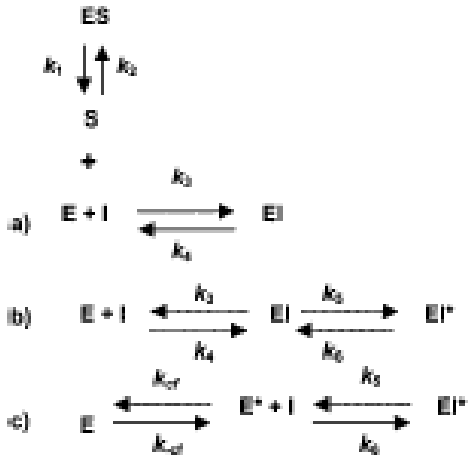
Figure 4: Determination of K_i by Dixon plot



The K_i value was estimated from the Dixon plot. Protolytic activity of VrAP was assayed using hemoglobin at 100 and 200µM with increasing concentrations of VrAPI at 37°C fro 30 minutes. The reciprocal of substrate hydrolysis was plotted against the inhibitor concentration. The straight lines indicate the best fit of the data using non linear regression analysis.

The apparent rate constant *k*, derived from the progress curves when plotted *versus* the inhibitor concentration, followed a hyperbolic function (Fig. 5), revealing that a fast equilibrium precedes the formation of the final slow dissociating enzyme-inhibitor complex (*EI**), indicating a two-step, slow-tight inhibition mechanism (Scheme I).

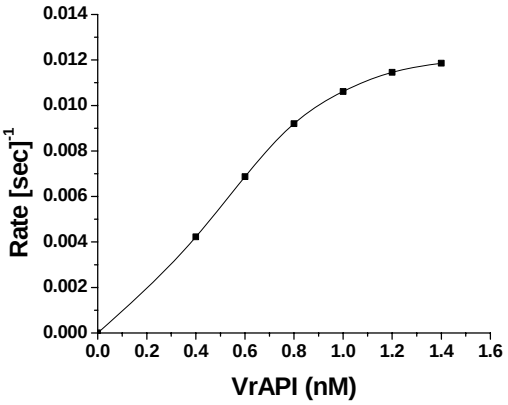
Scheme 1[a]



[a] E stands for the free enzyme, I is the free inhibitor, EI is a rapidly forming preequilibrium complex, and EI* is the final enzyme−inhibitor complex. Binding between the enzyme and inhibitor may either involve a single step, having slow association and dissociation rates (Scheme 1a), or have an initial fast binding step, followed by a slow reversible transformation of EI to another entity, EI* (Scheme 1b), or have an initial slow interconversion of the enzyme E into another form, E*, which binds to the inhibitor by a fast step (Scheme 1c).

Indeed, the data could be fitted to Equation 5 by non-linear regression analysis, which yielded the best estimate of the overall inhibition constant *Ki** of 1.1 x 10-9.

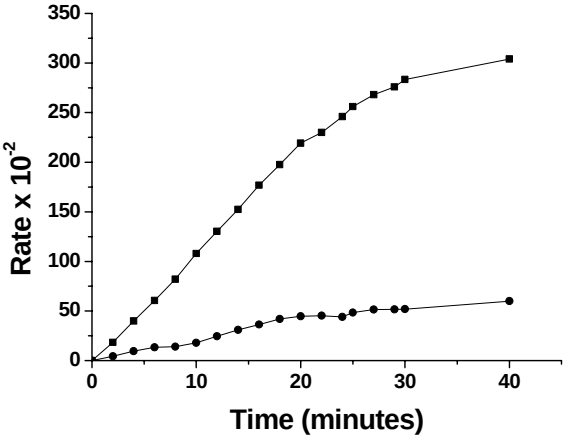
Figure 5: Effect of VrAPI on the rate of reaction (k) of the proteolytic activity of VrAP



The rate constants k was calculated from the progress curves recorded following the addition of VrAP to the reaction mixture containing the appropriate buffer and substrate. The solid line indicated the best fit of the data obtained.

In an independent method to determine *k*6, the rate constant for the conversion of EI* to EI, high concentrations of enzyme and inhibitor were preincubated for sufficient time to allow the system to reach equilibrium. Dilution of the enzyme inhibitor complex into a relatively large volume of assay mixture containing saturating substrate concentration causes dissociation of the enzyme-inhibitor complex and, thus, regeneration of enzymatic activity. Under these conditions, V_0 and the effective inhibitor concentration have been considered to be approximately equal to zero, and the k_6 was determined from the rate of activity regenerated. The values of k_6 determined for VrAP was 7.5 (± 0.5) x 10-6 s^{-1} by least-squares minimization of eq 2 (Figure 6) which clearly indicated a very slow dissociation of *EI**.

Figure 6: Dissociation rate (k_6) for VrAP-VrAPI complex.



VrAP (5µM) was preincubated without (▪) or with (•) equimolar concentrations of VrAPI for 180 minutes on ice in Gly HCl (0.05M, pH 3.0). Further the enzyme inhibitor mixture was

diluted 1000 fold into the assay mixture containing the substrate at 100Km. After preincubation, 5µl of the sample was removed and diluted to 30µl in the same buffer. At the specified time, aliquots were removed and assayed for the proteolytic activity using hemoglobin. The effective rate (k_6) was determined as described in the text.

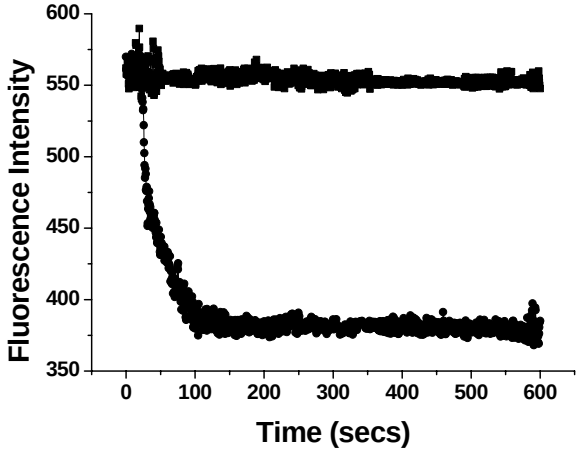
The final steady-state rate *Vs* was determined from the control that was preincubated without the inhibitor. The value of the rate constant *k*5, associated with the isomerization of *EI* to *EI** obtained from fits of Equation 3 to the onset of inhibition data using the experimentally determined values of *Ki* and *k*6 was 15.4 x 10-4 s_1. The overall inhibition constant *Ki** is a function of *k*6/ (*k*5 + *k*6) and is equal to the product of *Ki* and this function. The *k*6 value indicated a slower rate of dissociation of *EI** complex and the half-life *t*1/2, for the reactivation of EI* as determined from *k*6 values was 154 suggesting that the higher binding affinity did not deviate from linearity and the conversion of *EI* to *EI** was minimal.

There are two alternative models for the time-dependent inhibition by Scheme 1. An inhibition model in which the binding of the inhibitor to the enzyme is slow and tight, but occurs in a single step (Scheme 1a), is eliminated on the basis of the data of Table 1, because the inhibitor has a measurable effect on the initial rates before the onset of the slow-tight binding inhibition. An inhibition model where the inhibitor binds only to the free enzyme that has slowly adopted the transition-state configuration (Scheme 1c) can also be eliminated by the observed rates of onset of inhibition. From the observed results, we have concluded that the inhibition of the aspartic proteases followed the slowtight binding mechanism as described in Scheme 1b.

Fluorometric Analysis of Enzyme-Inhibitor Interactions

To delineate the conformational changes induced in the aspartic proteases due to the binding of VRAPI, the fluorescence spectra of the enzyme-inhibitor complexes were monitored. The tryptophanyl fluorescence spectra of VrAP and F-prot exhibited an emission maxima (λmax) at 342 nm, as a result of the radiative decay of the ð-ð* transition from the Trp residues (data not shown). The binding of VRAPI resulted in a concentration-dependent progressive quenching of the emission spectra of the enzymes. However, λmax of proteases indicated the absence of the blue or red shift in the intrinsic fluorescence, negating any drastic gross conformational changes in the three-dimensional structure of the enzymes. To monitor the isomerization of EI to EI*, we have followed the intrinsic tryptophanyl fluorescence of the complexes as a function of time. Upon the addition of VRAPI, a rapid decrease in the quantum yield of fluorescence was observed, followed by a slow decline to a final stable value over a period of 60 for VrAP (Figure 7), indicating an exponential decay of the fluorescence intensity.

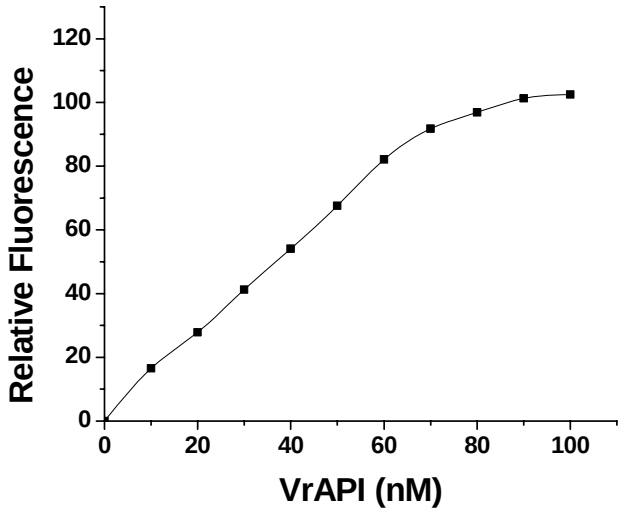
Figure 7: Time dependent effect of VrAPI on the fluorescence quenching of VrAP



VrAP (200µM) was treated at 30 seconds with 25 and 50µM of VrAPI respectively and the fluorescence emission was followed for 10 minutes at a data acquisition time of 0.5 s. The excitation wavelength was fixed at 280nm whereas the emission wavelength was 342 nm. The data were the average of ten scans with the correction for buffer and dilutions.

The magnitude of the rapid fluorescence decrease as a function of time was found to be similar to the total fluorescence quenching observed at a specific VRAPI concentration. Thus we have concluded that both VrAP-VRAPI and VrAP-VRAPI* complexes have the same intrinsic fluorescence. Further, titration was performed in which an increased concentration of VRAPI was added to the enzymes. The magnitude of the initial rapid fluorescence loss (F_0 - F) increased hyperbolically (Figure 8), corroborating the two-step, slow-tight binding inhibition of the aspartic proteases by VRAPI.

Figure 8: Effect of VrAPI on fluorescence of VrAP



A constant amount of VrAP (150nM) was treated with increasing concentrations of VrAPI. The fluorescence changes were measured at 30°C (excitation 280nm and emission, 342nm). Each measurement was repeated ten times and the average values of the fluorescence intensity at

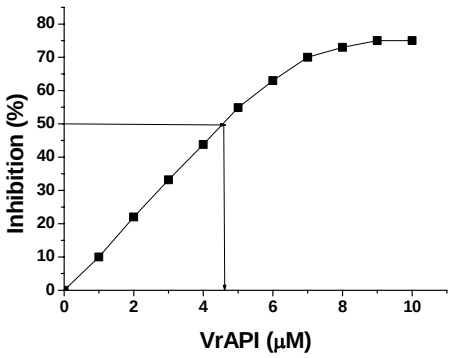
342 nm were recorded. Control experiments with the buffer and inhibitor were done under identical conditions. The fluorescence changes (F0- F) were plotted against the inhibitor concentrations. The hyperbola indicates the best fit of the data obtained.

The estimated value of *K*i determined by fitting the data for the magnitude of the rapid fluorescence decrease (F_0 - F) was (18.9 (± 0.5) X 10-9 M), and the k5 value determined from the data derived from the slow decrease in fluorescence was (8.3 (± 0.5) X 10^{-4} s^{-1}) for VrAP. These rate constants are in good agreement with that obtained from the kinetic analysis of VrAP; therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex EI, while the slow, time-dependent decrease reflected the accumulation of the tight-bound slow dissociating complex EI*.

Kinetics with FProt

The preliminary kinetic assessments revealed that 4.6µM of VrAPI inhibited 50% of the activity of the aspartic protease, F-prot from *Aspergillus saitoi* (Figure).

Figure 1: Determination of IC_{50} of VrAPI

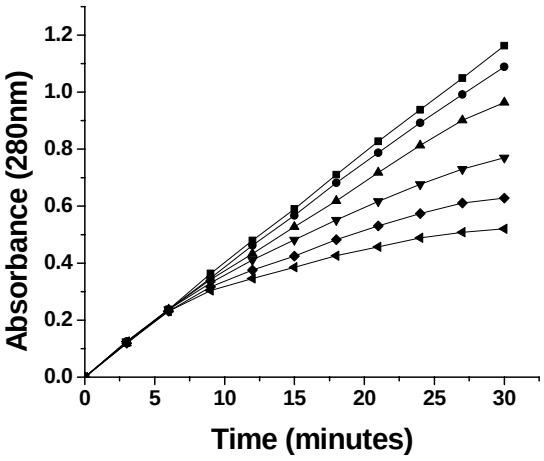


The IC_{50} value for inhibition of FProt was determined in the presence of 0-10µM VrAPI. The non linear regression analysis gave a value of 4.6µM.

Progress curve analysis

Assessment of the progress curves indicated a rapid achievement of steady state rate of proteolytic activity in the absence of VRAPI. In the presence of the inhibitor there was a time dependent decline in the rate (Figure 2).

Figure 2: Time dependent inhibition of FProt by VrAPI



The reaction solution for progress curve analysis contained FProt (15μM) in 0.05M Gly HCl pH 3.0 at increasing concentrations of VrAPI using hemoglobin (5mg/ ml). The reaction was initiated by the addition of the enzyme at 37°C. The points represent the hydrolysis of substrate as a function of time. The lines indicate the best fits of data obtained from equations 2 and 3. The concentrations of VrAPI were 0μM (■), 5μM (•), 10μM (▲), 15μM (▼), 20μM (◆) and 25μM (◄).

Analysis of the progress curves revealed that in the time range of 0-5 min the conversion of EI to EI* was minimal and classical competitive inhibition experiments could be performed within this time to determine the Ki value (Figure 2).

Estimation of Ki by Lineweaver Burk and Dixon plots

Determination of the inhibition rate constant *Ki* from the fits of data to the reciprocal equation of Lineweaver Burk was 9.6 x 10^{-6} M (Figure 3), which was corroborated by the Dixon method (Figure 4).

Figure 3: Determination of K_i by double reciprocal plots

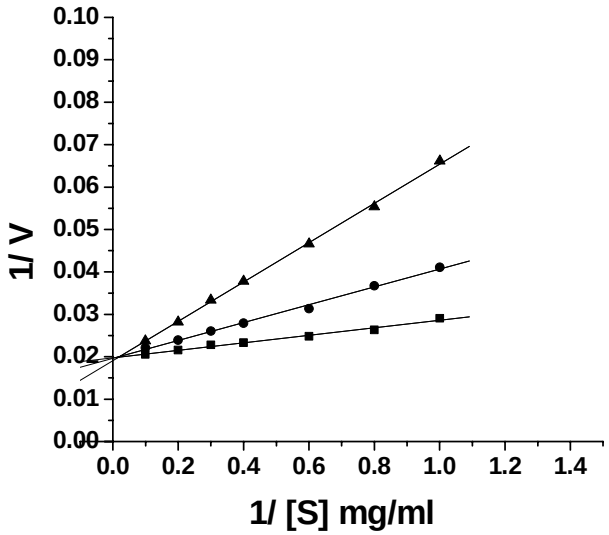
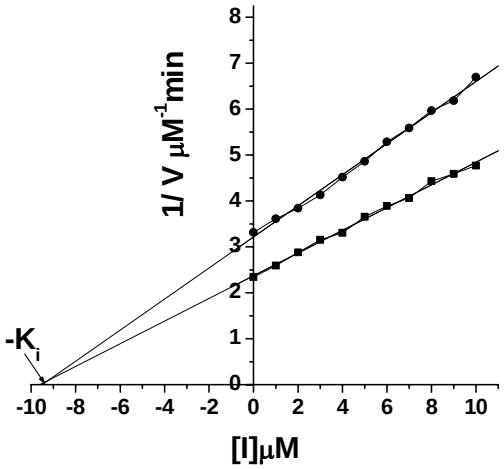


Figure 3: Initial rate of reaction in the presence of VrAPI. Initial rate of proteolysis by FProt were estimated using hemoglobin (5 mg/ml). The enzyme incubated without the inhibitor (▪) or with the inhibitor at 10μM (•) and 20μM (▲) was assayed at increasing substrate concentrations. The straight lines obtained in the double reciprocal plot indicated the best fit for the data obtained by nonlinear regression as analyzed by the Lineweaver-Burk reciprocal equation and the Km and Ki values were determined from the graphs. Velocities are in micromoles per minute per milligram.

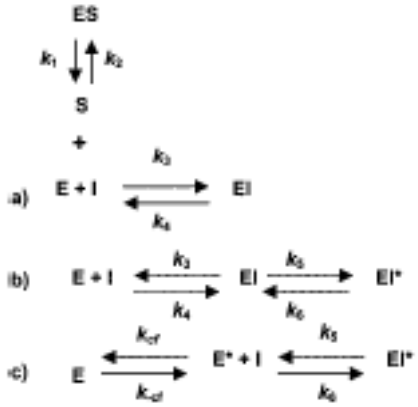
Figure 4: Determination of K_i by Dixon plot



The K_i value was estimated from the Dixon plot. Proteolytic activity of VrAP was assayed using hemoglobin at 100 and 200μM with increasing concentrations of VrAPI at 37°C fro 30 minutes. The reciprocal of substrate hydrolysis was plotted against the inhibitor concentration. The straight lines indicate the best fit of the data using non linear regression analysis.

The apparent rate constant *k*, derived from the progress curves when plotted *versus* the inhibitor concentration, followed a hyperbolic function (Fig. 5), revealing that a fast equilibrium precedes the formation of the final slow dissociating enzyme-inhibitor complex (*EI**), indicating a two-step, slow-tight inhibition mechanism.

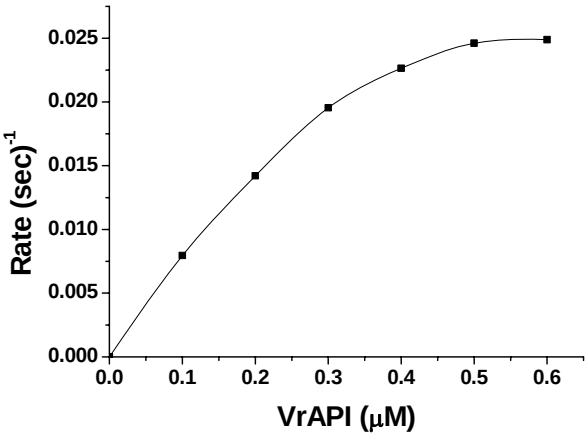
Scheme 1[a]



[a] E stands for the free enzyme, I is the free inhibitor, EI is a rapidly forming preequilibrium complex, and EI* is the final enzyme−inhibitor complex. Binding between the enzyme and inhibitor may either involve a single step, having slow association and dissociation rates (Scheme 1a), or have an initial fast binding step, followed by a slow reversible transformation of EI to another entity, EI* (Scheme 1b), or have an initial slow interconversion of the enzyme E into another form, E*, which binds to the inhibitor by a fast step (Scheme 1c).

Indeed, the data could be fitted to Equation 5 by non-linear regression analysis, which yielded the best estimate of the overall inhibition constant *Ki** of 1.56 x 10-8.

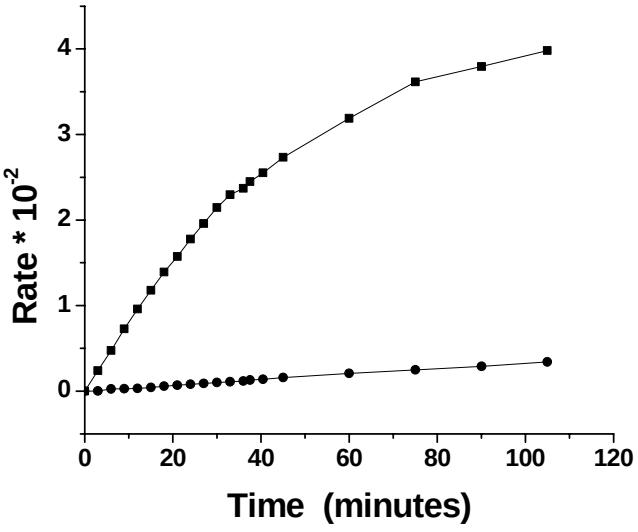
Figure 5: Effect of VrAPI on the rate of reaction (k) of the proteolytic activity of FProt



The rate constants k was calculated from the progress curves recorded following the addition of FProt to the reaction mixture containing the appropriate buffer and substrate. The solid line indicated the best fit of the data obtained.

In an independent method to determine *k*6, the rate constant for the conversion of EI* to EI, high concentrations of enzyme and inhibitor were preincubated for sufficient time to allow the system to reach equilibrium. Dilution of the enzyme inhibitor complex into a relatively large volume of assay mixture containing saturating substrate concentration causes dissociation of the enzyme-inhibitor complex and, thus, regeneration of enzymatic activity. Under these conditions, V_0 and the effective inhibitor concentration have been considered to be approximately equal to zero, and the k_6 was determined from the rate of activity regenerated. The values of k_6 determined for FProt was 7.6 (± 0.5) x 10^{-5} s^{-1} by least-squares minimization of eq 2 (Figure 6) which clearly indicated a very slow dissociation of *EI**.

Figure 6: Dissociation rate (k_6) for FProt-VrAPI complex



FProt (50μM) was preincubated without (▪) or with (•) equimolar concentrations of VrAPI for 180 minutes on ice in Gly HCl (0.05M, pH 3.0). Further the enzyme inhibitor mixture was diluted 100 fold into the assay mixture containing the substrate at 10Km. After preincubation, 5μl of the sample was removed and diluted to 30μl in the same buffer. At the specified time, aliquots were removed and assayed for the proteolytic activity using hemoglobin. The effective rate (k_6) was determined as described in the text.

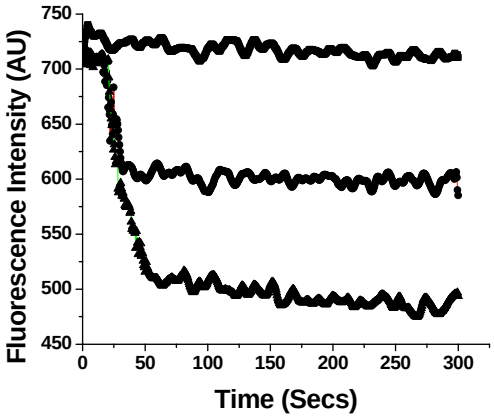
The final steady-state rate *Vs* was determined from the control that was preincubated without the inhibitor. The value of the rate constant *k*5, associated with the isomerization of *EI* to *EI** obtained from fits of Equation 3 to the onset of inhibition data using the experimentally determined values of *Ki* and *k*6 was 6.9×10^{-3}. The overall inhibition constant *Ki** is a function of *k*6/ (*k*5 + *k*6) and is equal to the product of *Ki* and this function. The *k*6 value indicated a slower rate of dissociation of *EI** complex and the half-life *t*1/2, for the reactivation of EI* as determined from *k*6 values was 15 suggesting that the higher binding affinity did not deviate from linearity and the conversion of *EI* to *EI** was minimal.

There are two alternative models for the time-dependent inhibition by Scheme 1 (*31*, *37*). An inhibition model in which the binding of the inhibitor to the enzyme is slow and tight, but occurs in a single step (Scheme 1a), is eliminated on the basis of the data of Table 1, because the inhibitor has a measurable effect on the initial rates before the onset of the slow-tight binding inhibition. An inhibition model where the inhibitor binds only to the free enzyme that has slowly adopted the transition-state configuration (Scheme 1c) can also be eliminated by the observed rates of onset of inhibition. From the observed results, we have concluded that the inhibition of the aspartic proteases followed the slow tight binding mechanism as described in Scheme 1b.

Fluorometric Analysis of Enzyme-Inhibitor Interactions

To delineate the conformational changes induced FProt due to the binding of VRAPI the fluorescence spectra of the enzyme-inhibitor complexes were monitored. The tryptophanyl fluorescence spectra of F-prot exhibited emission maxima (*λ*max) at 342 nm. The binding of VRAPI resulted in a concentration-dependent progressive quenching of the emission spectra of the enzymes. However, *λ*max of proteases indicated the absence of the blue or red shift in the intrinsic fluorescence, negating any drastic gross conformational changes in the three-dimensional structure of the enzymes. To monitor the isomerization of EI to EI*, we have followed the intrinsic tryptophanyl fluorescence of the complexes as a function of time. Upon the addition of VRAPI, a rapid decrease in the quantum yield of fluorescence was observed, followed by a slow decline to a final stable value over a period of 25 s for VrAP (Figure 7), indicating an exponential decay of the fluorescence intensity.

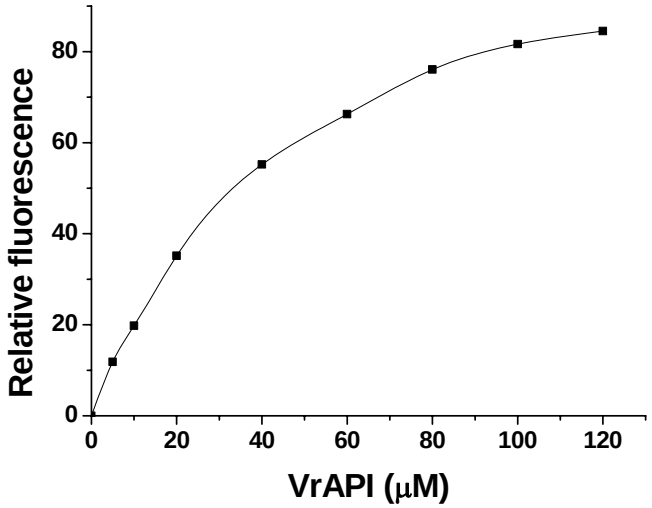
Figure 7: Time dependent effect of VrAPI on the fluorescence quenching of VrAP



FProt (50µM) was treated at 30 seconds with 25 and 50µM of VrAPI respectively and the fluorescence emission was followed for 10 minutes at a data acquisition time of 0.5 s. The excitation wavelength was fixed at 280nm whereas the emission wavelength was 342 nm. The data were the average of ten scans with the correction for buffer and dilutions.

The magnitude of the rapid fluorescence decrease as a function of time was found to be similar to the total fluorescence quenching observed at a specific VRAPI concentration. Thus we have concluded that both FProt-VRAPI and FProt-VRAPI* complexes have the same intrinsic fluorescence. Further, titration was performed in which an increased concentration of VRAPI was added to the enzymes. The magnitude of the initial rapid fluorescence loss (*F*0 - *F*) increased hyperbolically (Figure 8), corroborating the two-step, slow-tight binding inhibition of the aspartic proteases by VRAPI.

Figure 8: Effect of VrAPI on fluorescence of FProt



A constant amount of FProt (50µM) was treated with increasing concentrations of VrAPI. The fluorescence changes were measured at 30°C (excitation 280nm and emission, 342nm). Each measurement was repeated ten times and the average values of the fluorescence intensity at

342 nm were recorded. Control experiments with the buffer and inhibitor were done under identical conditions. The fluorescence changes (F_0- F) were plotted against the inhibitor concentrations. The hyperbola indicates the best fit of the data obtained.

The estimated value of *K*i determined by fitting the data for the magnitude of the rapid fluorescence decrease (F_0 - F) was (11 (± 0.5) X 10^{-6} M), and the *k*5 value determined from the data derived from the slow decrease in fluorescence was (7.3(± 0.5) X 10^{-3} s^{-1}) for FProt. These rate constants are in good agreement with that obtained from the kinetic analysis of FProt; therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex EI, while the slow, time-dependent decrease reflected the accumulation of the tight-bound slow dissociating complex EI*.

DISCUSSION

The inhibitors of aspartic proteases have been found to occupy a key position in the maintenance of normal physiology and life cycle of eukaryotic and prokaryotic systems. Detailed studies on the biochemical and kinetic properties of this class of enzymes and their inhibitors have been done on microbial and mammalian systems. There is a plethora of reports on the serine, cysteine and metalloproteases inhibitors but a lacuna of literature on aspartic protease inhibitors from plants.

In the present study the purification and biochemical characterization of an aspartic protease inhibitor from the seeds of *Vigna radiata* is reported. Seeds of different varieties of *Vigna radiata* were screened for the presence of the inhibitor. The results indicate the varietal difference could be due to the result of breeding. The wild variety was found to have maximum amount of the inhibitor wherein hybrids demonstrated decreased levels. The time dependent expression of the inhibitor demonstrated that the ungerminated seeds contained maximum of the inhibitor contributing to their resistant nature. The mung bean seeds are resistant to fungal or bacterial infection which could be ascribed to the antifungal activity of the inhibitor. The time dependent expression of VrAP and VrAPI indicate that the two never occur concurrently. In other words when the inhibitor was present a predominant absence of the enzyme was noted. The enzyme was found to be significant in germination of the seeds. That the expression of the inhibitor varies with that of the onset of germination and the subsequent expression of the enzyme point towards its significance in controlling seed germination. This inhibitor was purified to homogeneity and was found to be a low molecular weight peptide. To further probe into its physiological role in regulating the endogenous protease, the kinetic interactions between the enzyme and the inhibitor were performed. The inhibitor was

found to inhibit the aspartic protease from *Aspergillus saitoi*. This indicated that the inhibitor could be useful in controlling fungal growth since aspartic proteases are required for the successful completion of the life cycle of a fungus. The kinetic interactions with the enzyme were useful in understanding the basis of its antifungal property described elsewhere.

In order to understand the interactions between VrAPI and VrAP, they were subjected to kinetic analysis. The slow-binding inhibition phenomena on which the interactions were postulated are described in Scheme 1. Scheme 1a is based on the assumption that the formation of the EI complex is a single slow step and the magnitude of k_3I is less as compared to the rate constants for the conversion of substrate to product. Scheme 1b illustrates the two-step slow-binding inhibition, with the first step involving the rapid formation of a reversible EI complex and the second step being its slow isomerization to a stable, tightly bound enzyme-inhibitor complex, EI*. Inhibitors which inhibit the enzyme-catalyzed reactions at concentrations comparable to that of the enzyme and under conditions where the equilibria are set up rapidly are referred as tight-binding inhibitors. The establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes in slow-binding inhibition occurs slowly on the steady-state time scale (Morrison, 1982).

Considering the physiological importance of the aspartic proteases and their role in various diseases, there is a lacuna in the studies of the mechanism of inhibition by slow-binding inhibitors. To our knowledge, the best-known slow-binding inhibitors of FProt are ATBI (Dash, 2001), pepstatin (Umezawa, 1980) and its analogues. The data presented here showed that VrAPI, a peptidic inhibitor from *Vigna radiata* was a slow tight binding inhibitor of the endogenous aspartic protease, VrAP and the fungal

protease from *Aspergillus saitoi,* F-prot. The fluorescence studies revealed that the binding of VrAPI induced localized conformational changes in the proteases, as reflected during the isomerization of the EI complex to the EI* complex. The determined kinetic data for the enzyme-inhibitor interactions were linked with the conformational changes induced in the enzymes due to the inhibitor binding. Competitive inhibition of the enzymes was revealed during the initial kinetic analysis. The 1:1 molar ratio of the interaction of VrAPI with the enzymes made us classify it under the "tight-binding inhibitor" group (Williams and Morrison, 1979; Wolfenden, 1976). The major variable for slow-binding inhibition is *k*6, the first-order rate at which EI* complex reverts to EI. A comparable statement is that the apparent inhibitor constant, *K*i*, depends on the ability of the inhibitor to stabilize the secondary complex EI*. The longer half-life of EI* signified increased stability and the slow dissociating nature of these complexes.

The onset of slow-binding inhibition is caused by a normal conformational mode of the enzyme-inhibitor complex that attains the stable configuration. The slow-binding inhibitors combine at the active site and induce conformational changes that cause the enzyme to clamp down in the inhibitor, resulting in the formation of a stable enzyme-inhibitor complex. The time-dependent inhibition kinetics of VrAP and F-prot by VRAPI followed a two-step mechanism, which was also reflected in the quenching pattern of the fluorescence. On the basis of our fluorescence studies, we propose that the rapid fluorescence loss was due to the formation of the reversible EI complex, whereas the subsequent slower decrease was a result of the accumulation of the tightly bound EI* complex. The kinetically observable isomerization of EI to EI* does not involve a major alteration in the three-dimensional structure of the enzymes as reflected in the absence of any shift in the tryptophanyl fluorescence. Further, agreement of the rate constant values determined from kinetic and

fluorescence analysis prompted us to correlate the localized conformational changes to the isomerization of EI to EI*.

The possibility that can be considered for the above inhibitor induced fluorescence decrease is due to the presence of multiple sites binding at one induced rapid fluorescence change and at a second caused the slow fluorescence decrease. To verify this possibility, a fixed concentration of the enzymes was titrated with increasing concentrations of VRAPI. The proteolytic activity of VrAP and F-prot decreased linearly with increasing concentrations of VRAPI, yielding a stoichiometry close to 1:1 expected for the slow-tight binding inhibition. The effect of VRAPI concentration on the fluorescence quenching of the enzymes was also consistent with a 1:1 molar ratio.

The results of our investigation demonstrated that the inactivation of VrAP and F-prot by VRAPI followed the slow tight binding inhibition mechanism and can be conveniently monitored by fluorescence spectroscopy. The reversible nature of all the reactions indicates the flexibility with which VrAPI binds to the enzymes. The reversible interaction with VrAP indicates that germination will not occur in the presence of the inhibitor and the inhibitor is one of the ways devised by the plant to maintain seed dormancy. The reversible interactions with fungal protease signify its role in defense of the plant.

CHAPTER V

ASPARTIC PROTEASE INHIBITORS AS BIOCONTROL AGENTS

"The study of establishment and spread of plant disease is an exercise in ecology. The interactions involved are multidimensional and the partial control of such leads to disease management."

Robert Schein

ABSTRACT

Biocontrol agents are those which protect the plants from insect and pathogens attack. To date chemical control has been the strategy of choice for protecting the plants from pathogenic attack. The use of pesticides has resulted in severe environmental pollution and harm to the natural flora and fauna. Therefore alternative strategies are being sought to control their spread. This chapter focuses on the use of protease inhibitors as biocontrol agent. The effective management of powdery mildew of mung bean and fusarial wilt of pigeon pea has been demonstrated by laboratory, pot and field experiments. The successful use of the agent indicates that there are simple solutions to complicated problems in nature and we should look for alternatives from nature itself. The successful field trials indicate that without the use of any microorganism and using very simplified approaches plant diseases can be managed effectively. This study lends an altogether different perspective to the research on biocontrol of essential crop plants.

INTRODUCTION

Since the beginning of agriculture, farmers have had to cope with natural factors (biotic and abiotic) for attaining maximum yield of their crops. The biotic factors include pests, pathogens and weeds whereas the abiotic factors are lack or excess of water in the growth season, extreme temperatures, high or low irradiance and nutrient supply. Effective crop management by physical, biological and chemical measures leads to sustainable agriculture wherein the impact of these stresses on the crops is reduced. The current chapter focuses only on the pathogen related losses and their control by biological agents.

Current scenario of crop damage

The major biotic stressors responsible for huge crop losses are weeds, pests, viruses and fungi (Agrios, 1997). There are serious losses due to diseases especially in cereals, legumes and ornamentals. The annual global losses caused by pathogens amount to as much as US $300 billion (Thomas 1999).Extensive research has been conducted on the control of weeds and pests resulting in numerous control strategies. However the control of viruses and fungi has proved difficult due to location specific pathovars and constant emergence of resistant strains. There are no accurate estimates available for assessment of crop losses due to pathogens on a global basis. This is due to the inconsistency of data wherein infection due to pathogens often coincides with climatic changes such as irregular rainfall, increased humidity, or drought, which in themselves may lower crop output. Pathogen outbreaks may have a devastating impact in a given year, but cause only marginal losses in other years (Yudelman, Ratta, and Nygaard 1998). Some of the crop losses have been summarized by Oerke, 2006.

Table 1: Region wise distribution of estimated crop losses due to plant diseases

Region	US$ in billion	Percent of potential production
Asia	43.8	14.2
Former Sovlet Union	8.2	15.2
North America	7.1	9.7
Latin America	7.1	13.5
Europe	5.8	9.8
Africa	4.1	15.7

Source: Oerke et al. (2006).

Current control strategies

The use of chemical to protect crops from pathogen infection has led to short term benefits. Their use has led to contamination of soil, water and vegetation. Toxic residues in plants and agricultural produce like food grains, fruits and vegetables and even milk have built up. Human consumption of this contaminated food constitutes an important health hazard (Soon 1997). The unregulated use of chemical control agents has led to the development of resistant strains of pathogen (Thomas 1999). Thus the overall consensus is that the indiscriminate, excessive, and inefficient use of chemicals exacts too high a toll in terms of human health, environmental safety, and ultimate diminishing returns to justify any short-term increases in farm income or food output.

Although the emphasis is on anticipating pathogen outbreaks and preventing them from reaching economically damaging levels, some ecofriendly control strategies

have to be implemented for maximum crop production. Some such control strategies include:

- *Biological control*, such as protecting, enhancing, and releasing natural enemies of the pathogen;
- *Cultural practices*, such as ecological landscaping, crop rotation, improved crop residue management, better water management, and improved pathogen monitoring;
- *Use of naturally occurring chemicals,* with less reliance on synthetics in favor of biochemicals (naturally occurring chemicals that control pathogen incidence and spread); and
- *Genetic,* such as the use of naturally resistant varieties, new varieties bred for resistance, or transgenic varieties

The intense requirement for more environment friendly and sustainable approaches has oriented research in favor of biological control agents.

Biological control agents

Extensive research on pest control has led to the discovery and use of biocontrol agents against pest infestation. The research on pathogen control has led to the development of *Trichoderma, Gliocladium, Bacillus* and *Pseudomonas* on a commercial scale. These agents are however used in the form of spores or as live organisms in the field. This results in alteration of the natural flora and fauna. Therefore alternative bio-based strategies wherein the active biological principle can be developed as biocontrol agent are required.

Protease inhibitors in plant protection

Protease inhibitors (PIs) are ubiquitous in distribution with reports from microbial, animal and plant origin. The discussion will be restricted to plant protease inhibitors in plant defense. PIs are the most studied class of plant defense proteins which can be classified as biochemicals. They are abundantly present in seed storage tissues and represent up to 10% of total protein (Casaretto and Corcuera 1995). PIs have been studied in plants for various roles which among others also include their importance in pathogen and insect attack (Brzin and Kidric 1995). In many cases their accumulation in quantities far more than required for inhibiting endogenous proteases, underline their role as defense proteins. In others an absence of inhibitory activity against endogenous plant proteases with presence of activity against proteases of pests and pathogens confirms their role in defense.

Serine protease inhibitors

Of all the classes of PIs, the serine PIs are the most abundant and also the most studied proteins. Serine protease inhibitors have antinutritional effects against several lepidopteran insects. These inhibitors inhibit enzymes from species of *Tribolium*, *Callosobruchus*, *Manduca*, *Haematobia*, *Stomoxys*, *Spodoptera*, etc. A direct test of the roles of the inhibitors in plant leaves to defend against insects was first demonstrated by Hilder et al (Hilder et al., 1987). SBBTI isoinhibitors have been reported to inhibit completely the activities of mealworm (*Tenebrio molitor*), red flour beetle (*Tribolium castaneum*) and the migratory locust (*Locusta migratoria*). It has been shown that these are associated with resistance to insect and fungal attack and that the inhibitors can inhibit the proteases of microbes including pathogens. Peng and Black have observed that levels of trypsin inhibitors increase more in leaves in varieties of tomato that were resistant to *Phytophthora infestans* than in susceptible varieties. An increased proteinase inhibitory activity in melon plants infected with

Colletotrichum was noted by Roby et al (Roby et al., 1987). Reduced protease activity of proteases from *Fusarium solani* and *Colletotrichum* species was observed with protease inhibitors from healthy bean and tomato plants. Many protein inhibitors of trypsin and chymotrypsin isolated from seeds suppress microbial serine proteases like subtilisin and the proteases from molds like *Aspergillus.* Such inhibitors have been reported from families Gramineae, Cucurbitaceae, Amaranthaceae and Polygonaceae. These proteins are structurally similar to the potato proteinase inhibitor I which itself is active against many microbial enzymes (Valueva and Mosolov, 1999; Ryan, 1979). There are few reports of the effects of seed proteinase inhibitors on the enzymes of phytopathogenic microorganisms. Soyabean and kidney bean inhibitors from the SBBTI family were shown to suppress proteases of phytopathogenic fungi from the genus *Fusarium*. Buckwheat trypsin inhibitor suppressed the activity of protease of *Alternaria alternata* while those from sunflower seeds suppressed the activity of the proteases of the causative agent of gray mold. In several cases, plant resistance to microorganisms was found to correlate with the content of protease inhibitors as seen in wheat caryopses and lupine and soyabean trypsin inhibitors. Serine protease inhibitors have been reported from potato, barley, tomato, pumpkin, chick pea, cowpea, black beans, mung beans, adzuki beans, broad beans, jack beans (Ryan, 1990). The serine protease inhibitors having antifungal activity have the interesting property of inhibiting the α-amylase activity from insects but not from bacterial/ mammalian sources. These bifunctional inhibitors which inhibit fungal and insect growth have been reported from corn, ragi, wheat, barley, potato and daisy. Serine protease inhibitors have been used to produce transgenic plants. These transgenic studies were instrumental in establishing the importance of protease inhibitors in plant protection. One of the best demonstrated examples in this case was the transfer of the gene for the cowpea inhibitor. This inhibitor was proved to be a strong antimetabolite against insects from

the genera *Heliothis*, *Spodoptera*, *Diabrotica* and *Tribolium.* Hence the modified gene for this inhibitor was transferred to tobacco plants. The transgenic plants manifested higher resistance to insects from the genus *Heliothis* and *Manduca* as compared to the wild type plants. Thus the serine protease inhibitors class remains the best studied class of inhibitors in the plant kingdom for their participation in plant defense.

Cysteine protease inhibitors

The presence of this class of protease inhibitors in plants has been known for years. Their role in the plant defense against pest attack has been more recognized. These inhibitors are now referred to as cystatins and there are at least three distinct families (Barrett et al. 1987). The amino acid sequences of the pineapple and rice cysteine protease inhibitor have now been determined. Cysteine protease inhibitors have been reported from pineapple (Reddy et al., 1975), potato (Rodis and Hoff, 1984), corn (Abe and Whitaker, 1988), rice (Abe et al., 1987), cowpea (Rele et al., 1980), millet (Tashiro and Maki, 1986), mung bean (Baumgartner and Chrispeels, 1976), tomato, wheat, barley and rye (Fossom, 1970).

Cysteine proteases from insect larvae can be inhibited by both synthetic and naturally occurring cysteine protease inhibitors (Campos et al, 1989; Gatehouse et al., 1985; Kitch and Murdock, 1986; Lemos, 1987; Wieman and Nielsen, 1988). A specific cysteine protease inhibitor produced by *Aspergilllus japonicus*, called E—64 (trans-epoxysuccinyl-leucyl-augmentine), was shown to inhibit the digestive enzymes of several insect species including the Colorado potato beetle (Wolfson and Murdock, 1987), the common bean beetle (Wieman and Nielsen, 1985), the Mexican bean beetle, the red flour beetle and the cowpea weevil (Campos et al., 1989; Murdock, 1987; Murdock, 1988). E-64 caused a delay of development and larval

death, supporting the importance of cysteine protease inhibitors in the control of insect larvae on plants. An interesting observation was that the protease complex from the red flour beetle was inhibited by the same protein component from wheat endosperm, which inhibits the cysteine protease from wheat caryopses. The oryzocystatins from rice suppressed the activities of proteases from the intestines of rice weevil and the red flour beetles. Recently transgenic rice plants expressing the phytocystatine gene show wider specificity to the different cysteine proteases than rice oryzocystatin (Irie et al., 1996). Phytocystatin isolated from the transgenic tobacco plants inhibited papain and Cathepsin H as well as the cysteine proteases from weevil digestive complex. Many cysteine protease inhibitors have been demonstrated to show potentiating effects on the toxins responsible for insecticidal activity. The combined activities of both serine and cysteine proteases show a higher protective effect against insect larvae than either of them alone.

Aspartic protease inhibitors

The knowledge of aspartic protease inhibitors in plant protection is very scanty. There have been reports of aspartic proteases in insect guts, but conclusive evidence showing the role of the inhibitors in protection has not been reported. Pepstatin, a powerful and specific inhibitor strongly inhibited the gut enzymes of the Colorado potato beetle (Wolfson and Murdock, 1987). Potato tubers have shown the presence of a cathepsin D inhibitor that exhibits considerable amino acid homology with the soyabean trypsin inhibitor (Mares et al., 1989). In fact, many aspartic protease inhibitors from potato tubers have now been reported.

Metalloprotease inhibitor

Plants have evolved at least two families of metalloprotease inhibitors, the metallo-carboxypeptidase inhibitor family in potato (Rancour and Ryan, 1968) and tomato plants (Graham and Ryan, 1981; Hass and Ryan 1981), and a cathepsin D inhibitor family found in potatoes (Keilova and Tomasek, 1976). The cathepsin D inhibitor is unusual as it inhibits trypsin and chymotrypsin but it does not inhibit aspartic proteases. The metallo-carboxypeptidase inhibitors are polypeptides. These inhibitors are strong competitive inhibitors of a broad spectrum of carboxypeptidaases from animals and microorganisms but they do not inhibit the enzymes from yeasts and plants (Hass and Ryan, 1981). This inhibitor is found in potato tubers and leaves along with the potato inhibitor I and II families of serine protease inhibitors. Further study on these inhibitors needs to be carried out since there are not many reported.

Basis of selection of PIs for effective inhibition of the pathogen

The success of PI based strategy depends upon the selection of appropriate PIs and their proper expression. An ideal effective PI should have the following characteristics: (1) It should have activity against broad spectrum of pathogens (2) It should remain stable to degradation during the infective process of the pathogen. An added advantage is gained when the PI has additional inhibitory functions where it targets more than one enzyme of the pathogen. This is true if the PI in addition to proteases can inhibit, enzymes like xylanases, cellulases and pectinases which are commonly used by the pathogen for its invasion of plant tissues.

In summary, there is an urgent need to study and use molecules like PIs to control the problem of plant disease. The success of transgenic PI based strategies depends upon (i) identification of potent inhibitors, (ii) detailed in vitro and in vivo studies to

check their efficacy to inhibit pathogen growth, (iii) use of PIs in combinations. Devising a multifold strategy will retard pathogen growth, delay disease advance and arrest the growth of pathogen in the field thus minimizing the damage to crop yields. Thus there is tremendous potential for developing protease inhibitors as effective biocontrol agents either for individual application or as part of a multifold strategy in disease management.

SECTION I

BIOLOGICAL CONTROL OF WILT OF PIGEON PEA

Pigeon pea

Pigeon Pea (*Cajanus cajan, syn. Cajanus indicus*) is a member of the family Fabaceae. Other common names are red gram, toovar, toor, gandul, Congo pea, Gungo pea, and no-eye pea.

The cultivation of pigeon pea goes back at least 3000 years. The centre of origin is most likely Asia from where it traveled to East Africa and by means of the slave trade to the New World. Today pigeon peas are widely cultivated in all tropical and semi-tropical regions of both the Old and the New World.

Pigeon pea

Scientific classification

Kingdom	**Plantae**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Order	**Fabales**
Family	**Fabaceae**
Subfamily	**Faboideae**
Genus	***Cajanus***
Species	***cajan***
Binomial name	**Cajanus cajan (L.) Millspaugh**

General Description

Pigeon pea occurs in several varieties. The old varieties in cultivation and semi cultivation in India are semi deciduous, short-lived shrubs usually 1 to 4 m in height. They are usually single stemmed, freely branching, and become woody after a few months. The wood is moderately hard and brittle. They have a deep taproot (to 3 m) with lateral roots and nodulated fine roots. The branches and fine twigs support abundant light-green or yellow-green foliage. Spirally arranged, silky-pubescent, trifoliolate leaves have narrowly elliptic, lanceolate ore oblong leaflets, 2.5 to 9 cm long, the center leaflet being slightly longer than the laterals. The five- to 12-flowered racemes are axillary. Flowers are about 2 cm long, yellow, the standard often being orange to purple outside. The legumes, which are flattened, somewhat constricted between seeds, and 4 8 cm long, are mottled bronze-purple when immature, drying to brown. They contain two to nine mottled brown (white, red, brown, gray, or black in improved varieties) seeds, 7 to 8 mm long by 6 mm broad. There are 2n = 22, 44, or 66 chromosomes (Liogier 1988, Long and Lakela 1976).

Benefits

Pigeon peas are an important food in developing tropical countries. An excellent source of protein, the seeds (and sometimes the pods) are eaten as a vegetable, as a flour additive to other food, in soups, and with rice (Center for New Crops and Plants Products 2002). Although they vary slightly, typical nutritional values for seeds are: moisture, 10.1 percent, protein 19.2 percent, fat, 1.5 percent, carbohydrates, 57.3 percent, fiber 8.1 percent, and ash, 3.8 percent (Smartt 1976). The species is planted as a green manure crop, nurse crop, cover crop, a windbreak hedge, as a host for lac insects, and as food for silk worms. The stalks are used for fuel, thatch, and basketry (Center for New Crops and plants Products 2002). Pigeon pea forms root nodules in association with *Rhizobium* sp. Bacteria and is capable of

fixing 41 to 280 kg/ha of nitrogen (Red de Grupos de Agricultura de Cobertura 2002) thus becoming a nitrogen fixer for soil maintenance. Preparations of the leaves are used to treat jaundice, inflammation, and sores of the mouth (Parrotta 2001).

Overall pigeon pea (*Cajanus Cajan L*.) is one of the major grain legume crops of the subtropics and ranks sixth in world wide dryland legume production (Nene and Sheila, 1990). It is now widely grown in the Indian subcontinent which accounts for almost 90% of the world pigeon pea production. Pigeon pea can survive well in poor soils and an outstanding root system enables it to tolerate drought.

Disease

The crop is infected with *Fusarium udum* Butler causing soil borne wilt leading to 30-100% loss of the total crop yield (Kannaiyan et al., 1984). The pathogen invades the host by producing a germ tube from the mycelium / spores which penetrates seedlings through root tips, wounds or lateral roots. The vascular tissue gets infected leading to vascular plugging followed by wilting of the plant during flowering and pod-filling stages (Reddy et al., 1990, Beckman and Roberts, 1995).

Disease control

Chemical control of wilt is environmentally polluting and hazardous. High-yielding, disease-resistant crop varieties have been developed however they are not completely resistant to the pathogen at all the locations and resurgence of the pathogen is a recurring problem.

Research in the area of biocontrol has resulted in the use of fungal and bacterial species as biocontrol agents for sustainable agriculture (Baker., 1981; Marshall and Walters et al., 1994). Fungal species such as *Trichoderma, Gliocladium* and bacteria

like *Pseudomonas* and *Bacillus* have been reported in the control of *Fusarium* wilt and *Rhizoctonia* wet root rot of chick pea (Weindling and Emerson., 1936; Weindling., 1937; Weindling., 1941; Hanson and O'Leary., 1981; Papavizas., 1985). The development of organism free substitutes for the chemicals has lagged behind due to the paucity of reports on the biochemistry of the host-pathogen interactions. Even though it is known that pectinases, xylanases, cellulases and proteases are the enzymes secreted by the pathogens to invade plant tissues, their inhibitory molecules have not been studied as an approach for biocontrol. Also these biocontrol agents are applied in the form of spores or the whole live organism mixed with suitable carriers and applied as seed coats. This method of application is impractical as it offers protection only once when the plant is in the seed stage and not at the later stages.

Current work

In the present studies the effect of the aspartic protease inhibitors on disease management of pigeon pea wilt caused by *Fusarium udum* is summarized. The inhibition of xylanase and aspartic protease produced by the fungus indicates the effect of the protease inhibitors on limiting the entry of the pathogen in host tissues. The reversible inhibition of spore germination indicates the static effect of the inhibitors on transition from the spore to the mycelium stage. Taking into consideration the antifungal effect on the phytopathogenic fungus, the inhibitors were used for disease management of wilt on pigeon pea at the pot and the field level. An in depth statistical analysis of the field trial data is provided and a mechanism correlating the laboratory findings with the field trial data is suggested.

The development of formulations of the inhibitors, their applications in the field and the comparison with suitable controls wherein the classical method of drenching was utilized is also explicitly worked out. The formulations contained high amounts of the

inhibitors and were assessed for their efficacy at field level and the data for plant growth analyzed.

MATERIAL AND METHOD

Materials

Peptone, Yeast extract, tryptone, was obtained from Hi media Laboratories Ltd, wheat bran was purchased locally. All other chemicals used were of analytical grade. Carbendazim was obtained from the market on a commercial basis while *Trichoderma* and *Rhizobium* were prepared at the field site. Seeds of Kopergaon-1 were obtained from the local market.

Identification of Bacillus halodurans

The genomic DNA was purified as per the method mentioned in Sambrook and Maniatis. The 16S rDNA was sequenced and blasted at NCBI (www.ncbi.nih.gov/blast).

Purification of aspartic protease inhibitors

Bacillus halodurans was grown in a medium containing soyameal as the inducer for production of ATBI as mentioned in Dash et al., 2001. The inhibition of fungal protease of *Aspergillus saitoi* by ATBI was assessed at various stages of purification (Dash and Rao, 2001). HPLC purified ATBI was used for all laboratory experiments. VrAPI was purified from the dormant seeds of mung bean Kopergaon-1 variety as mentioned in Chapter IV. HPLC purified VrAPI was used for all laboratory experiments.

Antifungal activity of VrAPI

The antifungal activity of VrAPI was analyzed against the phytopathogenic fungi *Alternaria solani* (NCIM 887), *Aspergillus flavus* (NCIM 535), *Aspergillus niger* (NCIM 773), *Aspergillus oryzae* (NCIM 643), *Claviceps purpurea* (NCIM 1046), *Colletotricum*

(NCIM 1032), *Curvularia fallax* (NCIM 714), *Curvularia lunata* (NCIM 716), *Curvularia cymbopogonis* (NCIM 695), *Fusarium oxysporum* (NCIM 1043), *Fusarium moniliforme* (NCIM 1099), *Helminthosporium* (NCIM 1079), *Phomopsis* (NCIM 1324), *Penicillium fellulatum* (NCIM 1227), *Penicillium roqueforti* (NCIM 712), *Trichoderma reesei* (NCIM 1052) using the plate assay. For the plate assay freshly grown fungal mycelium of each fungal strain was spot inoculated at the center of a petri plate containing potato dextrose (PD) agar medium and incubated at 28°C for 24 to 48 h. Sterile filter paper disks (5-mm diameter) impregnated with 0.1 inhibitor units of VrAPI were placed in front of the growing fungal mycelium. The plates were further incubated at 28°C, and the zones of retarded mycelial growth were measured. The MICs for the fungal strains were determined by a broth dilution method. Serial dilutions of VrAPI were made in half-strength PD broth in microtiter plates. Each well was inoculated with 10 ml of the test organism containing 100 spores/ ml. The MIC was determined after overnight incubation of the plates and was taken as the lowest concentration of VrAPI at which growth was inhibited. A microspectrometric antifungal assay was performed for the quantitative demonstration of antifungal activity as described. Briefly, routine tests were performed with 10 ml of (filter [0.22-mm pore size]-sterilized) test solution and 40 ml of fungal spore suspension (100 spores/ml) in half-strength PD broth. Control microculture contained 10 ml of sterile distilled water and 40 ml of the fungal spore suspension. Unless otherwise stated, the incubation conditions for the experiments were 28°C for 48 h. Antifungal activity is expressed in terms of percent inhibition as defined by Dash et al, 2001.

Isolation of Fusarium udum

A virulent isolate of *Fusarium udum* was obtained from infected pigeon pea plants using potato dextrose agar (PDA) medium. The pathogenicity of the fungus was

tested by multiplying the fungus in sand-maize medium (Riker and Riker, 1936). Eight pigeon pea seeds were sown in each pot (700mm in diameter) containing sterilized soil with the fungal culture integrated into it at a ratio of 1:9 (Sand-maize inoculum:soil). The wilt incidence was evaluated and the pathogen was re-isolated using PDA. This isolate was confirmed to be *F. udum* by biochemical characterization and used for all further studies.

Antifungal activity assay for inhibition of *F. udum*

The antifungal activity of the inhibitors on *F. udum* was assayed by the plate assay and the spore suspension assay. The plate assay was performed as described earlier. For the spore inhibition assays, a spore suspension was prepared in distilled water from a 10 day old culture of *Fusarium udum* such that the final spore count was 1 x 10^8 spores/ml. 0.1ml aliquots of the spore suspension were taken in test tubes and incubated in the presence of three concentrations of the ATBI viz., 5, 30 and 60 inhibitor units of ATBI and two controls (sterile water and the universal inhibitor of aspartic proteases, pepstatin-A at a final concentration of 2µM) and analyzed for spore germination of *Fusarium udum*. The tubes were then incubated at 25 $\pm$ 2°C for 24 hours. 0.1ml aliquot was then stained with lactophenol cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the percent spore germination was calculated. A similar experimental set up was used with 4, 20 and 40 inhibitor units of VrAPI using the same controls. The kinetics of spore inhibition were performed wherein spores of *Fusarium udum* were obtained from a 10 day old culture and mixed with sterile distilled water to obtain a homogenous suspension of 1 x 10^8 spores/ml. 10µl of spore suspension was inoculated in 25, 50, 100 and 200 inhibitor units of the inhibitor in a test tube and a homogenous suspension (about 2 x 10^5 spores/ml) was made by inverting the tubes 3-4 times. After specific time intervals of 30, 60, 90, 150, 180 minutes, the reaction

mixtures were centrifuged and the spore pellet was washed two times with distilled water. The pellet was then resuspended in 50µl of sterile distilled water and incubated at 25 $\pm$ 2°C for 24 hours. 0.1ml aliquot was then stained with lactophenol cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the percent spore germination was calculated. Control sets were prepared in the same way in sterile distilled water. All the experiments were conducted in triplicates. Germination inhibition data of *Fusarium udum* obtained in spore germination assay was plotted according to Hill's equation (Gi/ Go-Gi vs log I, where Go and Gi are percent germination in the absence and presence of inhibitor) (Rana et. al., 1997). A parallel set up was used for VrAPI wherein 20, 40, 80 and 160 inhibitor units of VrAPI were used for the kinetic experiments.

Production of glucanases

An inoculum of *F. udum* was grown in a medium described by Mishra et al. (1984) containing 2% wheat bran / cellulose paper powder for 4 days at 30 °C and was added (10%) to the fermentation medium for the production of xylanase and cellulase respectively. The fermentation medium (Mishra et al., 1984) used contained 1% ammonium sulphate, 0.1% urea, 1.5% yeast extract, 0.15% tween 80 as surfactant, and wheat bran / cellulose paper powder (4%) as a carbon source. The culture was grown with continuous shaking on a rotary shaker (200rpm) at 30 °C for 8 days. At the end of the fermentation biomass was separated from the fermented broth by centrifugation and the filtrate was used as an enzyme source.

Inhibition of glucanases by the inhibitors

The activity of cellulase / xylanase was measured by incubating 1 ml of assay mixture containing 0.5 ml of 1% carboxy methyl cellulose / xylan and 0.5 ml of suitably diluted sample in 50 mM, pH 7.0, and phosphate buffer for 30 minutes at 30

°C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid (Miller, 1959). One international unit is defined as the amount of enzyme required to liberate 1µmole of glucose or xylose from carboxy methyl cellulose or xylan respectively in 1 minute under the said assay conditions. Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

Inhibition of aspartic protease

Proteolytic activity of the extracellular culture filtrate of *F. udum* was measured by assaying residual enzyme activity after incubating the enzyme and the substrate hemoglobin as given in Chapter II. The enzyme activity was measured in the absence or presence of inhibitor. The assay for inhibition was carried out as mentioned in Chapter IV.

Assay for pathogen levels

The field experiments were carried out during the years 2004 and 2005 in a field artificially infested with *F. udum* located at the Mahatma Phule Krishi Vidyapeeth, Rahuri, India. The soil type was black having pH 6.2. Experimental plots having inoculum levels of pathogen as high as log 8.90 colony forming units (cfu)/g of soil were chosen. The random population distribution of the pathogen in each plot was verified by drawing four samples from each plot (3m X 3 m) and plating 1 g after serial dilutions on *Fusarium* specific medium (Nash and Snyder, 1962). A Poisson distribution confirmed this statistically.

Pot trials

The 3 kDa filtrate containing 112 inhibitor units per liter of ATBI was used for the pot trial. The efficacy of the partially purified inhibitor in controlling pigeon pea wilt disease was assessed under experimental conditions at the pot level by applying the inhibitor as soil application. Pots containing field soil incorporated with *F. udum* culture grown on sand-maize medium described earlier were prepared. Eight seeds were sown per pot and eight pots per replication were maintained. There were three replications and the pots were arranged in a randomized manner. The seeds were then drenched with 10ml inhibitor solution containing 1.2 inhibitor units of the inhibitor at 0, 30, 60 days of sowing. The wilt incidence was observed at intervals. The pathogen was reisolated from the wilted plants. As a check, seeds were treated with the fungicide carbendazim at the rate of 2 g/kg seed and the soil was drenched with 0.1% concentration of carbendazim on the 30^{th} day after sowing. There was also an untreated control. The results obtained were used to decide on the doses for the subsequent field trial. A similar experimental set up was used with 120 inhibitor units per liter of VrAPI.

Field trials

The partially purified ATBI (3kDa filtrate) was sprayed on 10 plants of ICP 2376 variety of pigeonpea in an experimental trial. The extracellular culture filtrate containing 84 inhibitor units per liter was used for large-scale field trials. For the large scale trial a randomized block design experiment in a plot of size 4 x 2.4 metres with three replications was performed. Each experimental unit included 75 plants with a spacing of 50 X 10 cm. Dose dependent drenching was performed in 3 doses every 30 days. The disease incidence and the reduction over control were calculated. Carbendazim was used as a chemical control agent at the rate of 2 g/kg seed and additional drenching with 0.1% concentration of carbendazim. *Trichoderma*

and *Rhizobium* were used as biological controls wherein seeds were coated with the commercial formulations as per the manufacturer's recommendations. The results were analyzed by statistical tests as explained below. The percent disease intensity of wilt was calculated by the formula given below –

$$\text{Percent Disease intensity (P.D.I.)} = \frac{\text{Sum of all Numerical Ratings}}{\text{No. of plants observed} \times \text{Highest Rating}} \times 100$$

The Chi square test was carried out after subjecting the data to the Yates correction to test the null hypothesis that there is no effect of the inhibitor on the fungal pathogen. The dose dependent effect of the inhibitor on seed germination was evaluated on a percentage basis. The correlation of wilt incidence and inhibitor concentration was verified by carrying out nonlinear regression analysis and the t-test.

Similar experiments were performed with the variety ICPL 87 and the data analyzed accordingly.

The field trials were conducted at the Pulse Research Station, MPKV, Rahuri as shown in the figure below:

A similar experimental set up using 120 inhibitor units per liter of partially purified VrAPI was used to analyze the efficiency of VrAPI in disease control at the field level.

Development of powder formulations of aspartic protease inhibitor

Twenty milliliters of either of the inhibitors ATBI and VrAPI containing 84 and 120 inhibitor units per liter was added to 1 g of talcum powder and mixed well with 75 seeds of pigeonpea, ICP 2376 and ICPL 87. The treated seeds were sun dried and sown in a field containing soil incorporated with *F. udum* culture grown on the sand–maize medium. In addition to seed coating, the classical method of drenching was also employed along with an untreated control.

Methods of application

The efficacy of the formulations of the aspartic protease inhibitors in controlling pigeon pea wilt disease was assessed under field conditions. Application methods included drenching, seed coating and seed coating followed by drenching. Additional data on the growth of the plants as indicated by nodulation data, shoot length, root length, weight of plants was obtained till 150 days of sowing.

Field trials

Two field trials were conducted at the Pulse Research Station, MPKV, Rahuri. Pigeonpea ICP 2376 and ICPL 87 were used for the trials. The randomized block design was used throughout for all the trails. 720 seeds of each variety were used for the entire trial. 540 seeds of each variety were coated with 150ml extracellular culture filtrate mixed with 8g of talcum powder. The seeds were air dried and used for the experiments with coating and coating followed by drenching. The plot size was 1600m^2 with a bed size of 2 x 2 m. The seeds were sown with a spacing of 45x 10 cm (60 plants per bed). The application was repeated at 30, 60, 90 and 150 days after sowing. Drenching was done at the rate of 3 liters per bed. As a check, seeds were treated with carbendazim at 2 g / kg seeds and the fields were drenched with 0.1% carbendazim on the 30th day after sowing. The trials were laid out in a randomized replicated block design with three replications. The wilt incidence in the entire plot was assessed and expressed as a percentage of disease incidences. Grain yield was also recorded. The data were statistically analyzed by DMRT. This entire layout was used once with partially purified VrAPI and once with partially purified ATBI.

RESULTS

Control of pigeon pea wilt by ATBI

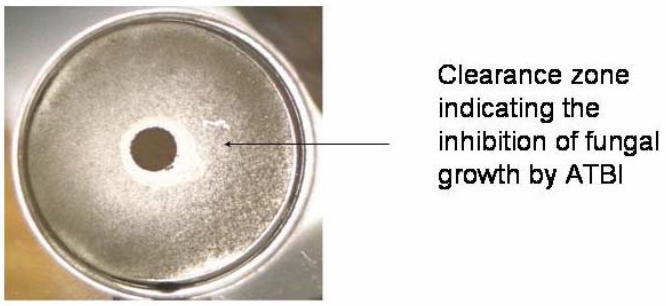
Inhibitor production and purification

ATBI was produced in batches of 20 liters, which was applied to the field in a single dose. Purified inhibitor was used for all the laboratory experiments.

Antifungal activity assay

The plate assay indicated a zone of inhibition of 4mm in diameter of *F. udum* in the presence of ATBI (Figure 1).

Figure 1: Effect of ATBI on mycelial growth of *F. udum*

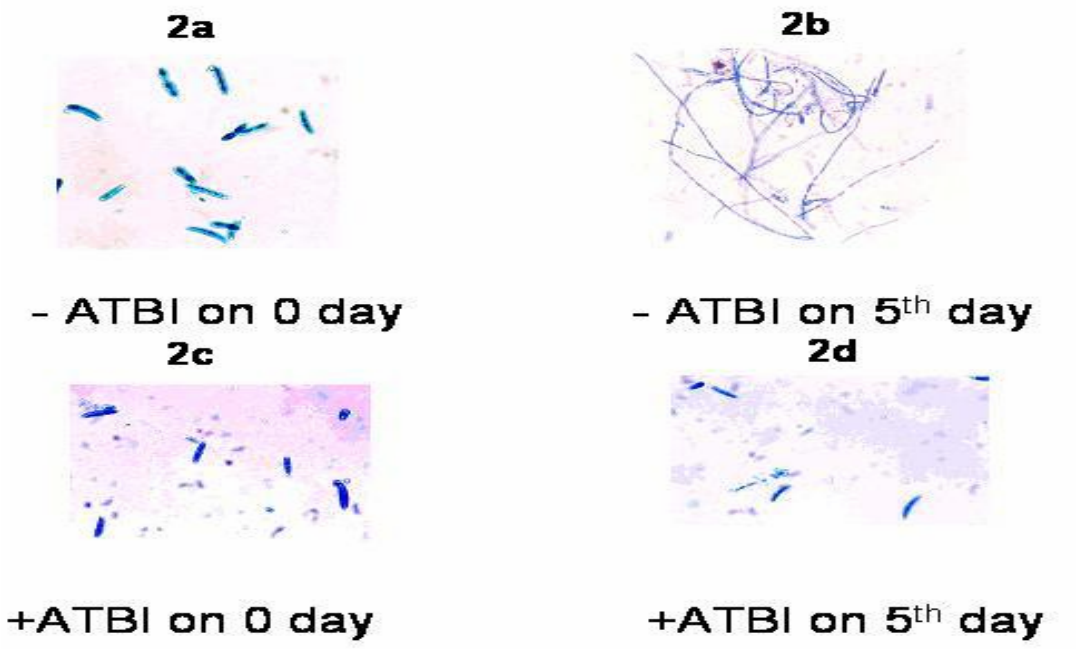


F. udum was grown on PDA plates for 24 hours at 30ºC. A sterile filter paper disc soaked in a solution containing 5 inhibitor units of ATBI was placed centrally. A zone of inhibition of growth (4mm in diameter) was observed on further incubation of 24 hours.

Figure 2 shows the spore germination assay of the *F.udum*. Pepstatin at the final concentration of 2µM inhibited spore germination for 5 days. A dose dependent inhibiton of spore germination was observed. The germ tubes produced at lower concentrations of ATBI were smaller those produced in water. Figure 2 shows the lactophenol stained spores of *F. udum* in the presence of 0.1 inhibitor units/ml of the

inhibitor. The control spores in water germinated with good mycelial growth (Figure 2 a and b) while the spores in the presence of the inhibitor remained ungerminated and swollen for 5 days (Figure 2 c and d). This indicates that the inhibitor is effective in stopping the germination of the spores and the subsequent formation of the mycelium.

Figure 2: Effect of ATBI on spore germination of *F. udum*



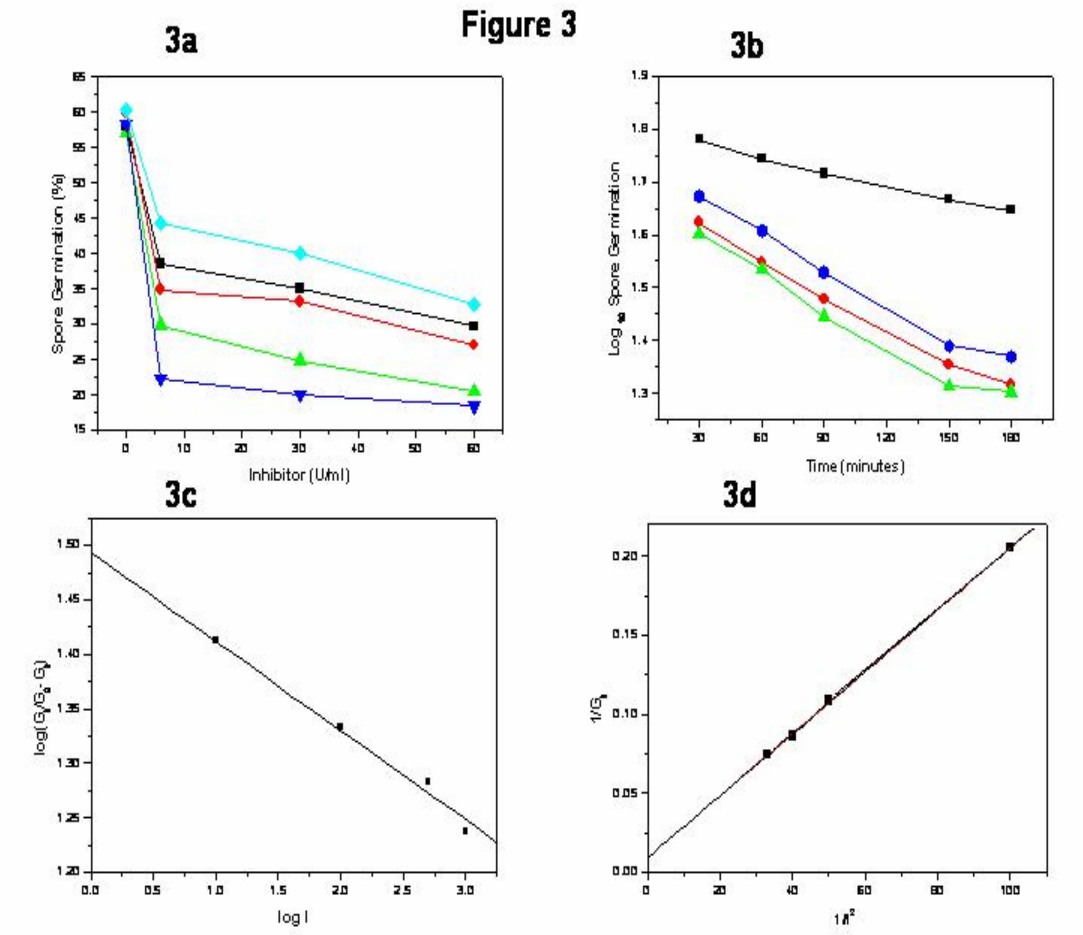
The spore germination was observed in the presence and absence of ATBI at a magnification of 400X. 2a: Spores of *F. udum* suspended in sterile water as observed on the 0 day of incubation. 2b: Spores suspended in water as observed on the 5th day of incubation at ambient temperature.2c: Spores suspended in solution of ATBI (0.1 inhibitor unit) as observed on the 0th day of incubation. 2d: Spores in the presence of ATBI on the 5th day of incubation.

Kinetics of inhibition of spores of F. udum

Figure 3a and 3b indicates the time and concentration dependent inhibitory effect of ATBI on germination of spores of *Fusarium udum*. The effect of ATBI was not

reversed even after 3 days of exposure as observed by continuous microscopic monitoring. The Hill plot of the data for *Fusarium udum* in Figure 3c gives a value of n=0.9254 and when a graph of reciprocal of percent inhibition (Figure 3d) is plotted against reciprocal of square of inhibitor concentration a straight line is observed.

Figure 3: Kinetics of inhibition of *F.udum* by ATBI



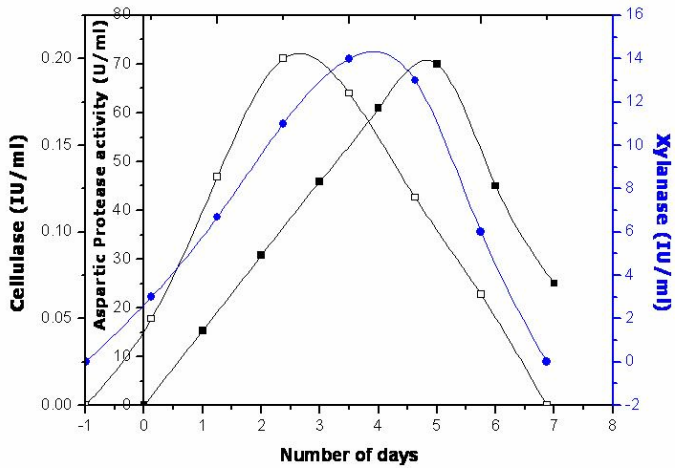
The kinetics of inhibition of spore germination was performed as explained in materials and methods. 3a: The concentration dependent effect of increasing concentrations of ATBI (5, 30 and 60 inhibitor units / ml) at 30 (⬥), 60 (■), 90 (●), 150 (▲) and 180 (▼) minutes on the spores of *F. udum* is indicated. 3b: Time dependent effect of 0 (■), 50 (●), 100 (●) and 150 (▲) inhibitor units / ml of ATBI over a period of time are indicated 3c: The Hill plot for the characterization of the mode of inhibition of spore germination by 10, 100, 500 and 1000

inhibitor units of ATBI is provided which indicates a complex mode of inhibition while the reciprocal plot for the inhibition of *F. udum* spore germination using ATBI confirms it (3d). G_0 and G_i are percent germination in the absence and presence of inhibitor respectively and I is the inhibitor concentration.

Production of hydrolytic enzymes by *Fusarium udum*

The time course of xylanase production was studied up to 7 days. A maximum of 14 IU/ml at 96 h was obtained in the presence of the inducer (Figure 4). The time course of cellulase production was studied up to 7 days. Cellulase production started without a lag and reached a maximum of 0.2 IU/ml at 72 h (Figure 4). The production profile of extracellular aspartic protease production as monitored for 8 days showed increased production in presence of soya meal as inducer. A maximum amount of 70 units of aspartic protease was observed at 120 h (Figure 4).

Figure 4: Time course of enzyme production



The time course following fermentation for 8 days was plotted. Cellulase (▫), xylanase (•) and aspartic protease production (■) was studied in the presence of cellulose paper powder, wheat bran and soyameal respectively.

Inhibition of hydrolytic enzymes of *F. udum*

A maximum inhibition of 21% of the extracellular xylanase was obtained with 0.1 inhibitor unit/ml of ATBI. The cellulase production was too low to estimate inhibition with ATBI. An inhibition of 32% of 10 units of aspartic protease was observed with 0.2 inhibitor units/ml of the inhibitor.

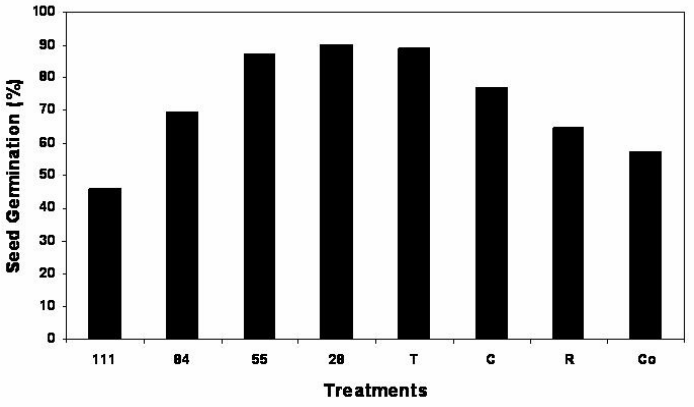
Assay for pathogen levels

The pathogen levels monitored in the wilt sick plot were in the range of 6-8 x 10^6 spores / ml. This data was obtained by random sampling of the soil at different time intervals.

Pot Trials

ATBI affected seed germination when used at a concentration of 112 inhibitor units per liter for drenching the soil around the pigeon pea plants. The effect on the seed germination was calculated on a percentage basis (Figure 5) to be 55%. This effect was seen to be minimized with 84 inhibitor units per liter of ATBI wherein a maximum of 71% inhibition of the growth of *F. udum* was obtained. Due to this significant decrease in the wilt percentage and the minimum effect on seed germination, further field trials were conducted with extracellular culture filtrate containing 84 inhibitor units of ATBI.

Figure 5: Effect of ATBI on seed germination in pot trials



Dose dependent drenching of soil containing spores of *F. udum* by ATBI (range used: 111, 84, 55, 28 inhibitor units per liter of inhibitor) in pots containing eight pigeon pea seeds per pot was conducted to see the efficacy of the inhibitor in controlling infection. Control treatments used were *Trichoderma* (T), Carbendazim (C), *Rhizobium* (R) and untreated pigeon pea (Co). The efficacy was evaluated by checking the seed germination in percent and the overall health of the plants.

Field trials

The dose dependent effect of the extracellular culture filtrate enriched in ATBI on wilt incidence in a highly wilt sick plot was analysed at the field level. A maximum disease intensity of 50% was observed in the absence of any control measures. The disease was controlled to an extent of 71% by 84 inhibitor units of ATBI as observed in Table 1. Carbendazim was effective in controlling 71% of the disease while the biocontrol agents *Trichoderma and Rhizobium* were successful in managing 53 and 61% of wilt respectively (Table 1). A dosage of 84 inhibitor units per liter of the inhibitor was found to be optimum for the treatment of the fungus in the field.

The dose dependent response on the wilt disease of pigeon pea is evident in the figure provided below. The 75% bacterial corresponds to the extracellular culture

filtrate containing 84 inhibitor units per liter of ATBI. The healthy pigeon pea plants bore flowers and subsequently the amount of seeds obtained i.e. yield was also substantially increased. Similar results were obtained with ICPL 87.

Table 1: Effect of extracellular culture filtrate enriched with ATBI on wilt of pigeon pea

Treatment	Number of Plants			Number of plants wilted			Percent Wilt			Mean % Wilt	Percent Disease Control
	RI	*RII*	*RIII*	*RI*	*RII*	*RIII*	*RI*	*RII*	*RIII*		
75% ATB ECF	12	54	53	3	2	8	25	3.70	15.09	14.59	70.6
50% ATB ECF	65	66	50	17	8	10	26.15	12.12	20	19.42	60.9
25% ATB ECF	53	53	71	13	9	13	24.52	35.84	18.30	26.22	47.21
Trichoderma viride	61	72	68	15	15	16	24.59	20.83	23.52	22.98	53.73
Rhizobium	51	50	74	8	14	10	15.68	28	13.51	19.06	61.62
Carbendazim	47	75	47	10	8	5	21.27	10.66	10.63	14.19	71.43
Control	46	53	31	23	20	19	50.00	37.73	61.29	49.67	-------

75% ATB ECF corresponds to the AlkaloThermophilic Bacillus Extracellular Culture Filtrate containing 84 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and *RIII* signify the replications in the randomized block design

The health and vigor of the plants can be observed in the following photographs:

Figure 6: Effect of ATBI on wilt of pigeon pea ICP 2376

A

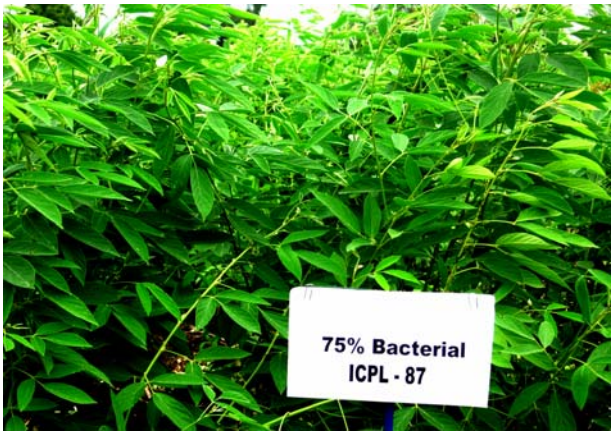
B

C

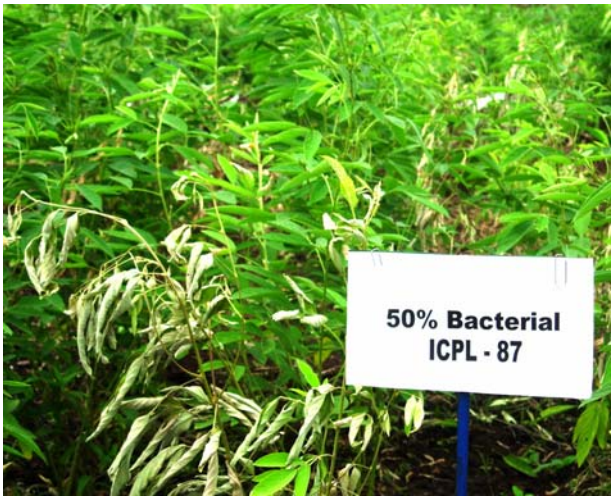
Field trials conducted with the extracellular culture filtrate of *Bacillus* resulted in the production of healthy plants. A: Untreated Control, B: ICP 2376 treated with extracellular culture filtrate of ATBI containing 84 inhibitor units per liter considered as 75% Bacterial, C: ICP 2376 treated with ATBI bearing flowers.

Figure 7: Effect of ATBI on wilt of pigeon pea ICPL 87

A



B



Field trials using the extracellular culture filtrate of *Bacillus* showed good healthy pigeon pea plants of ICPL 87. A: Pigeon pea plants treated with 75% partially purified ATBI B: Pigeon pea plants treated with 50% partially purified ATBI

The statistical analysis of the field trial data performed by the Chi test indicated a significant deviation from the expected value with 2 degrees of freedom. The data was subjected to the Yates correction and the degrees of freedom were therefore corrected to 1. The expected value of chi under the experimental conditions with a single degree of freedom is 10.83 with a confidence interval of 0.001 while the value obtained is 41.16. This deviation from the expected indicates that the null hypothesis that there is no effect of the inhibitor concentration on the wilting of plants is not true. Therefore we conclude that there is a significant effect of ATBI on the wilting of plants. The non linear regression analysis indicated a negative correlation signifying that as the inhibitor concentration increases the wilting percentage decreases. The t test is a popular test for the comparison of means of two groups. The groups used here were the treatments and the control. In the present study the value obtained 1.4058 is less than the expected value of 2.57 for the 2 sided t test as well as 2.01 for the 1 sided t test. This indicates that there is a significant difference in the means of the treated and the untreated samples and that this could be attributed to the inhibitor.

Formulation trials

The extra-cellular culture filtrate containing 84 inhibitor units / liter was found to be most effective in controlling wilt. This concentration was used to prepare the formulations wherein the solution containing inhibitor was mixed with talcum powder and used for coating the seeds of pigeon pea ICP 2376 and ICPL 87. Seed coating followed by drenching reduced wilt incidence compared to the control (Table 2).

Table 2: Effect of various formulations of the culture filtrate on the control of wilt of pigeon pea

Treatments	Disease Intensity (%) ICP 2376	Disease Control (%) ICP 2376	Disease Intensity (%) ICPL 87	Disease Control (%) ICPL 87
Seed Coating	30.42	45.47	26.18	25.70
Drenching	22.66	59.38	16.66	52.72
Seed Coating and Drenching	19.72	64.65	10.33	70.68
Control	55.79	-	35.24	-

Seed treatment as well as drenching was individually effective in achieving partial control; but additional soil application increased the efficacy (Figure 7). A maximum control of 65% with ICP 2376 and 71% with ICPL 87 was obtained with seed coating and additional drenching (Figure 8). The treatment of coating and drenching were on a par in their efficacy with carbendazim treatment (seed treatment followed by soil drenching with the fungicide). The reduction in wilt incidence was accompanied by increase in grain yield (data not shown). The health of the plants was additionally assessed by plant growth data. The nodulation data shoot and root length as also weight of the plants matched the trend obtained with the sampling analysis data (Tables 3 & 4).

Table 3: Effect of different formulations on nodulation, plant height, root and shoot length of pigeon pea after 150 days of treatment

Variety ICP 2376

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	11	128	29.00	98.00
Drenching	9.33	148.50	55.00	93.00
Seed coating and Drenching	7.66	162.50	37.00	125.00
Control	5.66	133.70	35.00	98.00

Variety ICPL 87

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	10.33	81.25	19.00	62.00
Drenching	10.33	82.40	29.00	53.00
Seed coating and Drenching	8.66	78.00	22.5	52.00
Control	5.66	133.70	19.00	61.00

Table 4: Effect of bioactive principles on weight of pigeon pea

Variety: ICP 2376

Treatment	Fresh weight			Mean	Dry weight			Mean
	I	II	III		I	II	III	
Seed coating	53.4	53.3	53.2	53.3	32.6	30.0	28	30.2
Drenching	96.8	98.6	99.5	98.3	53.8	49.9	51.2	51.63
Seed coating + drenching	70.2	70	75.5	71.56	42.8	38	39.5	40.10
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

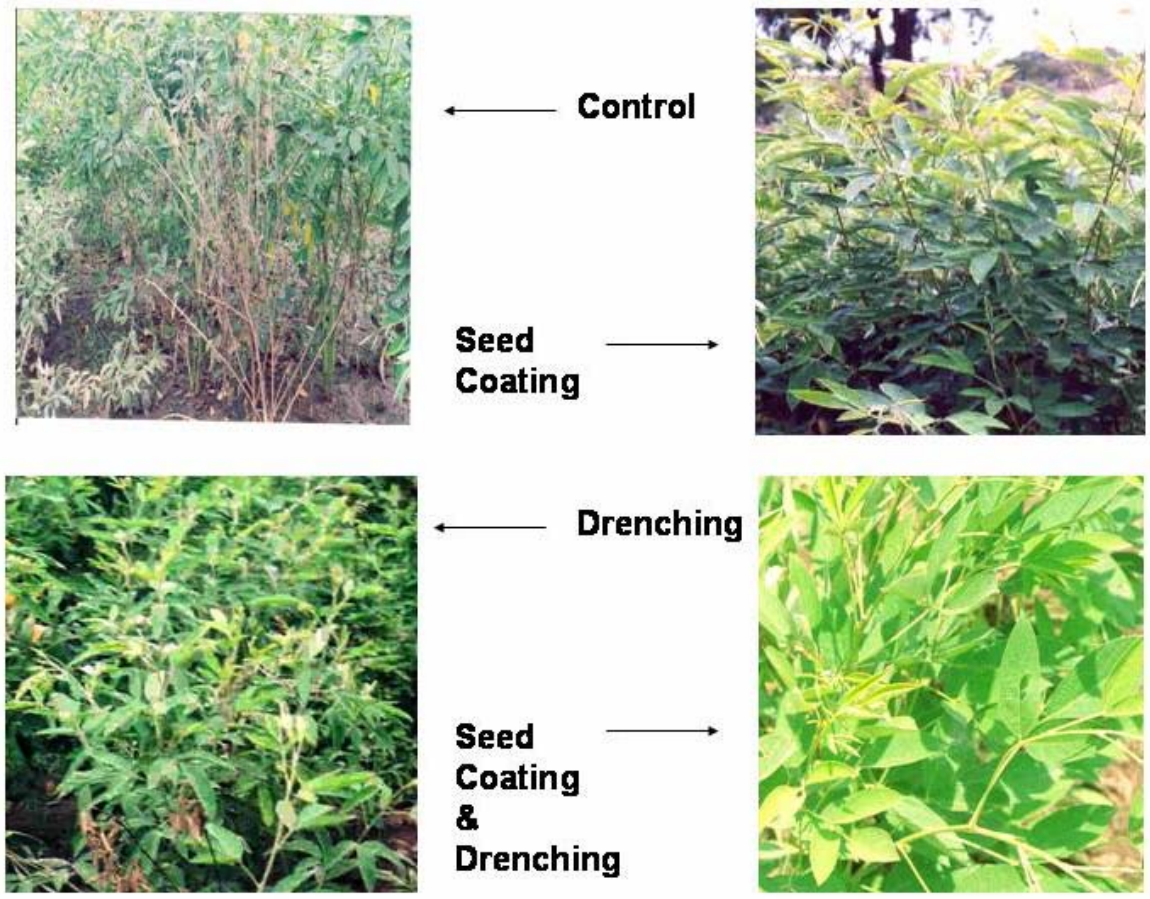
Variety: ICPL 87

Treatment	Fresh weight			Mean	Dry weight			Mean
	I	II	III		I	II	III	
Seed coating	32.4	32.0	32.1	32.16	24.20	22.00	21.70	22.63
Drenching	30.4	30.00	32.80	31.06	17.20	16.30	15.80	16.43
Seed coating + drenching	56.60	56.30	59.80	57.56	23.80	22.50	20.80	22.63
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

Figure 7: Effect of formulations on pigeon pea wilt of variety ICPL 87

Effect of formulations on pigeon pea wilt of variety ICPL 87 was analysed in field trials. The treatment of seed coating followed by booster doses of drenching resulted in maximum disease control of 71% as compared to the individual treatments of seed coating and drenching which resulted in disease control of 26 and 53%.

Figure 8: Effect of the formulations on the incidence of wilt on pigeon pea variety ICP 2376



Effect of formulations on pigeon pea wilt of variety ICP 2376 was analysed in field trials. The treatment of seed coating followed by booster doses of drenching resulted in maximum disease control of 65% as compared to the individual treatments of seed coating and drenching which resulted in disease control of 45 and 59%.

Drenching was more effective in enhancing the plant vigor and growth. The initial treatment of seed coating prevented the early onset of infection by the seed borne pathogen while drenching provided additional protection against soil borne pathogen.

The combined effect of seed coating and drenching was beneficial in preventing more than 60% of infection of plants of both varieties.

Control of wilt pf pigeon pea using VrAPI

Inhibitor production and purification

5kg of mung bean seeds of Kopergaon-1 variety were ground in water. The mixture was kept at room temperature for 3 hours after which the supernatant was obtained by centrifugation of the crude mixture. The supernatant was checked for inhibitor activity and used for all trials.

Antifungal activity assay

The partially purified inhibitor from mung bean seeds was evaluated for its potency as a fungal growth inhibitor. To assess the antifungal activity of the compound the molecule (VrAPI) was purified from the partially purified filtrate. During the purification process, the antifungal property of VrAPI was found to be co-purified with its aspartic protease-inhibitory activity. The antifungal activity of the purified VrAPI against 16 fungal strains was assessed in plate assays and the zone diameters were measured (Table 5).

Table 5: Inhibition of phytopathogenic fungi in plate assays as indicated by the diameter of the zone of inhibition

	Fungal Strain	Diameter of zone of inhibition (mm)
i)	*Alternaria solani*	1.2
ii)	*Aspergillus flavus*	4.5
iii)	*Aspergillus niger*	4.2
iv)	*Aspergillus oryzae*	4.0
v)	*Claviceps purpurea*	3.4
vi)	*Colletotricum*	2.5
vii)	*Curvularia fallax*	8.5
viii)	*Curvularia lunata*	7.5
ix)	*Curvularia cymbopogonis*	7.0
x)	*Fusarium oxysporum*	7.5
xi)	*Fusarium moniliforme*	8.0
xii)	*Helminthosporium*	6.5
xiii)	*Phomopsis*	5.5
xiv)	*Penicillium fellulatum*	4.0
xv)	*Penicillium roqueforti*	4.5
xvi)	*Trichoderma reesei*	6.0

VrAPI showed strong inhibitory activity against *C. cymbopogonis, Phomopsis sp., C. fallax, C. lunata, Helminthosporium, T. reesei, F. oxysporum, F. moniliforme and moderate activity against A. flavus, A. oryzae, A. solani, A. niger, P. roqueforti, P. fellulatum, sp.,* and *Colletotrichum* sp. as concluded from Table 5.

Fungal growth inhibition was also monitored in microscopic assay, wherein the spores of different fungal strains were cultured in the presence of varied concentrations of the inhibitor. After 24 h, the concentration of VrAPI required for 50% inhibition (IC_{50}) of fungal growth varied from 1.2 µg/ml for *C. fallax* to 6.5 µg/ml for *A. solani*, whereas the MIC ranged from 0.70 mg/ml to 5.70 mg/ml for the same. The phytopathogen *C. fallax* was found to be the most sensitive to ATBI, whereas *A. solani* was the least sensitive strain (Table 6).

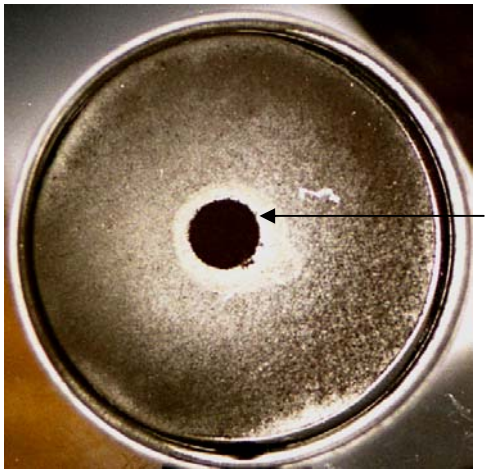
Table 6: IC_{50} and Minimum Inhibitory Concentration Values of VrAPI against phytopathogenic fungi

Fungus	**IC_{50} (µg/ml) of VrAPI**	**MIC (µg/ml) of VrAPI**
Alternaria solani	6.5	5.7
Aspergillus flavus	5.0	4.4
Aspergillus niger	5.3	4.6
Aspergillus oryzae	5.7	5.2
Claviceps purpurea	6.0	5.4
Colletotricum	6.3	5.6
Curvularia fallax	1.2	0.7
Curvularia lunata	2.5	1.5
Curvularia cymbopogonis	3.5	2.8
Fusarium oxysporum	3.0	2.5
Fusarium moniliforme	1.5	0.9
Helminthosporium	4.0	3.3
Phomopsis	4.6	4.1
Penicillium fellulatum	5.1	4.4

Penicillium roqueforti	5.8	4.9
Trichoderma reesei	4.3	3.7

The plate assay indicated a zone of inhibition of mycelial growth of *F. udum* of 6 mm in diameter in the presence of VrAPI (Figure 9).

Figure 9: Effect of VrAPI on mycelial growth of *F. udum*



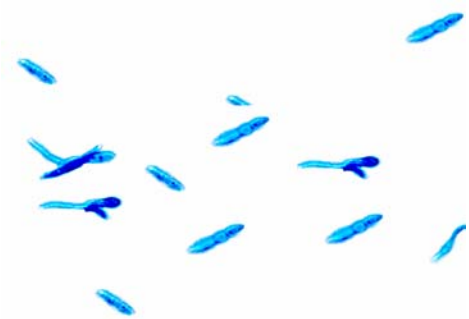
Clearance zone produced by VrAPI on the mycelium of *F. udum*.

F. udum was grown on PDA plates for 24 hours at 30ºC. A sterile filter paper disc soaked in a solution containing 7 inhibitor units of VrAPI was placed centrally on the plate. A zone of inhibition of growth (6mm in diameter) was observed on further incubation of 24 hours.

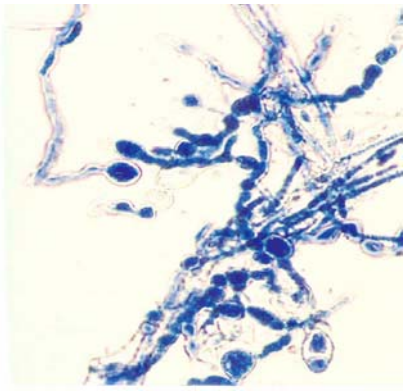
Figure 10 shows the spore germination assay of *F. udum*. Pepstatin at the final concentration of 2µM inhibited spore germination for 5 days. A dose dependent inhibition of germination of spores of *F.* u*dum* was observed. At lower concentrations of the inhibitor small germ tubes as compared to the control were produced. Figure 2 shows the lactophenol stained spores of *F. udum* in the presence of VrAPI. The spores in the presence of the inhibitor remained ungerminated and swollen for 5 days (Figure 2 c and d) while the control spores in water germinated with substantial mycelial growth (Figure 2 a and b). This indicates that the inhibitor is effective in

stopping the germination of the spores and the subsequent formation of the mycelium.

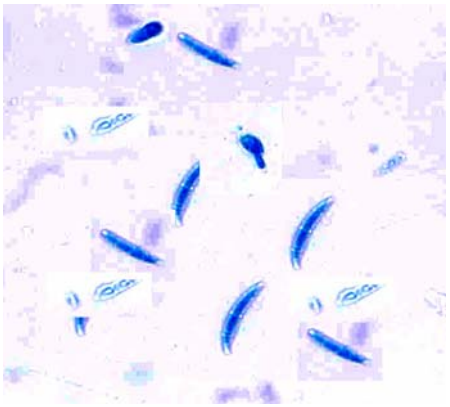
Figure 10: Effect of VrAPI on spore germination of *F. udum*



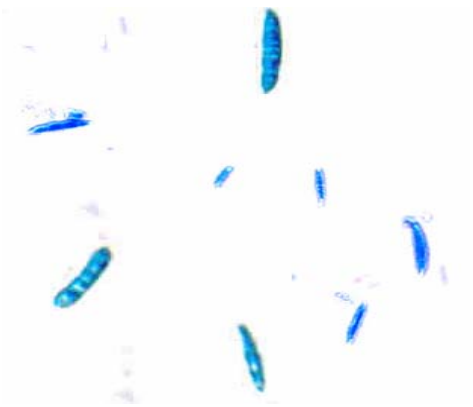
Water 0 day



Water 5th day



VrAPI 0 day



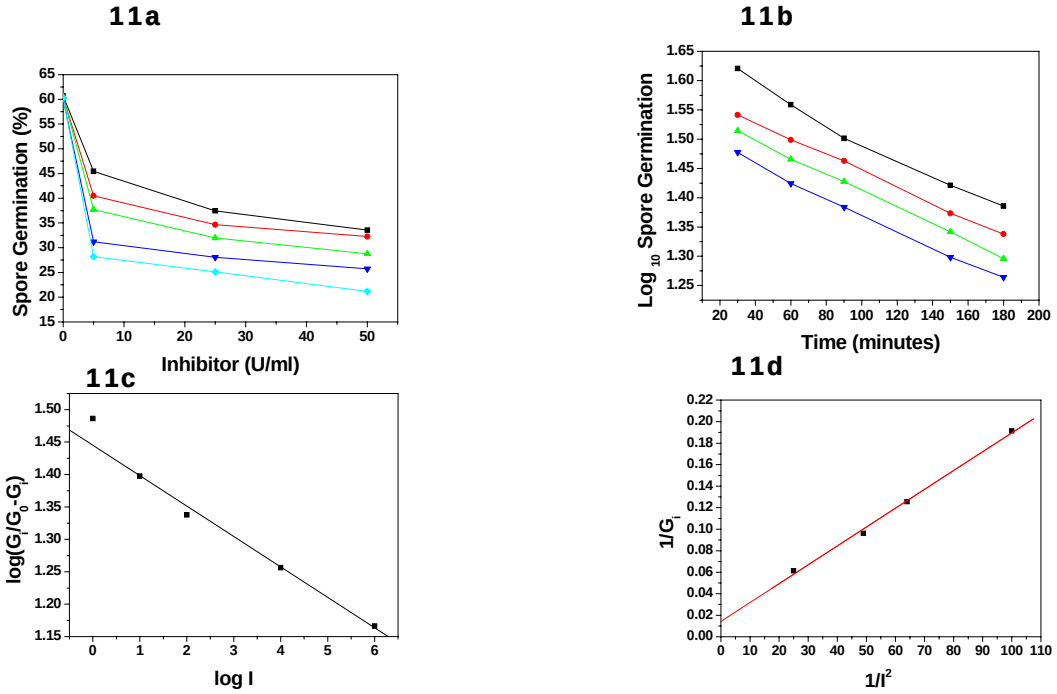
VrAPI 5th day

The spore germination was observed in the presence and absence of VrAPI at 400 X magnification. 10a: Spores of *F. udum* suspended in sterile water as observed on the 0 day of incubation. 10b: Spores suspended in water as observed on the 5th day of incubation at ambient temperature. 10c: Spores suspended in solution containing 0.1 inhibitor units of VrAPI as observed on the 0th day of incubation. 10d: Spores in the presence of VrAPI on the 5th day of incubation.

Kinetics of inhibition of germination of spores of F. udum

The effect time and concentration dependent inhibitory effect of VrAPI against germination of spores of *Fusarium udum* is shown in Figure 11a and 11b. The effect of VrAPI was not reversed even after 3 days of exposure as observed by continuous microscopic monitoring. The Hill plot of the data for *Fusarium udum* in Figure 11c gives a value of n=0.7542 and when a graph of reciprocal of percent inhibition (Figure 11d) is plotted against reciprocal of square of inhibitor concentration a straight line is observed.

Figure 11: Kinetics of inhibition of *F.udum* by VrAPI



The effect of VrAPI on inhibition of spore germination was assessed by carrying out kinetic experiments. 11a: The concentration dependent effect of increasing concentrations of VrAPI (5, 25 and 60 inhibitor units / ml) at 30 (■), 60 (•), 90 (▲), 150 (▼) and 180 (♦) minutes on the spores of *F. udum* is indicated. 11b: Time dependent effect of 0 (■), 40 (●), 80 (▲) and 160 (▼) inhibitor units / ml of VrAPI over a period of time are indicated 11c: The Hill plot for the characterization of the mode of inhibition of spore germination by 10, 100, 500 and 1000

inhibitor units of VrAPI is provided which indicates a complex mode of inhibition while the reciprocal plot (11d) for the inhibition of *F. udum* spore germination using VrAPI confirms it (11d). G_0 and G_i are percent germination in the absence and presence of inhibitor respectively and I is the inhibitor concentration.

Inhibition of hydrolytic enzymes of *F.udum*

Inhibition of the extracellular xylanase by VrAPI indicated that 0.08 unit/ml inhibitor units / ml inhibited 33% of the activity of the enzyme. 0.1 inhibitor units/ml of VrAPI inhibited 45% activity of 10 units of aspartic protease.

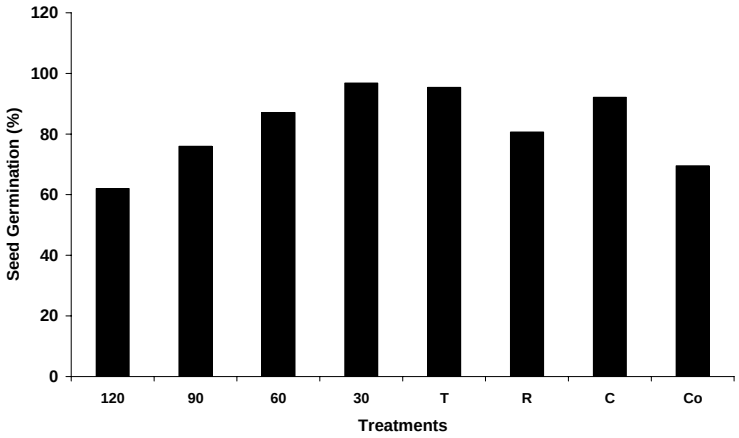
Assay for pathogen levels

The pathogen levels monitored in the wilt sick plot were in the range of 6-8 x 10^6 spores / ml. This data was obtained by random sampling of the soil at different time intervals.

Pot Trials

A maximum inhibition of 78% of wilt of pigeon pea ICP 2376 and ICPL 87 was observed with 120 inhibitor units of VrAPI which showed minimum effect of 37% on seed germination (Figure 12). Therefore further field trials were conducted with 120 inhibitor units of VrAPI.

Figure 12: Effect of VrAPI on seed germination in pot trials



Dose dependent drenching of soil containing spores of *F. udum* by VrAPI (range used: 120, 90, 60, 30 inhibitor units per liter of inhibitor) in pots containing eight pigeon pea seeds per pot was conducted to see the efficacy of the inhibitor in controlling infection. Control treatments used were *Trichoderma* (T), Carbendazim (C), *Rhizobium* (R) and untreated pigeon pea (Co). The efficacy was evaluated by checking the seed germination in percent and the overall health of the plants.

Field trials

Table 7 shows the dose dependent effect of the partially purified VrAPI on wilt incidence in a highly wilt sick plot. A maximum disease intensity of 50% was observed in the absence of any control measures. The disease was controlled to an extent of 78% by 120 inhibitor units of VrAPI as observed in Table 10. Carbendazim was effective in controlling 71% of the disease while the biocontrol agents *Trichoderma* and *Rhizobium* were successful in managing 53 and 61% of wilt respectively.

Table 7: Effect of VrAPI on wilt of pigeon pea

Sr No	Treatment	Number of Plants			Number of plants wilted			Percent Wilt			Mean % Wilt	Percent Disease Control
		RI	RII	RIII	RI	RII	RIII	RI	RII	RIII		
1	100 % PP VrAPI	35	44	61	3	10	1	8.57	22.72	1.64	10.97	77.91
2	75% PP VrAPI	47	46	90	7	10	18	14.89	21.73	20	18.87	62.00
3	50% PP VrAPI	57	28	79	10	11	18	17.54	39.28	22.78	26.53	46.58
4	25% PP VrAPI	65	45	59	18	20	17	27.69	44.44	28.81	33.64	32.27
5	*Trichoderma viride*	61	72	68	15	15	16	24.59	20.83	23.52	22.98	53.73
6	Carbendazim	47	75	47	10	8	5	21.27	10.66	10.63	14.19	71.43
7	*Rhizobium*	51	50	74	8	14	10	15.68	28	13.51	19.06	61.62

8	**Control**	**46**	**53**	**31**	**23**	**20**	**19**	**50.00**	**37.73**	**61.29**	**49.67**	**-------**

100% PP VrAPI corresponds to the Partially Purified *Vigna radiata* Protease Inhibitor containing 120 inhibitor units of VrAPI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, *RII* and *RIII* signify the replications in the randomized block design

A dosage of 120 inhibitor units per liter of the inhibitor was found to be optimum for the treatment of the fungus in the field. The healthy pigeon pea plants bore flowers and subsequently the amount of seeds obtained i.e. yield was also substantially increased. The health and vigor of the plants can be observed in the following photographs

Figure 13: Effect of VrAPI on pigeon pea wilt

A

B

Field trials were conducted to evaluate the efficacy of VrAPI on the pigeon pea variety ICP 2376 in field trials. A: Untreated Control ICP 2376; B: ICP 2376 treated with 100% VrAPI. The disease intensity was as high as 55% and an effective control of 78% was achieved with the inhibitor.

The same field trials were conducted with ICPL 87, one of the most popular varieties of pigeon pea and which is widely cultivated. Similar results were obtained with 77% control of wilt disease.

Figure 14: Effect of VrAPI on ICPL 87

The statistical analysis of the field trial data performed by the Chi test indicated a significant deviation from the expected value with 2 degrees of freedom. The data was subjected to the Yates correction and the degrees of freedom were therefore corrected to 1. The expected value of chi under the experimental conditions with a single degree of freedom is 10.83 with a confidence interval of 0.001 while the value obtained is 53.67. This deviation from the expected indicates that the null hypothesis that there is no effect of the inhibitor concentration on the wilting of plants is not true. Therefore we conclude that there is a significant effect of VrAPI on the wilting of plants. The non linear regression analysis indicated a negative correlation signifying that as the inhibitor concentration increases the wilting percentage

decreases. The t test is a popular test for the comparison of means of two groups. The groups used here were the treatments and the control. In the present study the value obtained 1.3265 is less than the expected value of 2.57 for the 2 sided t test as well as 2.01 for the 1 sided t test. This indicates that there is a significant difference in the means of the treated and the untreated samples and that this could be attributed to the inhibitor.

Effect of formulation studies of VrAPI on wilt

The results obtained with VrAPI indicated that 120 inhibitor units per liter were sufficient not only to control wilt occurrence but also in enhancing plant growth and vigor. The application of seed coating and booster doses of drenching was found to be better than any of the other applications in achieving a disease control of 64 and 70% in varieties ICP 2376 and ICPL 87 (Table 8). The increased vigor of the plants is evident in terms of the nodulation, fresh and dry weight, and root and shoots length data as also in the enhanced plant height data (Table 9, 10).

Table 8: Effect of formulations of VrAPI on pigeon pea wilt

Treatments	Disease Intensity (%) ICP 2376	Disease Control (%) ICP 2376	Disease Intensity (%) ICPL 87	Disease Control (%) ICPL 87
Seed Coating	29.42	44.47	27.18	24.70
Drenching	21.66	58.38	17.66	51.72
Seed Coating & Drenching	20.72	63.65	12.33	69.68
Control	55.79	-	35.24	-

Table 9: Effect of different formulations on nodulation, plant height, root and shoot length of pigeon pea after 150 days of treatment

Variety ICP 2376

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	5.7	89.50	24.00	105.00
Drenching	6.33	139.70	40.50	99.00
Seed coating and Drenching	7.33	149.00	34.00	135.00
Control	5.66	133.70	35.00	115.00

Variety ICPL 87

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	9.33	117.00	23.77	60.00
Drenching	7.66	88.40	35.91	57.00
Seed coating and Drenching	9	84.00	33.23	53.00
Control	5.66	133.70	31.06	59.00

Table 10: Effect of VrAPI on fresh and dry weight of pigeon pea

Variety: ICP 2376

Treatment	**Fresh weight**			**Mean**	**Dry weight**			Mean
	I	**II**	**III**		**I**	**II**	**III**	
Seed coating	44.8	44.2	49	46	22.5	20.5	19.2	20.73
Drenching	57.2	56.4	58.1	57.23	37.8	32.5	33.6	34.63
Seed coating + drenching	58	60	58.5	58.83	35.9	32	31.5	33.13
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

Variety: ICPL 87

Treatment	**Fresh weight**			**Mean**	**Dry weight**			**Mean**
	I	**II**	**III**		**I**	**II**	**III**	
Seed coating	86.2	89.1	86.4	87.23	44.2	41.0	42.0	42.73
Drenching	35.4	32.4	39.2	35.66	18.8	17	16.5	17.43
Seed coating + drenching	62	62.1	62.3	62.13	36.2	35.2	31.0	34.13
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

DISCUSSION

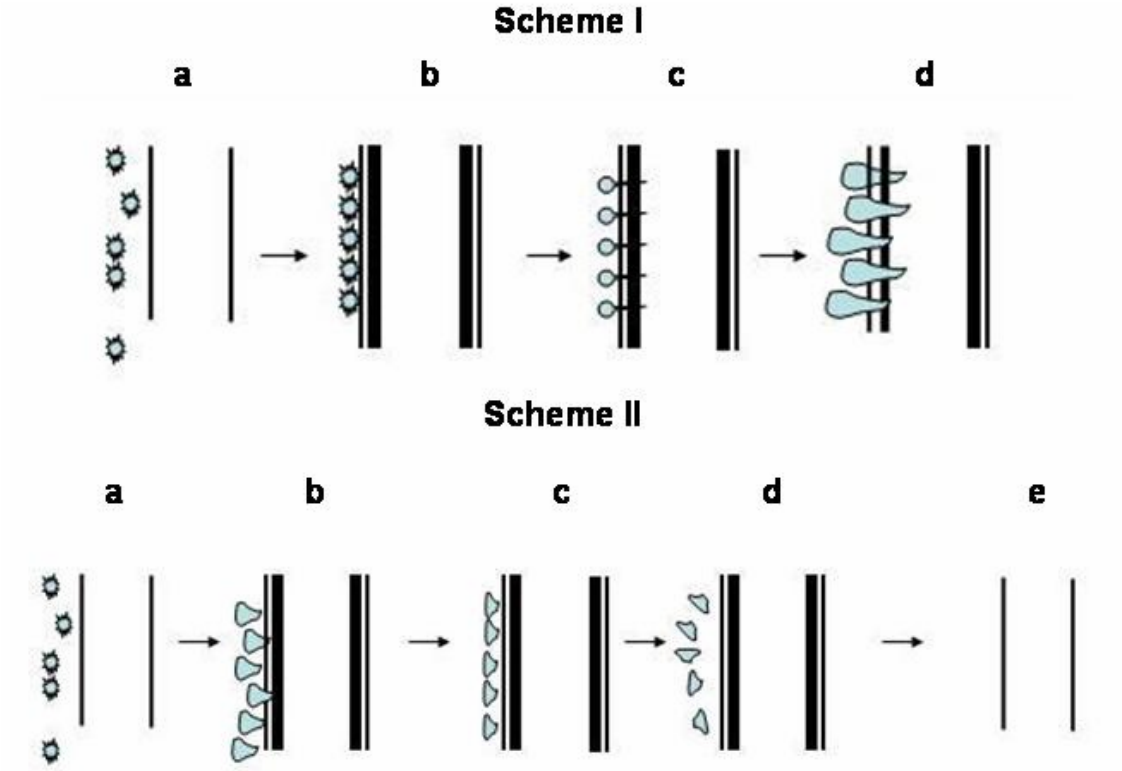
The biochemistry of mechanisms underlying host-pathogen interactions warrants study of the pathogen at the laboratory level. The inhibition of the growing phytopathogenic fungus and its enzymes b bioactive molecules may shed light on the strategies which could be employed for disease control. Xylanases, proteases and cellulases are some of the enzymes implicated in the invasion process of the pathogenic organisms (Whistler and Richards., 1970; Blake and Richards., 1971; Darvill et al., 1980., Cooper, R.M., 1983; Holden, F.R and Walton, J.D., 1992., Movahedi and Heale, 1990 a, b). Any successful pathogen produces a mixture of these enzymes for successful infection making them potential targets for the development of antifungal agents.

Protease inhibitors are an important class of defense proteins which have been implicated in a number of plant protection strategies. ATBI, a bifunctional inhibitor of xylanase and aspartic protease and VrAPI, an inhibitor of aspartic proteases were analyzed for their efficiency as an antifungal agent against *F. udum*, a pathogen of pigeon pea (Dash et al., 2001; Dash et al., 2001). The laboratory experiments included its effect on the growth of fungus at spore and mycelium stage and on the hydrolytic enzymes secreted by *Fusarium udum.* Both the inhibitors were efficient in stopping the growth of the fungus in plate assays thus proving its inhibitory action on the mycelia stage of *F. udum*. In addition the assays on germination of spores demonstrated their inhibitory effect of on the formation of germ tubes of the fungus. This indicated that the inhibitors were effective at both the mycelia as well as the spore stage. The complex multidimensional mode of inhibition could be visualized by the Hill and the reciprocal plots.

The inhibitors were found to inhibit the extracellular xylanase and aspartic protease produced by the fungus. These enzymes are critical in the germination of fungal spores and in the proliferation on the fungal mycelium. Their inhibition leads us to hypothesize that these critical processes are affected in the field also during pathogenesis of the host plant by the fungus. Thus the proposed mechanism of inhibition of *F. udum* by the inhibitors in the field is depicted in figure 15. In the soil, the spores of the fungus come into contact with the roots of the plant and adhere to it (Figure 15a, Scheme I). Upon adhesion the spores germinate forming the primary germ tubes which later form the penetrating mycelium, which proliferates inside the host tissues (Figure 15b-c, Scheme I). This leads to successful pathogenesis of the host plant leading subsequently to the occurrence of wilt (Figure 15d, Scheme I). This entire process is diagrammatically explained in Figure 15, Scheme I. In the presence of the inhibitor, the adhesion of the spores is unaffected. So, the spores succeed in adhering to the plant roots but they fail to form the germ tube (Figure 15a-b, Scheme II). Therefore invasion of host tissues is prevented (Figure 15c-d, Scheme II) leading to a healthy plant bereft of pathogen (Figure 15e, Scheme II). This mechanism is outlined in figure 15 Scheme II. The inhibition of growing mycelium by the inhibitors signifies that even if some of the spores germinate in soil their further proliferation is stopped. The reversibility of the inhibitory effect on the spores of *F. udum* indicates that it will not be killed in the soil but will be maintained in the environment in the spore form ensuring natural balance. The mechanisms outlined till date involve various modes of action, the most studied being mycoparasitism, competition and antibiosis (Elad, 1996). This bifunctional mode of action and its significance in the field provides a new area wherein further study will pinpoint the accuracy of the mechanism and will aid in the development of more novel molecules with potential as biocontrol agents.

The statistical analysis helped in scrutinizing the validity of the field experiments. The Chi test performed on the field trial data rejects the null hypothesis that there is no difference in disease occurrence between the control and the treatments by the inhibitors. The results indicate that there is a significant difference in the treatments and the control due to the use of the inhibitor. The non linear regression analysis evaluated the efficiency of the inhibitors in wilt control. The negative correlation obtained indicated that as the concentration of the inhibitor increased the intensity of wilt decreased. The t test was used to compare whether the groups indicated in the study i.e. the control and the treated plants group differ in their outcomes due to inhibitor effect and whether the difference is statistically valid. The null hypothesis was rejected and the alternative hypothesis that the groups do differ significantly was accepted at the 0.05 confidence level. This means that some of the difference between the control and the treated plants could be attributed to the treatment with the inhibitors.

Figure 15: Schematic mechanism of effect of inhibitors during infection by the pathogen in the field



In all schemes the plant roots are depicted by vertical lines and deposition of additional physical barriers by additional bold vertical lines. Scheme I depicts the normal method of progress of infection by spores of *F. udum*. a: The spores of *F. udum* are present in surrounding rhizosphere of the roots. b: They come in contact with the roots inducing additional physical barriers to be deposited. c: The spores form the primary germ tubes which invade the host tissues. d: Further mycelium formation and infection of host roots occurs.

Scheme II depicts the mechanism of the inhibitors in affecting the process of infection of roots of pigeon pea by *F. udum*. a: The spores are present in the vicinity of pigeon pea roots. b: The spores come in contact with the roots and try to form the primary germ tubes. c: Spores fail to form the germ tubes and are unable to initiate the infection process. d: The spores loose contact with the roots. e: The plant roots remain healthy and vigor increases.

Since the fungus is not killed it is maintained in the environment ensuring natural balance. The mechanisms outlined till date involve various modes of action, the most studied being mycoparasitism, competition and antibiosis (Elad, 1996). The present study outlines the control of wilt of pigeon pea by *F.udum* by aspartic protease inhibitors with a new mechanism providing a paradigm shift in biocontrol strategies wherein active biomolecules can be developed as biocontrol agents precluding the use of live organisms.

SECTION II

BIOLOGICAL CONTROL OF POWDERY MILDEW OF MUNG BEAN

Mung Bean

The genus *Vigna*, which is closely related to *Phaseolus* belongs to the family Fabaceae formerly known as Leguminosae. It is composed of more than 200 species that are native to the warm regions of the old and the new world. The genus contains several species that are of considerable economic importance especially in the developing countries. The species *V. radiata* was recognised as a separate species from *V. mungo* by Fery in 1980.

Vigna radiata

Scientific Classification:

Kingdom:	**Plantae**
Division:	**Magnoliophyta**
Class:	**Magnoliopsida**
Order:	**Fabales**
Family:	**Fabaceae**
Genus:	***Vigna***
Species:	***radiata***
Common Name:	**Mung Bean**
Former nomenclature:	***Phaseolus aureus***

Other names: nong taao, pua sha, moyashi-mame, lu tou, look dou, balatung, dau-xanh

Benefits

In India, *Vigna radiata* (mung bean) is one of the most important pulse crops from the agro-economic and nutritional point of view. India is the largest producer of mung bean accounting for 45% of the world's share of the area under cultivation and production. Area, production and productivity were 3.09 m ha, 1.12 m tones and 364 kg/ha respectively. It provides high quality protein, vitamins, carbohydrates and minerals especially for the vegetarian diet. Apart from human consumption it is used as a feed for the livestock and contributes substantially to soil nitrogen and phosphate solubilization. It is extensively grown in the Indian subcontinent, America and Africa. The overall significance of the crop especially the seeds and their importance in ayurveda makes mung bean a primary target for crop and yield improvement. This pulse is still today used as an opportunity crop in rotation with cereals. The plants are erect with few branches carrying pods borne in clusters near the top of the plant. Pods contain 8-15 green seeds. Its main advantages are that being a legume it does not require nitrogen fertilizer application and it has a short (75-90 days) growth duration which means that it requires less water and can be easily fitted into rotation programs. The seeds which are the edible part of the plant are small, cylindrical bean with a bright green skin.

Nutritional significance of mung bean

Nutrient	Units / kg
Water	734g
Energy	1060 kcal
Protein	75 g
Total lipid (fat)	4 g
Carbohydrate (by difference)	195 g
Fiber, total dietary	75 g
Sugars, total	20 g
Minerals	2.6 g – 0.05 mg
Vitamins	28 – 0.5 mg
Lipids	1 g
Cholesterol	0 mg
Amino acids	0.5 – 13 g
Beta carotene	140 mcg

USDA National Nutrient Database for standard Reference, Release 18 (2005)

Diseases

The productivity of the crop is restricted due to several abiotic and biotic stresses. In the recent past a large number of varieties having high yield potential, short maturity duration and resistant/tolerant to major diseases have been developed but their spread is slow due to non availability of seeds of improved varieties and poor knowledge of farmers about them.

The major diseases of mung bean are the yellow vein mosaic disease caused by a virus and the powdery mildew disease due to an obligate fungal pathogen. Insect pests especially bruchids are a persistent threat to the plants.

Powdery mildew

Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world. The name "powdery mildew" reflects the powdery tarnish on the plant surface and the tricking spores that are the result of the non-sexual reproductive phase of the fungus. Powdery mildew is a common and widespread plant disease that is caused by obligate biotrophic ecto-parasite. Powdery mildew fungi are classified in Kingdom, *Fungi*; Phylum, *Ascomycota;* Class, *Plectomycetes;* Order, *Erysiphales* and Family, *Erysiphaceae*. The family Erysiphaceae is divided into six main genera that are identifiable from one another by their differing clesothecial appendages and varying numbers of asci per cleistothecium. The genus *Erysiphe* is widely spread and contains many phytopathogens. It is identified by the cleistothecia which are dark brown, globose, having many unbranched appendages. There are multiple asci within each cleistothecium. This genus causes powdery mildew disease on plants such as clover, cucurbits, beans, soybeans, delphinium, hydrangea, zinnia, phlox, aster, crepe myrtle, and tulip poplar. The representative species *Erysiphe polygoni* ***c***auses powdery mildew disease of beans, soybeans, clovers, and other legumes, cabbage, beets, cucumber, canaloupe, delphinium, and hydrangea.

Disease Control

Fungicidal products containing dithiocarbamate, quinomethianate and sulphur have long been known as active against powdery mildew. However they are predominantly protective and their persistence and activity are often relatively small, thus requiring frequent applications. In the early 1970s, the situation changed dramatically with the

introduction of systemic products that established new standards of control for powdery mildews. The mode of action of these systemic fungicides involved specific inhibition of physiological processes and they were effective at lower concentrations. The first systemic fungicides used against powdery mildew were benomyl, mopholines, tridemorph and dodemorph, and the 2- aminophyrimidines, dimethirimol and ethirimol. Intesive research throughout the agrochemical industry expanded options for powdery mildew control in the 1980s through introduction of several triazoles (sterol demethylation inhibitors, DMIs) and two additional members of the morpholines group, fenpropimorph and fenpropidin. Recent new chemistry has improved standards of disease control still further through the development of strobilurins (Clough and Godfrey, 1998), azoxystrobin, kresoxim methyl, and trifloxystrobin (Margot et al., 1994), a "morpholine" type compound, spiroxamine (Dutzmann et al., 1996), and a phenoxyquinoline, quinoxyfen (Longhurst et al., 1996). One consequence of using of systemic fungicides was to reduce dose rates. This allowed chemistry to seriously challenge plant breeding as a cost effective way to control powdery mildews in cereals.

The impact of chemical control has been tempered by the ease of powdery mildews developing resistance, quickly rendering many systemic fungicides ineffective (Holloman and Wheeler, 1999; Limpert et al. 1996; Lyr et al., 1999). Indeed, powdery mildew has a number of characteristics, which favor a rapid adaptation rate, such as its relatively short generation time, with sexual recombination throughout the year, and the nature of its spread, as newly adapted pathotypes can be carried relatives quickly by wind over a wide area. For instance, sterol demethylation inhibitors (DMIs) are not used as single compounds against powdery mildew anymore. Likewise, inhibitors of sterol reductase and isomerase, the morpholines, have shown some sensitivity shifts (spiroketalmines are also affected due to cross

resistance). Isolates of wheat powdery mildew resistant to both strobilurins and the quinoline fungicides, quinoxyfen were detected at a low frequency in some parts of Europe. Many different resistance mechanisms are possible:

The modes of action of different fungicides within each of the groups are very similar or the same. Any pathogen population that is resistant to one fungicide within a group will almost certainly be resistant to other members of that same group. The issue of cross-resistance adds a dimension that limits the flexibility for managing resistance. Once resistance develops to one fungicide, others in that group are likely to also become less effective or useless.

In contrast to cross-resistance, pathogen populations have been shown to develop resistance to fungicides in more than one chemical group. The intensive use of at-risk fungicides in different chemical groups without following resistance management principles can result in the development of multiple resistances. Couple multiple resistances across groups with cross-resistance within groups, and the loss of efficacy for a large number of fungicides is possible.

The hazards of chemical control agents are evident in the form of environmental damage and accumulation of toxic residues within the body tissues of animals and humans. This has warranted the need to search for less hazardous and more eco-friendly alternatives in management of diseases of mung bean.

Current work

This section reports the development of aspartic protease as biocontrol agents in the disease management of powdery mildew of mung bean. There are no reports of control of this disease on mung bean by a biological agent. The obligate nature of

the pathogen inhibits the development of biological controls since laboratory studies cannot be performed. However, this study is based on the assumption that all fungi require more or less aspartic proteases and xylanases for their development and successful pathogenesis. Therefore the inhibitors of these enzymes were thought to be effective in controlling the growth of the fungi in field. Since laboratory studies could not be carried out, the inhibitors were directly analysed at the pot and the field stage. Another dimension was that if any agent could be found to be even partially successful in controlling the disease then it could be used in the partial substitution of the hazardous chemical agents especially sulphur which are used on a large scale.

MATERIAL AND METHODS

Production of inhibitors

The alkalothermophilic *Bacillus* species was grown for 48 hours as described in Dash *et al*, 2001. Extracellular culture filtrate obtained by centrifugation of the growth after 48 hours at 5000 rpm for 20 minutes was used for pot and the large-scale field trials. The seed of Kopergaon-1 variety were ground in 4 volumes of Gly HCl buffer (0.05M, pH 3.0) and used for the preparation of partially purified inhibitor as described in the section on pigeon pea.

Pot Trials

For the pot trials of 21 pots, a randomized block design with two plants of the susceptible variety Kopergaon-1 of mung bean per pot was used. For all the trials conducted sulfur and carbendazim were used as controls. Treatments began 1 week after the first observation of mildew lesions and continued for 8 weeks. Treatments were carried out with a 5-liter backpack sprayer. Per plant 50ml liquid was sprayed so that there was no drip off as indicated in the diagram below:

Figure 16: Spraying of inhibitors on mung bean crop

Field trials

For the large scale field trial for analyzing dose dependent inhibition of powdery mildew of mung bean a randomized block design experiment in a plot of size 4 x 2.4 metres with three replications was performed as given in Table 11.

Table11: Experimental Design

Coding of Treatments

Treatments	Code
100% PIS	T1
75% PIS	T2
50% PIS	T3
25% PIS	T4
Wettable Sulphur (0.2%)	T5
Carbendazim	T6
Control	T7

PIS: Protease Inhibitor solution

Randomized block design

RIII	T3	T5	T1	T7	T2	T6	T4
RII	T7	T6	T5	T4	T3	T2	T1
RI	T1	T2	T3	T4	T7	T6	T5

Variety used is Kopergaon-1; RI, RII and RIII represents the number of replicate (T1-T7); one bed (ex. T1) consist of five line i.e. 15-20 seed with plot size of 2 X 2 (4 sqm) with spacing of 10cm between plants and 30 cm between rows.

Plant distance was 10cm in the rows and 30cm between the rows. Each experimental unit included 75 plants of Kopergaon-1, the highly susceptible variety of mung bean. 3 sprayings were performed every 15 days with a low-pressure sprayer in a dose dependent manner. The plants were observed for the powdery mildew disease

symptoms. The inhibitor sprayed plants were compared with the suitable controls and the entire data was subjected to statistical analysis.

In each treatment 10 plants of mungbean were selected and 5 leaves were observed for mildew observations on 30 DAS and there after at 5 days interval upto 60 Days by following 0-9 scale. Thus the percentage of leaf area covered by mildew was assessed using a key with 9 disease classes (0= 0% mildew; 3= 6-10%; 5= 21-30%; 7= 41-60% and 9= 81-75%. The key provided most detail in the severity range 1-40%, the more relevant part of the total range 0-75%. The assessments were made weekly on a "middle" leaf level (about leaf 15) and a "high" leaf level (about leaf 28). From each experimental unit, mung bean was harvested and weighed to give the yield per plot. An average value of the replications is reported.

For statistical analysis the mildew assessments (means of scored classes) were averaged per plot. Observed responses (severities) were rearranged in derived responses (Campbell and Madden, 1990): AUDPC, EGR and MML. The percent disease intensity of powdery mildew was calculated by the formula given below –

$$\text{Percent Disease intensity (P.D.I.)} = \frac{\text{Sum of all Numerical Ratings}}{\text{No. of plants observed} \times \text{Highest Rating}} \times 100$$

The figures indicate that out of a population of 100 plants what it the proportion of the wilted ones.

The AUDPC (Area Under Disease Progress Curve) was calculated by the formula given below.

AUDPC = K [(Si + Si-1) / 2] x D

Where, Si. = Disease intensity at i^{th} day of evaluation

K = No. of successive evaluation

D = Interval between i and i=1 evaluation of disease.

MMLs were determined by taking the means of the last three assessments per experiment. The EGRs were calculated by linear regression through three data points per plot. An √EGR + 0.5) transformation was carried out before analysis of variance. Analysis of variance and the Tukey test were carried out with arc sine square root transformed data. Treatment effects on the cumulative yield of mung bean heavier than 150g per plot per experimental unit were tested by ANOVA and the Tukey test.

RESULTS

Area under disease progress curve

The AUDPC of mung bean summarize mildew progress during the 8 weeks of experimentation (Table 12). 75% dilution of the culture filtrate gave significant reduction of mildew. The AUDPC values for the other dilutions were significantly smaller than those of 75% dilution. There was significant reduction (79 and 86%) in the incidence of mildew with carbendazim and sulfur respectively as compared to control (Table 12).

Table 12: Effect of ATBI on powdery mildew of mung bean

Treatments (inhibitor units per liter)	**Disease Intensity (%)**			**Mean**	**Disease Control (%)**	**AUDPC**
	RI	RII	RIII			
75%ECF T2	19.95	15.53	22.84	19.44	69.59	273.83
50%ECF T3	19.95	15.53	24.84	20.10	68.55	353.60
25%ECF T4	27.08	31	28.44	28.84	54.88	424.6
Wettable Sulphur (0.2%) T5	7.55	8.88	9.99	8.77	86.26	135.94
Carbendazim T6	11.11	13.33	16.4	13.61	78.71	168.05
Control T7	66.8	60.50	64.50	63.93	-	649.13

75% ECF corresponds to the extracellular culture filtrate of Bacillus sp. containing 112 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

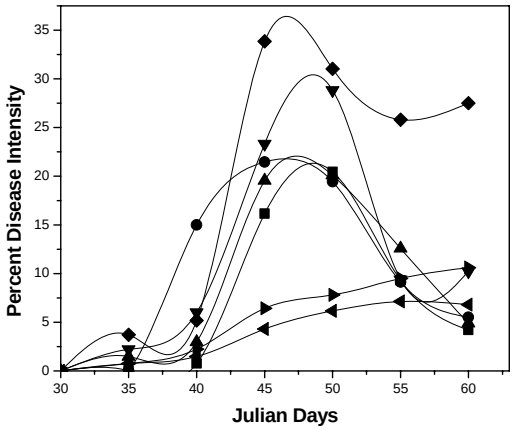
Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and RIII signify the replications in the randomized block design

Mildew Progress Curves (MPC's)

Figure 13 shows the MPC's of the field trial on the susceptible mung bean cv Kopergaon-1. Effective control of PM was obtained with 75% bacterial culture filtrate containing 112 inhibitor units per liter of the inhibitor. Although treatments with sulphur and carbendazim were more effective than with any dosage of the culture filtrate, the study indicated that biological control agents could be developed. After 40 days after sowing when the effect of the culture filtrate was reduced one booster dose of antifungal agent could be included to give a combined strategy for inhibition and for reducing the application of harmful pesticides. Similar results were obtained with the MML levels attained in the presence of the inhibitor and the chemical control agents. It is thought that an earlier start wherein the biocontrol agent is applied right from day 1 would have been a better alternative. Also it would have been better to use increased concentration of the bioactive principles in the culture filtrate by effectively concentrating the culture filtrate.

Figure 13: Mildew Progress Curves for powdery mildew of mung bean



The mildew progress curves for powdery mildew of mung bean indicated that substantial disease control was achieved with 100% ECF (▼), 75% ECF (●), 50% ECF (▲), 25% ECF (■), sulphur (▶) and carbendazim (◀)as compared to the control (◆).

Early Growth Rates and MMRs

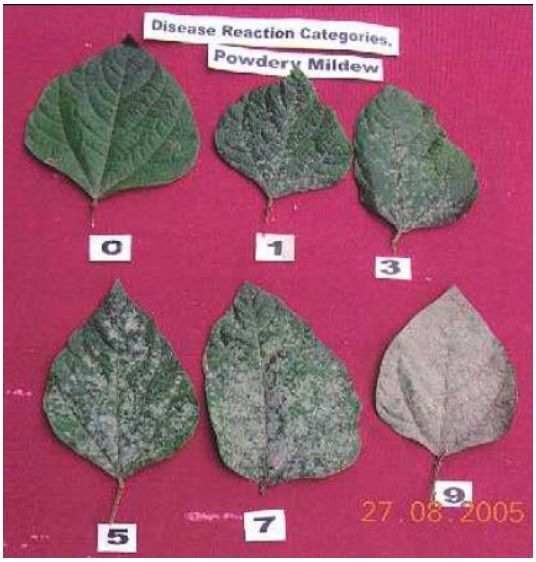
As observed in Figure 13, the early growth rate of mildew fungus was slow in case of all the treatments. After the 35th day of observation, mildew levels were seen to rise. As compared to the control the growth rate was less in the treatments with the biocontrol agent especially with higher doses of the agent. Maximum mildew levels attained in the control were 35-40% which increased to 70% by the end of the season. The maximum mildew level attained with sulphur remained low at 10% while that with the highest dosage of the biocontrol agent remained at 35%.

Mildew Observations

Figure 14 illustrates the intensity of the powdery mildew disease under the local climatic conditions indicating more than 51% leaf area to be infected by the

pathogen. This percentage figure was obtained by using the disease scale for the disease reaction categories.

Figure 14: Disease Reaction Categories for powdery mildew of mung bean



The disease intensity of powdery mildew was scaled on a scale of 0-9 as

0 : No powdery growth on leaves.

1 : Small scattered powdery speaks covering 1% or less area of leaf.

3 : Small scattered powdery speaks covering 1-10% of the leaf area.

5 : Powdery leaf scions enlarging with gray coloured powdery mass.

7 : Gray coloured powdery growth covering 26-50% of the leaf area.

9 : Gray coloured pathogen of powdery growth covering 51% or more leaf area.

Field trial

Figure 14 denotes the effectiveness of ATBI in controlling the infection of powdery mildew in the pot trials. The dose dependent field trials indicate that 84 inhibitor units of the inhibitor were able to control 70.00% of the disease (Figure 14, Table 13). Chemical control with carbendazim and wettable sulphur showed that 79% and

86% of the disease could be managed. Also, the inhibitor being extracellular, the organism can be effectively separated from the bio-formulation which becomes eco- and user friendly.

Figure 14: Effect of the extracellular culture filtrate of *Bacillus* on powdery mildew of mung bean

A: A disease intensity of 64% was observed in the absence of any control measures.

B: The disease was controlled to an extent of 70% using the extracellular culture filtrate of *Bacillus* containing 112 inhibitor units of ATBI.

Yield

The mean yield showed a two fold increase with 75% of the culture filtrate as compared to the yield in control. Carbendazim and Sulfur showed almost equal increase in the yield. The mean increase in yield for the biocontrol agent was comparable to the chemical control agents used as seen in Table 14.

Table 14: Effect of application of extracellular culture filtrate on yield of mung bean infected with powdery mildew

Treatments (inhibitor units per liter)		Yield of mung bean (g)			Mean
		RI	RII	RIII	
75%ECF	T2	317	295.18	317.01	309.73
50%ECF	T3	291.86	321.05	294.08	302.33
25%ECF	T4	257	249	238	248
Wettable Sulphur (0.2%)	T5	361.95	346.08	379.17	326.4
Carbendazim	T6	340.2	325.6	337	334.26
Control	T7	178	166.80	160.02	168

75% ECF corresponds to the extracellular culture filtrate of Bacillus sp. containing 112 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and RIII signify the replications in the randomized block design

Aspartic protease from plant source

Area under disease progress curve

The AUDPC of mung bean summarizes mildew progress during the 8 weeks of experimentation (Table 15). 100% of the partially purified VrAPI solution gave 60% reduction in mildew. There was significant reduction (79 and 86%) in the incidence

of mildew with carbendazim and sulfur respectively as compared to control (Table 15).

Table 15: Effect of VrAPI on powdery mildew of mung bean

SN	Aspartic Protease Inhibitors	Percent disease intensity (%)	% Disease control	AUDPC
1	**100 % PP VrAPI**	25.62 (30.36)	59.92	338.39
2	**75% PP VrAPI**	32.09 (34.48)	52.00	405.15
3	**50% PP VrAPI**	32.63 (34.20)	48.95	436.71
4	**25% PP VrAPI**	34.37 (35.89)	46.26	435.17
5	**Wett sulphur (0.25%)**	8.77 (17.17)	86.26	135.94
6	**Carbendazim (0.1 %)**	13.61 (21.59)	78.71	168.05
7	**Unsprayed control**	63.93 (53.10)	---	649.13
	SE +/- CD at 5 % CV (%)	1.25 3.68 7.05		

100% PP VrAPI corresponds to the Partially Purified *Vigna radiata* Protease Inhibitor containing 120 inhibitor units of VrAPI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

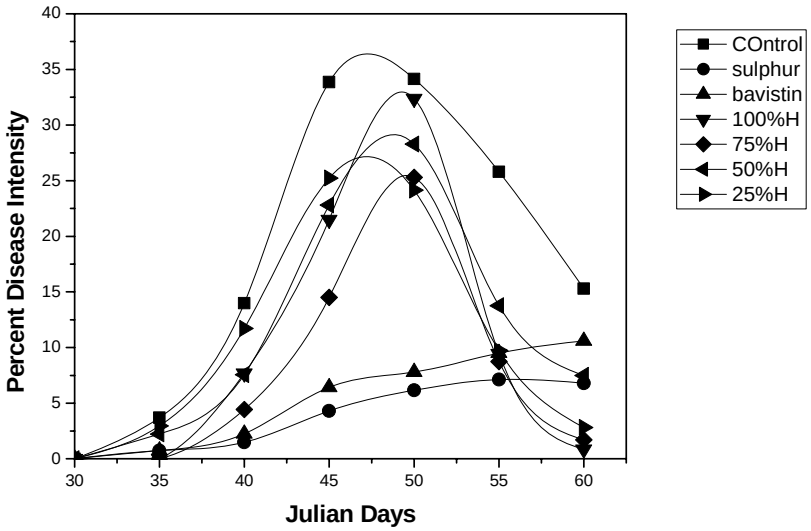
RI, *RII* and *RIII* signify the replications in the randomized block design

Figures in parentheses are arc sin values.

Mildew Progress Curves (MPC's)

Figure 15 shows the MPC's of the field trial on the susceptible mung bean cv Kopergaon-1. Effective control of PM was obtained with 100% partially purified VrAPI containing 120 inhibitor units per liter of the inhibitor. Although treatments with sulphur and carbendazim were more effective than with any dosage of the culture filtrate, the study indicated that biological control agents could be developed. After 40 days after sowing when the effect of the culture filtrate was reduced one booster dose of antifungal agent could be included to give a combined strategy for inhibition and for reducing the application of harmful pesticides. Similar results were obtained with the MML levels attained in the presence of the inhibitor and the chemical control agents.

Figure 15: Mildew progress curves for VrAPI



The mildew progress curves for powdery mildew of mung bean indicated that substantial disease control was achieved with the control agents as compared to the untreated control.

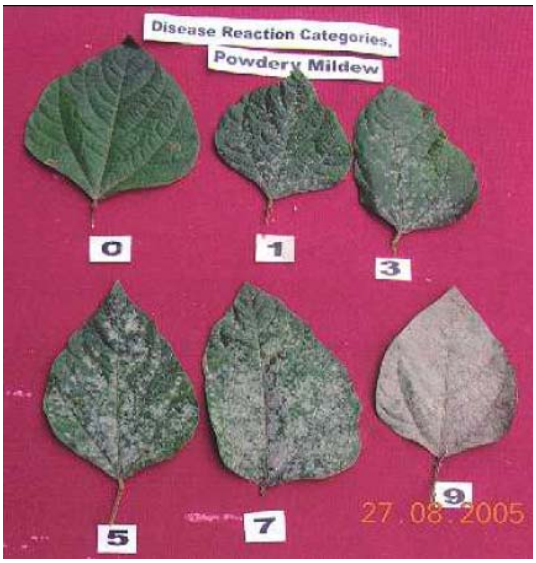
Early Growth Rates and MMRs

As observed in Figure 15, the early growth rate of mildew fungus was slow in case of all the treatments. After the 35th day of observation, mildew levels were seen to rise. As compared to the control the growth rate was less in the treatments with the biocontrol agent especially with higher doses of the agent. Maximum mildew levels attained in the control were 35-40% which increased to 70% by the end of the season. The maximum mildew level attained with sulphur remained low at 10% while that with the highest dosage of the biocontrol agent remained at 35%.

Mildew Observations

Figure 16 illustrates the intensity of the powdery mildew disease under the local climatic conditions indicating more than 51% leaf area to be infected by the pathogen. This percentage figure was obtained by using the disease scale for the disease reaction categories.

Figure 16: Disease Reaction Categories for powdery mildew of mung bean



The disease intensity of powdery mildew was scaled on a scale of 0-9 as

0 : No powdery growth on leaves.

1 : Small scattered powdery speaks covering 1% or less area of leaf.

3 : Small scattered powdery speaks covering 1-10% of the leaf area.

5 : Powdery leaf scions enlarging with gray coloured powdery mass.

7 : Gray coloured powdery growth covering 26-50% of the leaf area.

9 : Gray coloured pathogen of powdery growth covering 51% or more leaf area.

Field trial

Figure 17 denotes the effectiveness of VrAPI in controlling the infection of powdery mildew in the field trials. The dose dependent field trials indicate that 120 inhibitor units per liter of the inhibitor were able to control 60% of the disease (Figure 3, Table 4). Chemical control with carbendazim and wettable sulphur showed that 79% and 86% of the disease could be managed.

Figure 17: Effect of VrAPI on powdery mildew

The figure indicates the positive control obtained with 100% partially purified VrAPI (right). All the healthy plants bore flowers and fruits. The control remained the same as with ATBI (left).

More yields were obtained in the presence of inhibitor. This indicates the positive effect of VrAPI on health of the plants.

DISCUSSION

Phytopathogens are responsible for enormous losses ($30-$50 billion annually) in cultivated and stored crops and are a major impediment to effective food distribution worldwide[1]. Countervailing measures in the form of antimicrobials and pesticides not only significantly increase production costs and are regarded as serious environmental contaminants, they contribute to the increase in antimicrobial-resistant species. Consequently, there has been considerable activity in developing disease resistance strategies; however, most of these have been fairly narrow with respect to the microbial spectrum of protection.

Antimicrobial peptides play a primary role in host defense. To most researchers, they immediately call to mind protein fragments as antigens, or signaling molecules between the cellular components of innate and/or adaptive immunity. However, all life forms also produce peptides with a direct capacity to inactivate microorganisms, as a central component of innate defense (Fossom; 1970). These antimicrobial peptides (AMPs) have an ancient lineage and have likely participated in the relentless battle between host and pathogen throughout evolution, so helping to shape the relationship between host and microbe, be it commensal or pathogen (Broekaert et al., 1997). Plant protection has seen significant progress in the use of antimicrobial peptides (Weindling and Emerson., 1936; Weindling., 1937; Weindling., 1941; Hanson and O'Leary., 1981; Papavizas., 1985., Ignoffo and Anderson., 1979). However the nature of these antimicrobial peptides has not been outlined and studied in detail. Even though biocontrol agents have been developed against many other crop pathogens, there are no biocontrol agents reported for powdery mildew of mung bean.

Powdery mildew is a devastating disease on a major crop plants. The obligate nature of the pathogen has however hampered progress in the development of biocontrol agents against the fungus, *Erysiphe polygoni*. Since no laboratory studies can be carried out for the development of specific agents against the fungus, the control of powdery mildew has lagged behind. The development of the fungus on the host plant occurs via the formation of haustoria or the sucking roots which penetrate the host tissues and form the mycelium which further leads to proliferation inside the plant ultimately ending in disease. During the formation of haustoria from the spores of the fungus, xylanases and proteases are required. Therefore the inhibitors of these enzymes which do not affect the plant enzymes could be developed as effective biocontrol agents against the fungus. This is all the more possible since the process of haustoria formation occurs only after the spores sit on the host tissues and get an ambient condition for germination. If at this stage inhibitors of the critical enzymes are applied then the spores are unable to germinate and form haustoria. Therefore they cannot invade the plant tissues. This leads to effective disease control.

Both ATBI and VrAPI were found to be effective against xylanases and proteases of phytopathogenic fungi such as *F. udum*. Therefore these could be responsible for the prevention of haustoria formation leading to effective powdery mildew management. Although partial control could be achieved which was less as compared to the chemical control agent, sulphur; it was thought that these inhibitors could be developed further such that the use of sulphur could be reduced by combinatorial treatments.

CHAPTER V

ASPARTIC PROTEASE INHIBITORS AS BIOCONTROL AGENTS

"The study of establishment and spread of plant disease is an exercise in ecology. The interactions involved are multidimensional and the partial control of such leads to disease management."

Robert Schein

ABSTRACT

Biocontrol agents are those which protect the plants from insect and pathogens attack. To date chemical control has been the strategy of choice for protecting the plants from pathogenic attack. The use of pesticides has resulted in severe environmental pollution and harm to the natural flora and fauna. Therefore alternative strategies are being sought to control their spread. This chapter focuses on the use of protease inhibitors as biocontrol agent. The effective management of powdery mildew of mung bean and fusarial wilt of pigeon pea has been demonstrated by laboratory, pot and field experiments. The successful use of the agent indicates that there are simple solutions to complicated problems in nature and we should look for alternatives from nature itself. The successful field trials indicate that without the use of any microorganism and using very simplified approaches plant diseases can be managed effectively. This study lends an altogether different perspective to the research on biocontrol of essential crop plants.

INTRODUCTION

Since the beginning of agriculture, farmers have had to cope with natural factors (biotic and abiotic) for attaining maximum yield of their crops. The biotic factors include pests, pathogens and weeds whereas the abiotic factors are lack or excess of water in the growth season, extreme temperatures, high or low irradiance and nutrient supply. Effective crop management by physical, biological and chemical measures leads to sustainable agriculture wherein the impact of these stresses on the crops is reduced. The current chapter focuses only on the pathogen related losses and their control by biological agents.

Current scenario of crop damage

The major biotic stressors responsible for huge crop losses are weeds, pests, viruses and fungi (Agrios, 1997). There are serious losses due to diseases especially in cereals, legumes and ornamentals. The annual global losses caused by pathogens amount to as much as US $300 billion (Thomas 1999).Extensive research has been conducted on the control of weeds and pests resulting in numerous control strategies. However the control of viruses and fungi has proved difficult due to location specific pathovars and constant emergence of resistant strains. There are no accurate estimates available for assessment of crop losses due to pathogens on a global basis. This is due to the inconsistency of data wherein infection due to pathogens often coincides with climatic changes such as irregular rainfall, increased humidity, or drought, which in themselves may lower crop output. Pathogen outbreaks may have a devastating impact in a given year, but cause only marginal losses in other years (Yudelman, Ratta, and Nygaard 1998). Some of the crop losses have been summarized by Oerke, 2006.

Table 1: Region wise distribution of estimated crop losses due to plant diseases

Region	US$ in billion	Percent of potential production
Asia	43.8	14.2
Former Sovlet Union	8.2	15.2
North America	7.1	9.7
Latin America	7.1	13.5
Europe	5.8	9.8
Africa	4.1	15.7

Source: Oerke et al. (2006).

Current control strategies

The use of chemical to protect crops from pathogen infection has led to short term benefits. Their use has led to contamination of soil, water and vegetation. Toxic residues in plants and agricultural produce like food grains, fruits and vegetables and even milk have built up. Human consumption of this contaminated food constitutes an important health hazard (Soon 1997). The unregulated use of chemical control agents has led to the development of resistant strains of pathogen (Thomas 1999). Thus the overall consensus is that the indiscriminate, excessive, and inefficient use of chemicals exacts too high a toll in terms of human health, environmental safety, and ultimate diminishing returns to justify any short-term increases in farm income or food output.

Although the emphasis is on anticipating pathogen outbreaks and preventing them from reaching economically damaging levels, some ecofriendly control strategies

have to be implemented for maximum crop production. Some such control strategies include:

- *Biological control*, such as protecting, enhancing, and releasing natural enemies of the pathogen;
- *Cultural practices*, such as ecological landscaping, crop rotation, improved crop residue management, better water management, and improved pathogen monitoring;
- *Use of naturally occurring chemicals,* with less reliance on synthetics in favor of biochemicals (naturally occurring chemicals that control pathogen incidence and spread); and
- *Genetic,* such as the use of naturally resistant varieties, new varieties bred for resistance, or transgenic varieties

The intense requirement for more environment friendly and sustainable approaches has oriented research in favor of biological control agents.

Biological control agents

Extensive research on pest control has led to the discovery and use of biocontrol agents against pest infestation. The research on pathogen control has led to the development of *Trichoderma, Gliocladium, Bacillus* and *Pseudomonas* on a commercial scale. These agents are however used in the form of spores or as live organisms in the field. This results in alteration of the natural flora and fauna. Therefore alternative bio-based strategies wherein the active biological principle can be developed as biocontrol agent are required.

Protease inhibitors in plant protection

Protease inhibitors (PIs) are ubiquitous in distribution with reports from microbial, animal and plant origin. The discussion will be restricted to plant protease inhibitors in plant defense. PIs are the most studied class of plant defense proteins which can be classified as biochemicals. They are abundantly present in seed storage tissues and represent up to 10% of total protein (Casaretto and Corcuera 1995). PIs have been studied in plants for various roles which among others also include their importance in pathogen and insect attack (Brzin and Kidric 1995). In many cases their accumulation in quantities far more than required for inhibiting endogenous proteases, underline their role as defense proteins. In others an absence of inhibitory activity against endogenous plant proteases with presence of activity against proteases of pests and pathogens confirms their role in defense.

Serine protease inhibitors

Of all the classes of PIs, the serine PIs are the most abundant and also the most studied proteins. Serine protease inhibitors have antinutritional effects against several lepidopteran insects. These inhibitors inhibit enzymes from species of *Tribolium*, *Callosobruchus*, *Manduca*, *Haematobia*, *Stomoxys*, *Spodoptera*, etc. A direct test of the roles of the inhibitors in plant leaves to defend against insects was first demonstrated by Hilder et al (Hilder et al., 1987). SBBTI isoinhibitors have been reported to inhibit completely the activities of mealworm (*Tenebrio molitor*), red flour beetle (*Tribolium castaneum*) and the migratory locust (*Locusta migratoria*). It has been shown that these are associated with resistance to insect and fungal attack and that the inhibitors can inhibit the proteases of microbes including pathogens. Peng and Black have observed that levels of trypsin inhibitors increase more in leaves in varieties of tomato that were resistant to *Phytophthora infestans* than in susceptible varieties. An increased proteinase inhibitory activity in melon plants infected with

Colletotrichum was noted by Roby et al (Roby et al., 1987). Reduced protease activity of proteases from *Fusarium solani* and *Colletotrichum* species was observed with protease inhibitors from healthy bean and tomato plants. Many protein inhibitors of trypsin and chymotrypsin isolated from seeds suppress microbial serine proteases like subtilisin and the proteases from molds like *Aspergillus.* Such inhibitors have been reported from families Gramineae, Cucurbitaceae, Amaranthaceae and Polygonaceae. These proteins are structurally similar to the potato proteinase inhibitor I which itself is active against many microbial enzymes (Valueva and Mosolov, 1999; Ryan, 1979). There are few reports of the effects of seed proteinase inhibitors on the enzymes of phytopathogenic microorganisms. Soyabean and kidney bean inhibitors from the SBBTI family were shown to suppress proteases of phytopathogenic fungi from the genus *Fusarium*. Buckwheat trypsin inhibitor suppressed the activity of protease of *Alternaria alternata* while those from sunflower seeds suppressed the activity of the proteases of the causative agent of gray mold. In several cases, plant resistance to microorganisms was found to correlate with the content of protease inhibitors as seen in wheat caryopses and lupine and soyabean trypsin inhibitors. Serine protease inhibitors have been reported from potato, barley, tomato, pumpkin, chick pea, cowpea, black beans, mung beans, adzuki beans, broad beans, jack beans (Ryan, 1990). The serine protease inhibitors having antifungal activity have the interesting property of inhibiting the α-amylase activity from insects but not from bacterial/ mammalian sources. These bifunctional inhibitors which inhibit fungal and insect growth have been reported from corn, ragi, wheat, barley, potato and daisy. Serine protease inhibitors have been used to produce transgenic plants. These transgenic studies were instrumental in establishing the importance of protease inhibitors in plant protection. One of the best demonstrated examples in this case was the transfer of the gene for the cowpea inhibitor. This inhibitor was proved to be a strong antimetabolite against insects from

the genera *Heliothis*, *Spodoptera*, *Diabrotica* and *Tribolium.* Hence the modified gene for this inhibitor was transferred to tobacco plants. The transgenic plants manifested higher resistance to insects from the genus *Heliothis* and *Manduca* as compared to the wild type plants. Thus the serine protease inhibitors class remains the best studied class of inhibitors in the plant kingdom for their participation in plant defense.

Cysteine protease inhibitors

The presence of this class of protease inhibitors in plants has been known for years. Their role in the plant defense against pest attack has been more recognized. These inhibitors are now referred to as cystatins and there are at least three distinct families (Barrett et al. 1987). The amino acid sequences of the pineapple and rice cysteine protease inhibitor have now been determined. Cysteine protease inhibitors have been reported from pineapple (Reddy et al., 1975), potato (Rodis and Hoff, 1984), corn (Abe and Whitaker, 1988), rice (Abe et al., 1987), cowpea (Rele et al., 1980), millet (Tashiro and Maki, 1986), mung bean (Baumgartner and Chrispeels, 1976), tomato, wheat, barley and rye (Fossom, 1970).

Cysteine proteases from insect larvae can be inhibited by both synthetic and naturally occurring cysteine protease inhibitors (Campos et al, 1989; Gatehouse et al., 1985; Kitch and Murdock, 1986; Lemos, 1987; Wieman and Nielsen, 1988). A specific cysteine protease inhibitor produced by *Aspergilllus japonicus*, called E—64 (trans-epoxysuccinyl-leucyl-augmentine), was shown to inhibit the digestive enzymes of several insect species including the Colorado potato beetle (Wolfson and Murdock, 1987), the common bean beetle (Wieman and Nielsen, 1985), the Mexican bean beetle, the red flour beetle and the cowpea weevil (Campos et al., 1989; Murdock, 1987; Murdock, 1988). E-64 caused a delay of development and larval

death, supporting the importance of cysteine protease inhibitors in the control of insect larvae on plants. An interesting observation was that the protease complex from the red flour beetle was inhibited by the same protein component from wheat endosperm, which inhibits the cysteine protease from wheat caryopses. The oryzocystatins from rice suppressed the activities of proteases from the intestines of rice weevil and the red flour beetles. Recently transgenic rice plants expressing the phytocystatine gene show wider specificity to the different cysteine proteases than rice oryzocystatin (Irie et al., 1996). Phytocystatin isolated from the transgenic tobacco plants inhibited papain and Cathepsin H as well as the cysteine proteases from weevil digestive complex. Many cysteine protease inhibitors have been demonstrated to show potentiating effects on the toxins responsible for insecticidal activity. The combined activities of both serine and cysteine proteases show a higher protective effect against insect larvae than either of them alone.

Aspartic protease inhibitors

The knowledge of aspartic protease inhibitors in plant protection is very scanty. There have been reports of aspartic proteases in insect guts, but conclusive evidence showing the role of the inhibitors in protection has not been reported. Pepstatin, a powerful and specific inhibitor strongly inhibited the gut enzymes of the Colorado potato beetle (Wolfson and Murdock, 1987). Potato tubers have shown the presence of a cathepsin D inhibitor that exhibits considerable amino acid homology with the soyabean trypsin inhibitor (Mares et al., 1989). In fact, many aspartic protease inhibitors from potato tubers have now been reported.

Metalloprotease inhibitor

Plants have evolved at least two families of metalloprotease inhibitors, the metallo-carboxypeptidase inhibitor family in potato (Rancour and Ryan, 1968) and tomato plants (Graham and Ryan, 1981; Hass and Ryan 1981), and a cathepsin D inhibitor family found in potatoes (Keilova and Tomasek, 1976). The cathepsin D inhibitor is unusual as it inhibits trypsin and chymotrypsin but it does not inhibit aspartic proteases. The metallo-carboxypeptidase inhibitors are polypeptides. These inhibitors are strong competitive inhibitors of a broad spectrum of carboxypeptidaases from animals and microorganisms but they do not inhibit the enzymes from yeasts and plants (Hass and Ryan, 1981). This inhibitor is found in potato tubers and leaves along with the potato inhibitor I and II families of serine protease inhibitors. Further study on these inhibitors needs to be carried out since there are not many reported.

Basis of selection of PIs for effective inhibition of the pathogen

The success of PI based strategy depends upon the selection of appropriate PIs and their proper expression. An ideal effective PI should have the following characteristics: (1) It should have activity against broad spectrum of pathogens (2) It should remain stable to degradation during the infective process of the pathogen. An added advantage is gained when the PI has additional inhibitory functions where it targets more than one enzyme of the pathogen. This is true if the PI in addition to proteases can inhibit, enzymes like xylanases, cellulases and pectinases which are commonly used by the pathogen for its invasion of plant tissues.

In summary, there is an urgent need to study and use molecules like PIs to control the problem of plant disease. The success of transgenic PI based strategies depends upon (i) identification of potent inhibitors, (ii) detailed in vitro and in vivo studies to

check their efficacy to inhibit pathogen growth, (iii) use of PIs in combinations. Devising a multifold strategy will retard pathogen growth, delay disease advance and arrest the growth of pathogen in the field thus minimizing the damage to crop yields. Thus there is tremendous potential for developing protease inhibitors as effective biocontrol agents either for individual application or as part of a multifold strategy in disease management.

SECTION I

BIOLOGICAL CONTROL OF WILT OF PIGEON PEA

Pigeon pea

Pigeon Pea (*Cajanus cajan, syn. Cajanus indicus*) is a member of the family Fabaceae. Other common names are red gram, toovar, toor, gandul, Congo pea, Gungo pea, and no-eye pea.

The cultivation of pigeon pea goes back at least 3000 years. The centre of origin is most likely Asia from where it traveled to East Africa and by means of the slave trade to the New World. Today pigeon peas are widely cultivated in all tropical and semi-tropical regions of both the Old and the New World.

Pigeon pea

Scientific classification

Kingdom	**Plantae**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Order	**Fabales**
Family	**Fabaceae**
Subfamily	**Faboideae**
Genus	***Cajanus***
Species	***cajan***
Binomial name	**Cajanus cajan (L.) Millspaugh**

General Description

Pigeon pea occurs in several varieties. The old varieties in cultivation and semi cultivation in India are semi deciduous, short-lived shrubs usually 1 to 4 m in height. They are usually single stemmed, freely branching, and become woody after a few months. The wood is moderately hard and brittle. They have a deep taproot (to 3 m) with lateral roots and nodulated fine roots. The branches and fine twigs support abundant light-green or yellow-green foliage. Spirally arranged, silky-pubescent, trifoliolate leaves have narrowly elliptic, lanceolate ore oblong leaflets, 2.5 to 9 cm long, the center leaflet being slightly longer than the laterals. The five- to 12-flowered racemes are axillary. Flowers are about 2 cm long, yellow, the standard often being orange to purple outside. The legumes, which are flattened, somewhat constricted between seeds, and 4 8 cm long, are mottled bronze-purple when immature, drying to brown. They contain two to nine mottled brown (white, red, brown, gray, or black in improved varieties) seeds, 7 to 8 mm long by 6 mm broad. There are 2n = 22, 44, or 66 chromosomes (Liogier 1988, Long and Lakela 1976).

Benefits

Pigeon peas are an important food in developing tropical countries. An excellent source of protein, the seeds (and sometimes the pods) are eaten as a vegetable, as a flour additive to other food, in soups, and with rice (Center for New Crops and Plants Products 2002). Although they vary slightly, typical nutritional values for seeds are: moisture, 10.1 percent, protein 19.2 percent, fat, 1.5 percent, carbohydrates, 57.3 percent, fiber 8.1 percent, and ash, 3.8 percent (Smartt 1976). The species is planted as a green manure crop, nurse crop, cover crop, a windbreak hedge, as a host for lac insects, and as food for silk worms. The stalks are used for fuel, thatch, and basketry (Center for New Crops and plants Products 2002). Pigeon pea forms root nodules in association with *Rhizobium* sp. Bacteria and is capable of

fixing 41 to 280 kg/ha of nitrogen (Red de Grupos de Agricultura de Cobertura 2002) thus becoming a nitrogen fixer for soil maintenance. Preparations of the leaves are used to treat jaundice, inflammation, and sores of the mouth (Parrotta 2001).

Overall pigeon pea (*Cajanus Cajan L*.) is one of the major grain legume crops of the subtropics and ranks sixth in world wide dryland legume production (Nene and Sheila, 1990). It is now widely grown in the Indian subcontinent which accounts for almost 90% of the world pigeon pea production. Pigeon pea can survive well in poor soils and an outstanding root system enables it to tolerate drought.

Disease

The crop is infected with *Fusarium udum* Butler causing soil borne wilt leading to 30-100% loss of the total crop yield (Kannaiyan et al., 1984). The pathogen invades the host by producing a germ tube from the mycelium / spores which penetrates seedlings through root tips, wounds or lateral roots. The vascular tissue gets infected leading to vascular plugging followed by wilting of the plant during flowering and pod-filling stages (Reddy et al., 1990, Beckman and Roberts, 1995).

Disease control

Chemical control of wilt is environmentally polluting and hazardous. High-yielding, disease-resistant crop varieties have been developed however they are not completely resistant to the pathogen at all the locations and resurgence of the pathogen is a recurring problem.

Research in the area of biocontrol has resulted in the use of fungal and bacterial species as biocontrol agents for sustainable agriculture (Baker., 1981; Marshall and Walters et al., 1994). Fungal species such as *Trichoderma, Gliocladium* and bacteria

like *Pseudomonas* and *Bacillus* have been reported in the control of *Fusarium* wilt and *Rhizoctonia* wet root rot of chick pea (Weindling and Emerson., 1936; Weindling., 1937; Weindling., 1941; Hanson and O'Leary., 1981; Papavizas., 1985). The development of organism free substitutes for the chemicals has lagged behind due to the paucity of reports on the biochemistry of the host-pathogen interactions. Even though it is known that pectinases, xylanases, cellulases and proteases are the enzymes secreted by the pathogens to invade plant tissues, their inhibitory molecules have not been studied as an approach for biocontrol. Also these biocontrol agents are applied in the form of spores or the whole live organism mixed with suitable carriers and applied as seed coats. This method of application is impractical as it offers protection only once when the plant is in the seed stage and not at the later stages.

Current work

In the present studies the effect of the aspartic protease inhibitors on disease management of pigeon pea wilt caused by *Fusarium udum* is summarized. The inhibition of xylanase and aspartic protease produced by the fungus indicates the effect of the protease inhibitors on limiting the entry of the pathogen in host tissues. The reversible inhibition of spore germination indicates the static effect of the inhibitors on transition from the spore to the mycelium stage. Taking into consideration the antifungal effect on the phytopathogenic fungus, the inhibitors were used for disease management of wilt on pigeon pea at the pot and the field level. An in depth statistical analysis of the field trial data is provided and a mechanism correlating the laboratory findings with the field trial data is suggested.

The development of formulations of the inhibitors, their applications in the field and the comparison with suitable controls wherein the classical method of drenching was utilized is also explicitly worked out. The formulations contained high amounts of the

inhibitors and were assessed for their efficacy at field level and the data for plant growth analyzed.

MATERIAL AND METHOD

Materials

Peptone, Yeast extract, tryptone, was obtained from Hi media Laboratories Ltd, wheat bran was purchased locally. All other chemicals used were of analytical grade. Carbendazim was obtained from the market on a commercial basis while *Trichoderma* and *Rhizobium* were prepared at the field site. Seeds of Kopergaon-1 were obtained from the local market.

Identification of Bacillus halodurans

The genomic DNA was purified as per the method mentioned in Sambrook and Maniatis. The 16S rDNA was sequenced and blasted at NCBI (www.ncbi.nih.gov/blast).

Purification of aspartic protease inhibitors

Bacillus halodurans was grown in a medium containing soyameal as the inducer for production of ATBI as mentioned in Dash et al., 2001. The inhibition of fungal protease of *Aspergillus saitoi* by ATBI was assessed at various stages of purification (Dash and Rao, 2001). HPLC purified ATBI was used for all laboratory experiments. VrAPI was purified from the dormant seeds of mung bean Kopergaon-1 variety as mentioned in Chapter IV. HPLC purified VrAPI was used for all laboratory experiments.

Antifungal activity of VrAPI

The antifungal activity of VrAPI was analyzed against the phytopathogenic fungi *Alternaria solani* (NCIM 887), *Aspergillus flavus* (NCIM 535), *Aspergillus niger* (NCIM 773), *Aspergillus oryzae* (NCIM 643), *Claviceps purpurea* (NCIM 1046), *Colletotricum*

(NCIM 1032), *Curvularia fallax* (NCIM 714), *Curvularia lunata* (NCIM 716), *Curvularia cymbopogonis* (NCIM 695), *Fusarium oxysporum* (NCIM 1043), *Fusarium moniliforme* (NCIM 1099), *Helminthosporium* (NCIM 1079), *Phomopsis* (NCIM 1324), *Penicillium fellulatum* (NCIM 1227), *Penicillium roqueforti* (NCIM 712), *Trichoderma reesei* (NCIM 1052) using the plate assay. For the plate assay freshly grown fungal mycelium of each fungal strain was spot inoculated at the center of a petri plate containing potato dextrose (PD) agar medium and incubated at 28°C for 24 to 48 h. Sterile filter paper disks (5-mm diameter) impregnated with 0.1 inhibitor units of VrAPI were placed in front of the growing fungal mycelium. The plates were further incubated at 28°C, and the zones of retarded mycelial growth were measured. The MICs for the fungal strains were determined by a broth dilution method. Serial dilutions of VrAPI were made in half-strength PD broth in microtiter plates. Each well was inoculated with 10 ml of the test organism containing 100 spores/ ml. The MIC was determined after overnight incubation of the plates and was taken as the lowest concentration of VrAPI at which growth was inhibited. A microspectrometric antifungal assay was performed for the quantitative demonstration of antifungal activity as described. Briefly, routine tests were performed with 10 ml of (filter [0.22-mm pore size]-sterilized) test solution and 40 ml of fungal spore suspension (100 spores/ml) in half-strength PD broth. Control microculture contained 10 ml of sterile distilled water and 40 ml of the fungal spore suspension. Unless otherwise stated, the incubation conditions for the experiments were 28°C for 48 h. Antifungal activity is expressed in terms of percent inhibition as defined by Dash et al, 2001.

Isolation of Fusarium udum

A virulent isolate of *Fusarium udum* was obtained from infected pigeon pea plants using potato dextrose agar (PDA) medium. The pathogenicity of the fungus was

tested by multiplying the fungus in sand-maize medium (Riker and Riker, 1936). Eight pigeon pea seeds were sown in each pot (700mm in diameter) containing sterilized soil with the fungal culture integrated into it at a ratio of 1:9 (Sand-maize inoculum:soil). The wilt incidence was evaluated and the pathogen was re-isolated using PDA. This isolate was confirmed to be *F. udum* by biochemical characterization and used for all further studies.

Antifungal activity assay for inhibition of *F. udum*

The antifungal activity of the inhibitors on *F. udum* was assayed by the plate assay and the spore suspension assay. The plate assay was performed as described earlier. For the spore inhibition assays, a spore suspension was prepared in distilled water from a 10 day old culture of *Fusarium udum* such that the final spore count was 1 x 10^8 spores/ml. 0.1ml aliquots of the spore suspension were taken in test tubes and incubated in the presence of three concentrations of the ATBI viz., 5, 30 and 60 inhibitor units of ATBI and two controls (sterile water and the universal inhibitor of aspartic proteases, pepstatin-A at a final concentration of 2µM) and analyzed for spore germination of *Fusarium udum*. The tubes were then incubated at 25 $\pm$ 2°C for 24 hours. 0.1ml aliquot was then stained with lactophenol cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the percent spore germination was calculated. A similar experimental set up was used with 4, 20 and 40 inhibitor units of VrAPI using the same controls. The kinetics of spore inhibition were performed wherein spores of *Fusarium udum* were obtained from a 10 day old culture and mixed with sterile distilled water to obtain a homogenous suspension of 1 x 10^8 spores/ml. 10µl of spore suspension was inoculated in 25, 50, 100 and 200 inhibitor units of the inhibitor in a test tube and a homogenous suspension (about 2 x 10^5 spores/ml) was made by inverting the tubes 3-4 times. After specific time intervals of 30, 60, 90, 150, 180 minutes, the reaction

mixtures were centrifuged and the spore pellet was washed two times with distilled water. The pellet was then resuspended in 50µl of sterile distilled water and incubated at 25 $\pm$ 2°C for 24 hours. 0.1ml aliquot was then stained with lactophenol cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the percent spore germination was calculated. Control sets were prepared in the same way in sterile distilled water. All the experiments were conducted in triplicates. Germination inhibition data of *Fusarium udum* obtained in spore germination assay was plotted according to Hill's equation (Gi/ Go-Gi vs log I, where Go and Gi are percent germination in the absence and presence of inhibitor) (Rana et. al., 1997). A parallel set up was used for VrAPI wherein 20, 40, 80 and 160 inhibitor units of VrAPI were used for the kinetic experiments.

Production of glucanases

An inoculum of *F. udum* was grown in a medium described by Mishra et al. (1984) containing 2% wheat bran / cellulose paper powder for 4 days at 30 °C and was added (10%) to the fermentation medium for the production of xylanase and cellulase respectively. The fermentation medium (Mishra et al., 1984) used contained 1% ammonium sulphate, 0.1% urea, 1.5% yeast extract, 0.15% tween 80 as surfactant, and wheat bran / cellulose paper powder (4%) as a carbon source. The culture was grown with continuous shaking on a rotary shaker (200rpm) at 30 °C for 8 days. At the end of the fermentation biomass was separated from the fermented broth by centrifugation and the filtrate was used as an enzyme source.

Inhibition of glucanases by the inhibitors

The activity of cellulase / xylanase was measured by incubating 1 ml of assay mixture containing 0.5 ml of 1% carboxy methyl cellulose / xylan and 0.5 ml of suitably diluted sample in 50 mM, pH 7.0, and phosphate buffer for 30 minutes at 30

°C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The reducing sugar formed was estimated by dinitrosalicylic acid (Miller, 1959). One international unit is defined as the amount of enzyme required to liberate 1µmole of glucose or xylose from carboxy methyl cellulose or xylan respectively in 1 minute under the said assay conditions. Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

Inhibition of aspartic protease

Proteolytic activity of the extracellular culture filtrate of *F. udum* was measured by assaying residual enzyme activity after incubating the enzyme and the substrate hemoglobin as given in Chapter II. The enzyme activity was measured in the absence or presence of inhibitor. The assay for inhibition was carried out as mentioned in Chapter IV.

Assay for pathogen levels

The field experiments were carried out during the years 2004 and 2005 in a field artificially infested with *F. udum* located at the Mahatma Phule Krishi Vidyapeeth, Rahuri, India. The soil type was black having pH 6.2. Experimental plots having inoculum levels of pathogen as high as log 8.90 colony forming units (cfu)/g of soil were chosen. The random population distribution of the pathogen in each plot was verified by drawing four samples from each plot (3m X 3 m) and plating 1 g after serial dilutions on *Fusarium* specific medium (Nash and Snyder, 1962). A Poisson distribution confirmed this statistically.

Pot trials

The 3 kDa filtrate containing 112 inhibitor units per liter of ATBI was used for the pot trial. The efficacy of the partially purified inhibitor in controlling pigeon pea wilt disease was assessed under experimental conditions at the pot level by applying the inhibitor as soil application. Pots containing field soil incorporated with *F. udum* culture grown on sand-maize medium described earlier were prepared. Eight seeds were sown per pot and eight pots per replication were maintained. There were three replications and the pots were arranged in a randomized manner. The seeds were then drenched with 10ml inhibitor solution containing 1.2 inhibitor units of the inhibitor at 0, 30, 60 days of sowing. The wilt incidence was observed at intervals. The pathogen was reisolated from the wilted plants. As a check, seeds were treated with the fungicide carbendazim at the rate of 2 g/kg seed and the soil was drenched with 0.1% concentration of carbendazim on the 30^{th} day after sowing. There was also an untreated control. The results obtained were used to decide on the doses for the subsequent field trial. A similar experimental set up was used with 120 inhibitor units per liter of VrAPI.

Field trials

The partially purified ATBI (3kDa filtrate) was sprayed on 10 plants of ICP 2376 variety of pigeonpea in an experimental trial. The extracellular culture filtrate containing 84 inhibitor units per liter was used for large-scale field trials. For the large scale trial a randomized block design experiment in a plot of size 4 x 2.4 metres with three replications was performed. Each experimental unit included 75 plants with a spacing of 50 X 10 cm. Dose dependent drenching was performed in 3 doses every 30 days. The disease incidence and the reduction over control were calculated. Carbendazim was used as a chemical control agent at the rate of 2 g/kg seed and additional drenching with 0.1% concentration of carbendazim. *Trichoderma*

and *Rhizobium* were used as biological controls wherein seeds were coated with the commercial formulations as per the manufacturer's recommendations. The results were analyzed by statistical tests as explained below. The percent disease intensity of wilt was calculated by the formula given below –

$$\text{Percent Disease intensity (P.D.I.)} = \frac{\text{Sum of all Numerical Ratings}}{\text{No. of plants observed} \times \text{Highest Rating}} \times 100$$

The Chi square test was carried out after subjecting the data to the Yates correction to test the null hypothesis that there is no effect of the inhibitor on the fungal pathogen. The dose dependent effect of the inhibitor on seed germination was evaluated on a percentage basis. The correlation of wilt incidence and inhibitor concentration was verified by carrying out nonlinear regression analysis and the t-test.

Similar experiments were performed with the variety ICPL 87 and the data analyzed accordingly.

The field trials were conducted at the Pulse Research Station, MPKV, Rahuri as shown in the figure below:

A similar experimental set up using 120 inhibitor units per liter of partially purified VrAPI was used to analyze the efficiency of VrAPI in disease control at the field level.

Development of powder formulations of aspartic protease inhibitor

Twenty milliliters of either of the inhibitors ATBI and VrAPI containing 84 and 120 inhibitor units per liter was added to 1 g of talcum powder and mixed well with 75 seeds of pigeonpea, ICP 2376 and ICPL 87. The treated seeds were sun dried and sown in a field containing soil incorporated with *F. udum* culture grown on the sand–maize medium. In addition to seed coating, the classical method of drenching was also employed along with an untreated control.

Methods of application

The efficacy of the formulations of the aspartic protease inhibitors in controlling pigeon pea wilt disease was assessed under field conditions. Application methods included drenching, seed coating and seed coating followed by drenching. Additional data on the growth of the plants as indicated by nodulation data, shoot length, root length, weight of plants was obtained till 150 days of sowing.

Field trials

Two field trials were conducted at the Pulse Research Station, MPKV, Rahuri. Pigeonpea ICP 2376 and ICPL 87 were used for the trials. The randomized block design was used throughout for all the trails. 720 seeds of each variety were used for the entire trial. 540 seeds of each variety were coated with 150ml extracellular culture filtrate mixed with 8g of talcum powder. The seeds were air dried and used for the experiments with coating and coating followed by drenching. The plot size was 1600m^2 with a bed size of 2 x 2 m. The seeds were sown with a spacing of 45x 10 cm (60 plants per bed). The application was repeated at 30, 60, 90 and 150 days after sowing. Drenching was done at the rate of 3 liters per bed. As a check, seeds were treated with carbendazim at 2 g / kg seeds and the fields were drenched with 0.1% carbendazim on the 30th day after sowing. The trials were laid out in a randomized replicated block design with three replications. The wilt incidence in the entire plot was assessed and expressed as a percentage of disease incidences. Grain yield was also recorded. The data were statistically analyzed by DMRT. This entire layout was used once with partially purified VrAPI and once with partially purified ATBI.

RESULTS

Control of pigeon pea wilt by ATBI

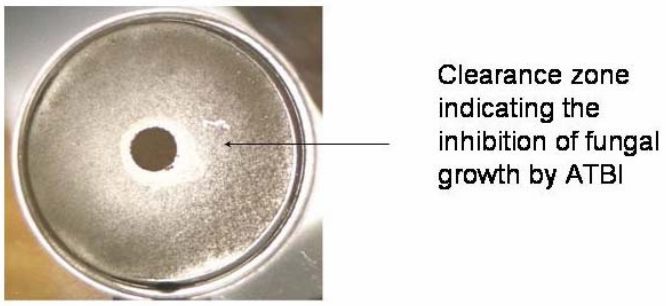
Inhibitor production and purification

ATBI was produced in batches of 20 liters, which was applied to the field in a single dose. Purified inhibitor was used for all the laboratory experiments.

Antifungal activity assay

The plate assay indicated a zone of inhibition of 4mm in diameter of *F. udum* in the presence of ATBI (Figure 1).

Figure 1: Effect of ATBI on mycelial growth of *F. udum*

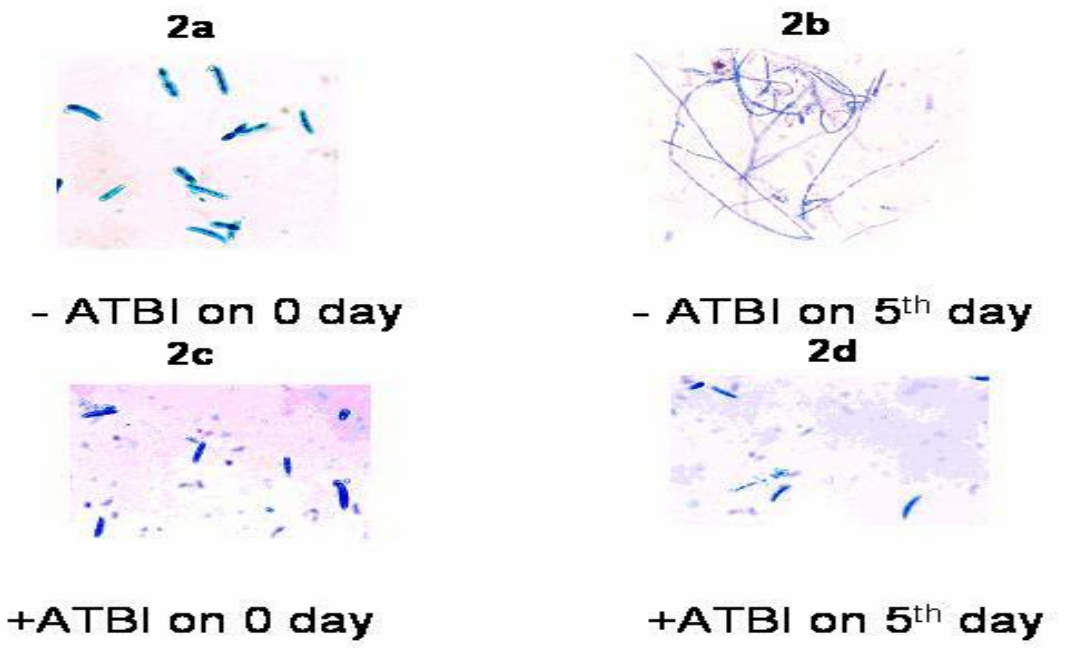


F. udum was grown on PDA plates for 24 hours at 30ºC. A sterile filter paper disc soaked in a solution containing 5 inhibitor units of ATBI was placed centrally. A zone of inhibition of growth (4mm in diameter) was observed on further incubation of 24 hours.

Figure 2 shows the spore germination assay of the *F.udum*. Pepstatin at the final concentration of 2µM inhibited spore germination for 5 days. A dose dependent inhibiton of spore germination was observed. The germ tubes produced at lower concentrations of ATBI were smaller those produced in water. Figure 2 shows the lactophenol stained spores of *F. udum* in the presence of 0.1 inhibitor units/ml of the

inhibitor. The control spores in water germinated with good mycelial growth (Figure 2 a and b) while the spores in the presence of the inhibitor remained ungerminated and swollen for 5 days (Figure 2 c and d). This indicates that the inhibitor is effective in stopping the germination of the spores and the subsequent formation of the mycelium.

Figure 2: Effect of ATBI on spore germination of *F. udum*



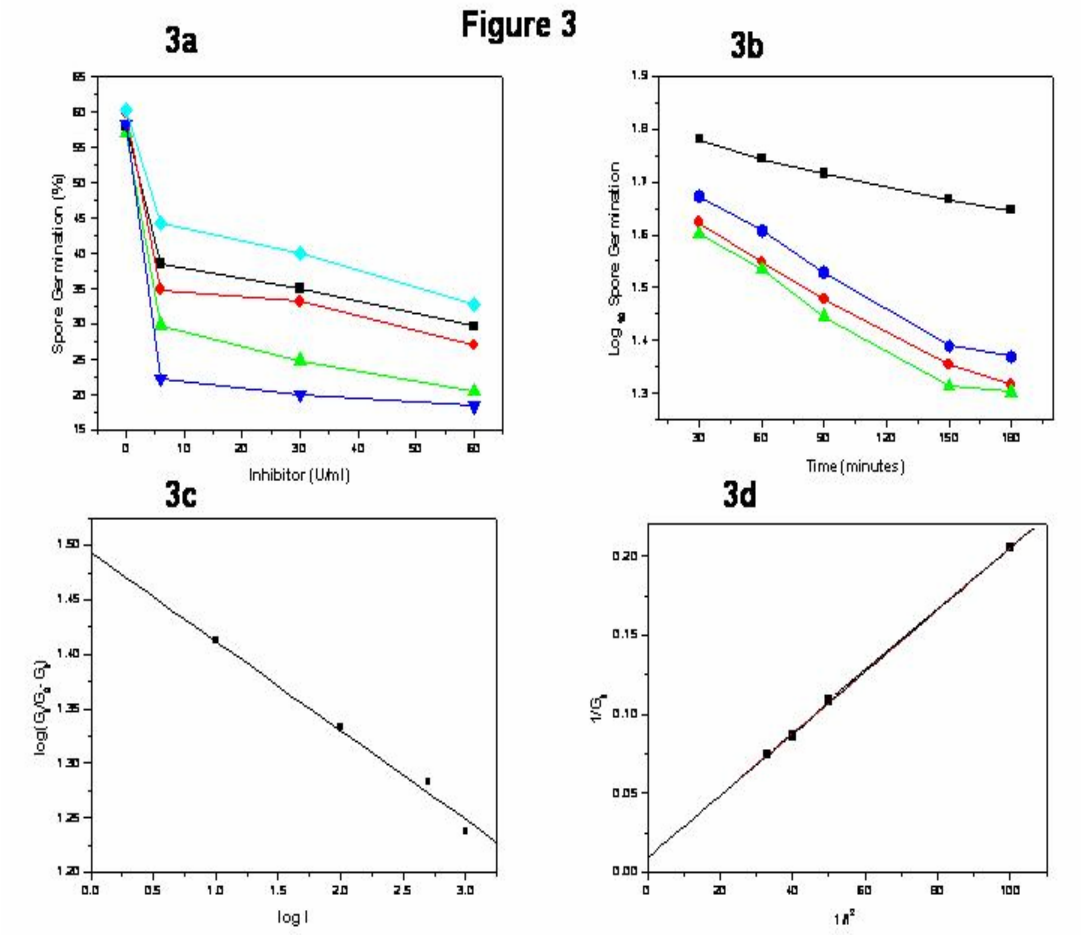
The spore germination was observed in the presence and absence of ATBI at a magnification of 400X. 2a: Spores of *F. udum* suspended in sterile water as observed on the 0 day of incubation. 2b: Spores suspended in water as observed on the 5th day of incubation at ambient temperature.2c: Spores suspended in solution of ATBI (0.1 inhibitor unit) as observed on the 0th day of incubation. 2d: Spores in the presence of ATBI on the 5th day of incubation.

Kinetics of inhibition of spores of F. udum

Figure 3a and 3b indicates the time and concentration dependent inhibitory effect of ATBI on germination of spores of *Fusarium udum*. The effect of ATBI was not

reversed even after 3 days of exposure as observed by continuous microscopic monitoring. The Hill plot of the data for *Fusarium udum* in Figure 3c gives a value of n=0.9254 and when a graph of reciprocal of percent inhibition (Figure 3d) is plotted against reciprocal of square of inhibitor concentration a straight line is observed.

Figure 3: Kinetics of inhibition of *F.udum* by ATBI



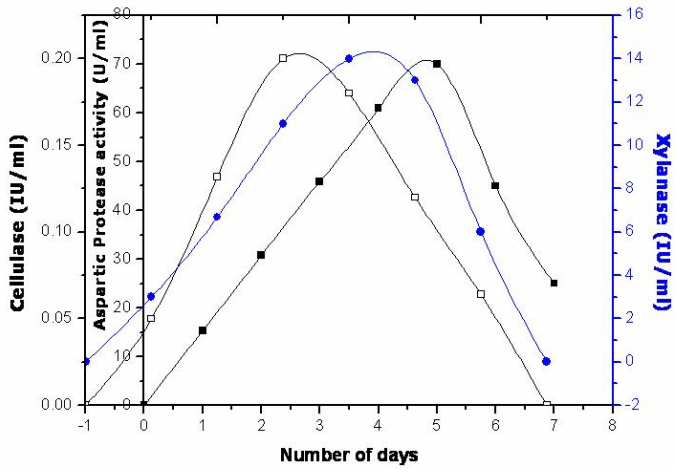
The kinetics of inhibition of spore germination was performed as explained in materials and methods. 3a: The concentration dependent effect of increasing concentrations of ATBI (5, 30 and 60 inhibitor units / ml) at 30 (⬥), 60 (■), 90 (●), 150 (▲) and 180 (▼) minutes on the spores of *F. udum* is indicated. 3b: Time dependent effect of 0 (■), 50 (●), 100 (●) and 150 (▲) inhibitor units / ml of ATBI over a period of time are indicated 3c: The Hill plot for the characterization of the mode of inhibition of spore germination by 10, 100, 500 and 1000

inhibitor units of ATBI is provided which indicates a complex mode of inhibition while the reciprocal plot for the inhibition of *F. udum* spore germination using ATBI confirms it (3d). G_0 and G_i are percent germination in the absence and presence of inhibitor respectively and I is the inhibitor concentration.

Production of hydrolytic enzymes by *Fusarium udum*

The time course of xylanase production was studied up to 7 days. A maximum of 14 IU/ml at 96 h was obtained in the presence of the inducer (Figure 4). The time course of cellulase production was studied up to 7 days. Cellulase production started without a lag and reached a maximum of 0.2 IU/ml at 72 h (Figure 4). The production profile of extracellular aspartic protease production as monitored for 8 days showed increased production in presence of soya meal as inducer. A maximum amount of 70 units of aspartic protease was observed at 120 h (Figure 4).

Figure 4: Time course of enzyme production



The time course following fermentation for 8 days was plotted. Cellulase (▫), xylanase (•) and aspartic protease production (■) was studied in the presence of cellulose paper powder, wheat bran and soyameal respectively.

Inhibition of hydrolytic enzymes of *F. udum*

A maximum inhibition of 21% of the extracellular xylanase was obtained with 0.1 inhibitor unit/ml of ATBI. The cellulase production was too low to estimate inhibition with ATBI. An inhibition of 32% of 10 units of aspartic protease was observed with 0.2 inhibitor units/ml of the inhibitor.

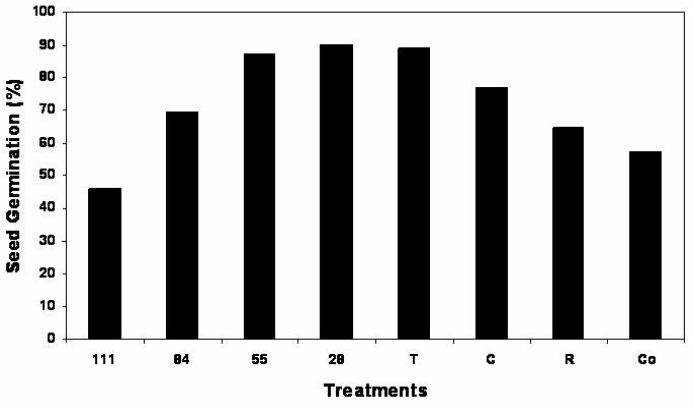
Assay for pathogen levels

The pathogen levels monitored in the wilt sick plot were in the range of 6-8 x 10^6 spores / ml. This data was obtained by random sampling of the soil at different time intervals.

Pot Trials

ATBI affected seed germination when used at a concentration of 112 inhibitor units per liter for drenching the soil around the pigeon pea plants. The effect on the seed germination was calculated on a percentage basis (Figure 5) to be 55%. This effect was seen to be minimized with 84 inhibitor units per liter of ATBI wherein a maximum of 71% inhibition of the growth of *F. udum* was obtained. Due to this significant decrease in the wilt percentage and the minimum effect on seed germination, further field trials were conducted with extracellular culture filtrate containing 84 inhibitor units of ATBI.

Figure 5: Effect of ATBI on seed germination in pot trials



Dose dependent drenching of soil containing spores of *F. udum* by ATBI (range used: 111, 84, 55, 28 inhibitor units per liter of inhibitor) in pots containing eight pigeon pea seeds per pot was conducted to see the efficacy of the inhibitor in controlling infection. Control treatments used were *Trichoderma* (T), Carbendazim (C), *Rhizobium* (R) and untreated pigeon pea (Co). The efficacy was evaluated by checking the seed germination in percent and the overall health of the plants.

Field trials

The dose dependent effect of the extracellular culture filtrate enriched in ATBI on wilt incidence in a highly wilt sick plot was analysed at the field level. A maximum disease intensity of 50% was observed in the absence of any control measures. The disease was controlled to an extent of 71% by 84 inhibitor units of ATBI as observed in Table 1. Carbendazim was effective in controlling 71% of the disease while the biocontrol agents *Trichoderma and Rhizobium* were successful in managing 53 and 61% of wilt respectively (Table 1). A dosage of 84 inhibitor units per liter of the inhibitor was found to be optimum for the treatment of the fungus in the field.

The dose dependent response on the wilt disease of pigeon pea is evident in the figure provided below. The 75% bacterial corresponds to the extracellular culture

filtrate containing 84 inhibitor units per liter of ATBI. The healthy pigeon pea plants bore flowers and subsequently the amount of seeds obtained i.e. yield was also substantially increased. Similar results were obtained with ICPL 87.

Table 1: Effect of extracellular culture filtrate enriched with ATBI on wilt of pigeon pea

Treatment	Number of Plants			Number of plants wilted			Percent Wilt			Mean % Wilt	Percent Disease Control
	RI	*RII*	*RIII*	*RI*	*RII*	*RIII*	*RI*	*RII*	*RIII*		
75% ATB ECF	12	54	53	3	2	8	25	3.70	15.09	14.59	70.6
50% ATB ECF	65	66	50	17	8	10	26.15	12.12	20	19.42	60.9
25% ATB ECF	53	53	71	13	9	13	24.52	35.84	18.30	26.22	47.21
Trichoderma viride	61	72	68	15	15	16	24.59	20.83	23.52	22.98	53.73
Rhizobium	51	50	74	8	14	10	15.68	28	13.51	19.06	61.62
Carbendazim	47	75	47	10	8	5	21.27	10.66	10.63	14.19	71.43
Control	46	53	31	23	20	19	50.00	37.73	61.29	49.67	-------

75% ATB ECF corresponds to the AlkaloThermophilic Bacillus Extracellular Culture Filtrate containing 84 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and *RIII* signify the replications in the randomized block design

The health and vigor of the plants can be observed in the following photographs:

Figure 6: Effect of ATBI on wilt of pigeon pea ICP 2376

A

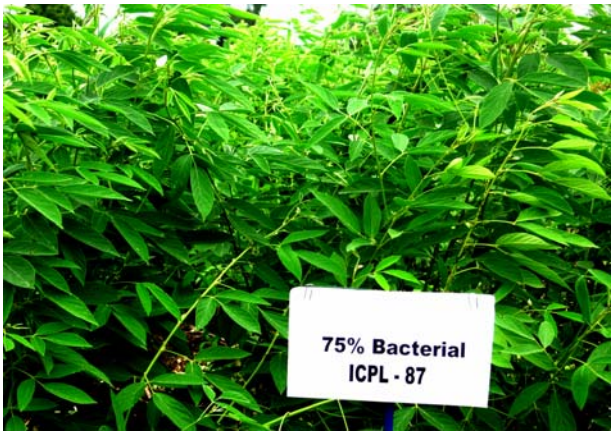
B

C

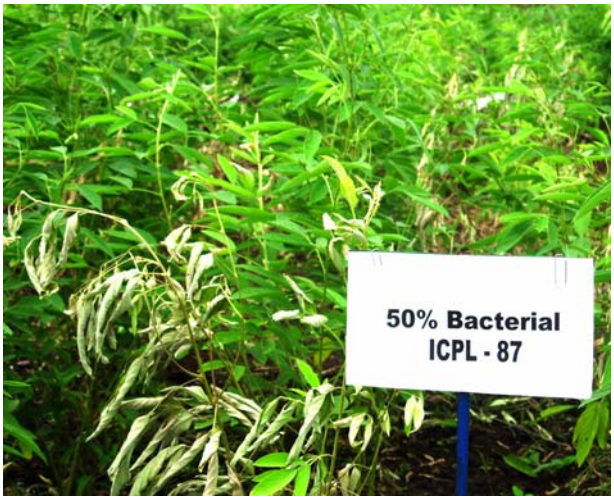
Field trials conducted with the extracellular culture filtrate of *Bacillus* resulted in the production of healthy plants. A: Untreated Control, B: ICP 2376 treated with extracellular culture filtrate of ATBI containing 84 inhibitor units per liter considered as 75% Bacterial, C: ICP 2376 treated with ATBI bearing flowers.

Figure 7: Effect of ATBI on wilt of pigeon pea ICPL 87

A



B



Field trials using the extracellular culture filtrate of *Bacillus* showed good healthy pigeon pea plants of ICPL 87. A: Pigeon pea plants treated with 75% partially purified ATBI B: Pigeon pea plants treated with 50% partially purified ATBI

The statistical analysis of the field trial data performed by the Chi test indicated a significant deviation from the expected value with 2 degrees of freedom. The data was subjected to the Yates correction and the degrees of freedom were therefore corrected to 1. The expected value of chi under the experimental conditions with a single degree of freedom is 10.83 with a confidence interval of 0.001 while the value obtained is 41.16. This deviation from the expected indicates that the null hypothesis that there is no effect of the inhibitor concentration on the wilting of plants is not true. Therefore we conclude that there is a significant effect of ATBI on the wilting of plants. The non linear regression analysis indicated a negative correlation signifying that as the inhibitor concentration increases the wilting percentage decreases. The t test is a popular test for the comparison of means of two groups. The groups used here were the treatments and the control. In the present study the value obtained 1.4058 is less than the expected value of 2.57 for the 2 sided t test as well as 2.01 for the 1 sided t test. This indicates that there is a significant difference in the means of the treated and the untreated samples and that this could be attributed to the inhibitor.

Formulation trials

The extra-cellular culture filtrate containing 84 inhibitor units / liter was found to be most effective in controlling wilt. This concentration was used to prepare the formulations wherein the solution containing inhibitor was mixed with talcum powder and used for coating the seeds of pigeon pea ICP 2376 and ICPL 87. Seed coating followed by drenching reduced wilt incidence compared to the control (Table 2).

Table 2: Effect of various formulations of the culture filtrate on the control of wilt of pigeon pea

Treatments	Disease Intensity (%) ICP 2376	Disease Control (%) ICP 2376	Disease Intensity (%) ICPL 87	Disease Control (%) ICPL 87
Seed Coating	30.42	45.47	26.18	25.70
Drenching	22.66	59.38	16.66	52.72
Seed Coating and Drenching	19.72	64.65	10.33	70.68
Control	55.79	-	35.24	-

Seed treatment as well as drenching was individually effective in achieving partial control; but additional soil application increased the efficacy (Figure 7). A maximum control of 65% with ICP 2376 and 71% with ICPL 87 was obtained with seed coating and additional drenching (Figure 8). The treatment of coating and drenching were on a par in their efficacy with carbendazim treatment (seed treatment followed by soil drenching with the fungicide). The reduction in wilt incidence was accompanied by increase in grain yield (data not shown). The health of the plants was additionally assessed by plant growth data. The nodulation data shoot and root length as also weight of the plants matched the trend obtained with the sampling analysis data (Tables 3 & 4).

Table 3: Effect of different formulations on nodulation, plant height, root and shoot length of pigeon pea after 150 days of treatment

Variety ICP 2376

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	11	128	29.00	98.00
Drenching	9.33	148.50	55.00	93.00
Seed coating and Drenching	7.66	162.50	37.00	125.00
Control	5.66	133.70	35.00	98.00

Variety ICPL 87

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	10.33	81.25	19.00	62.00
Drenching	10.33	82.40	29.00	53.00
Seed coating and Drenching	8.66	78.00	22.5	52.00
Control	5.66	133.70	19.00	61.00

Table 4: Effect of bioactive principles on weight of pigeon pea

Variety: ICP 2376

Treatment	Fresh weight			Mean	Dry weight			Mean
	I	II	III		I	II	III	
Seed coating	53.4	53.3	53.2	53.3	32.6	30.0	28	30.2
Drenching	96.8	98.6	99.5	98.3	53.8	49.9	51.2	51.63
Seed coating + drenching	70.2	70	75.5	71.56	42.8	38	39.5	40.10
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

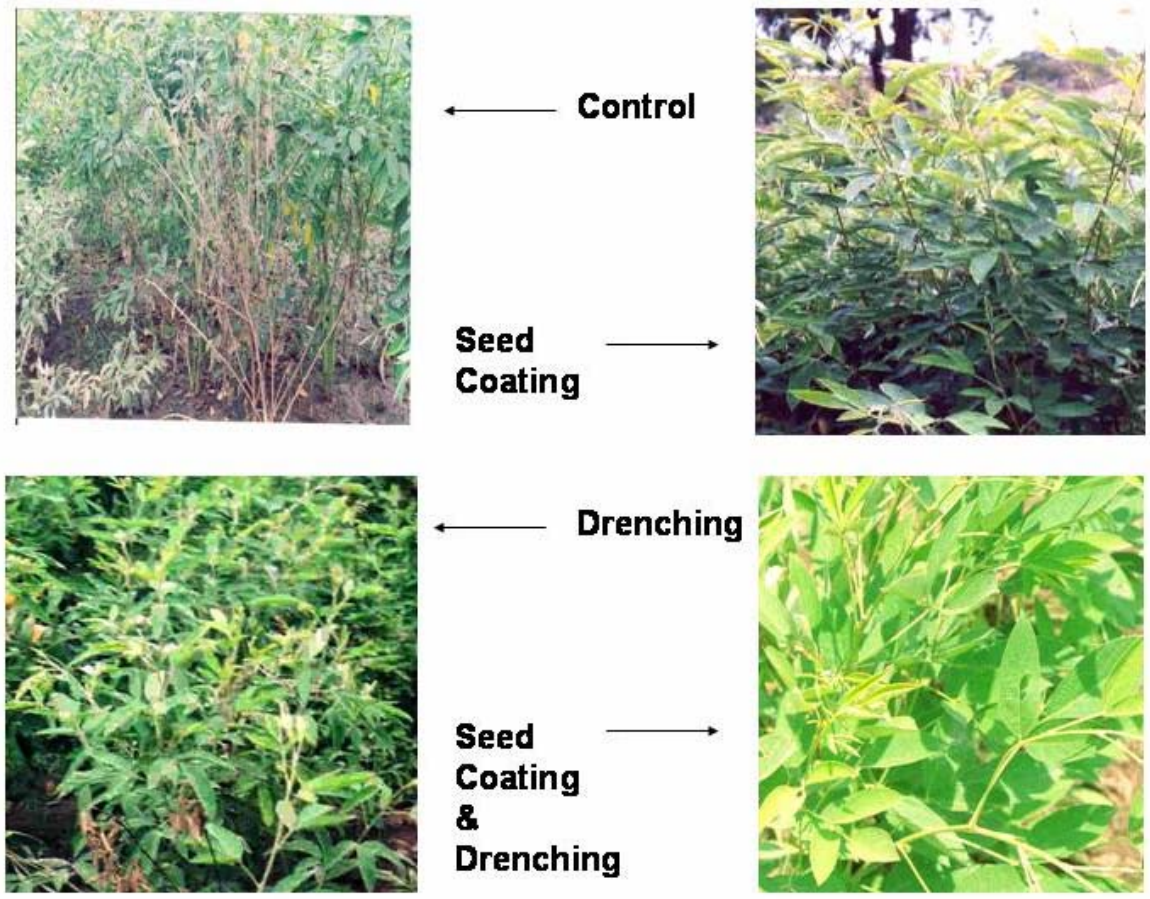
Variety: ICPL 87

Treatment	Fresh weight			Mean	Dry weight			Mean
	I	II	III		I	II	III	
Seed coating	32.4	32.0	32.1	32.16	24.20	22.00	21.70	22.63
Drenching	30.4	30.00	32.80	31.06	17.20	16.30	15.80	16.43
Seed coating + drenching	56.60	56.30	59.80	57.56	23.80	22.50	20.80	22.63
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

Figure 7: Effect of formulations on pigeon pea wilt of variety ICPL 87

Effect of formulations on pigeon pea wilt of variety ICPL 87 was analysed in field trials. The treatment of seed coating followed by booster doses of drenching resulted in maximum disease control of 71% as compared to the individual treatments of seed coating and drenching which resulted in disease control of 26 and 53%.

Figure 8: Effect of the formulations on the incidence of wilt on pigeon pea variety ICP 2376



Effect of formulations on pigeon pea wilt of variety ICP 2376 was analysed in field trials. The treatment of seed coating followed by booster doses of drenching resulted in maximum disease control of 65% as compared to the individual treatments of seed coating and drenching which resulted in disease control of 45 and 59%.

Drenching was more effective in enhancing the plant vigor and growth. The initial treatment of seed coating prevented the early onset of infection by the seed borne pathogen while drenching provided additional protection against soil borne pathogen.

The combined effect of seed coating and drenching was beneficial in preventing more than 60% of infection of plants of both varieties.

Control of wilt pf pigeon pea using VrAPI

Inhibitor production and purification

5kg of mung bean seeds of Kopergaon-1 variety were ground in water. The mixture was kept at room temperature for 3 hours after which the supernatant was obtained by centrifugation of the crude mixture. The supernatant was checked for inhibitor activity and used for all trials.

Antifungal activity assay

The partially purified inhibitor from mung bean seeds was evaluated for its potency as a fungal growth inhibitor. To assess the antifungal activity of the compound the molecule (VrAPI) was purified from the partially purified filtrate. During the purification process, the antifungal property of VrAPI was found to be co-purified with its aspartic protease-inhibitory activity. The antifungal activity of the purified VrAPI against 16 fungal strains was assessed in plate assays and the zone diameters were measured (Table 5).

Table 5: Inhibition of phytopathogenic fungi in plate assays as indicated by the diameter of the zone of inhibition

	Fungal Strain	Diameter of zone of inhibition (mm)
i)	*Alternaria solani*	1.2
ii)	*Aspergillus flavus*	4.5
iii)	*Aspergillus niger*	4.2
iv)	*Aspergillus oryzae*	4.0
v)	*Claviceps purpurea*	3.4
vi)	*Colletotricum*	2.5
vii)	*Curvularia fallax*	8.5
viii)	*Curvularia lunata*	7.5
ix)	*Curvularia cymbopogonis*	7.0
x)	*Fusarium oxysporum*	7.5
xi)	*Fusarium moniliforme*	8.0
xii)	*Helminthosporium*	6.5
xiii)	*Phomopsis*	5.5
xiv)	*Penicillium fellulatum*	4.0
xv)	*Penicillium roqueforti*	4.5
xvi)	*Trichoderma reesei*	6.0

VrAPI showed strong inhibitory activity against *C. cymbopogonis, Phomopsis sp., C. fallax, C. lunata, Helminthosporium, T. reesei, F. oxysporum, F. moniliforme and moderate activity against A. flavus, A. oryzae, A. solani, A. niger, P. roqueforti, P. fellulatum, sp.,* and *Colletotrichum* sp. as concluded from Table 5.

Fungal growth inhibition was also monitored in microscopic assay, wherein the spores of different fungal strains were cultured in the presence of varied concentrations of the inhibitor. After 24 h, the concentration of VrAPI required for 50% inhibition (IC_{50}) of fungal growth varied from 1.2 µg/ml for *C. fallax* to 6.5 µg/ml for *A. solani*, whereas the MIC ranged from 0.70 mg/ml to 5.70 mg/ml for the same. The phytopathogen *C. fallax* was found to be the most sensitive to ATBI, whereas *A. solani* was the least sensitive strain (Table 6).

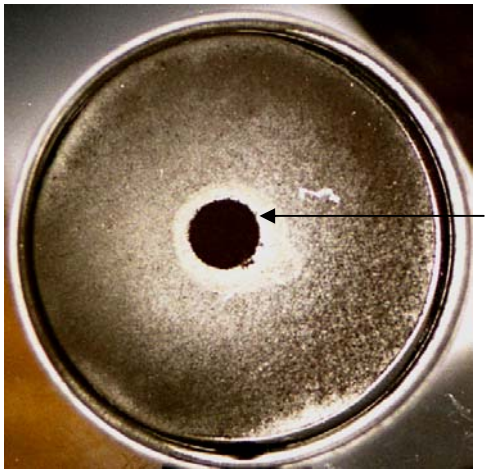
Table 6: IC_{50} and Minimum Inhibitory Concentration Values of VrAPI against phytopathogenic fungi

Fungus	**IC_{50} (µg/ml) of VrAPI**	**MIC (µg/ml) of VrAPI**
Alternaria solani	6.5	5.7
Aspergillus flavus	5.0	4.4
Aspergillus niger	5.3	4.6
Aspergillus oryzae	5.7	5.2
Claviceps purpurea	6.0	5.4
Colletotricum	6.3	5.6
Curvularia fallax	1.2	0.7
Curvularia lunata	2.5	1.5
Curvularia cymbopogonis	3.5	2.8
Fusarium oxysporum	3.0	2.5
Fusarium moniliforme	1.5	0.9
Helminthosporium	4.0	3.3
Phomopsis	4.6	4.1
Penicillium fellulatum	5.1	4.4

Penicillium roqueforti	5.8	4.9
Trichoderma reesei	4.3	3.7

The plate assay indicated a zone of inhibition of mycelial growth of *F. udum* of 6 mm in diameter in the presence of VrAPI (Figure 9).

Figure 9: Effect of VrAPI on mycelial growth of *F. udum*



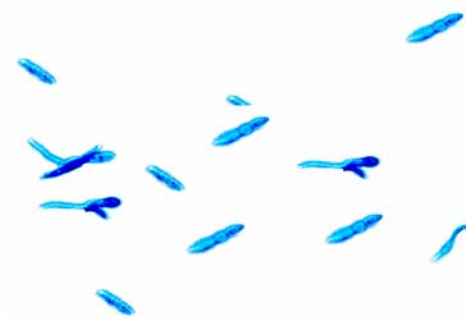
Clearance zone produced by VrAPI on the mycelium of *F. udum*.

F. udum was grown on PDA plates for 24 hours at 30ºC. A sterile filter paper disc soaked in a solution containing 7 inhibitor units of VrAPI was placed centrally on the plate. A zone of inhibition of growth (6mm in diameter) was observed on further incubation of 24 hours.

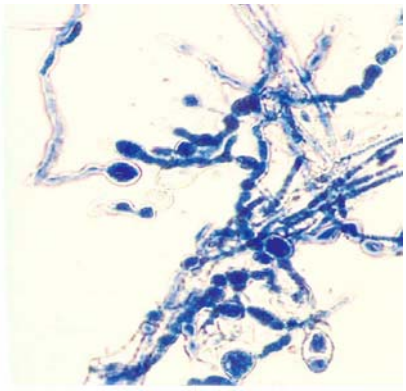
Figure 10 shows the spore germination assay of *F. udum*. Pepstatin at the final concentration of 2µM inhibited spore germination for 5 days. A dose dependent inhibition of germination of spores of *F.* u*dum* was observed. At lower concentrations of the inhibitor small germ tubes as compared to the control were produced. Figure 2 shows the lactophenol stained spores of *F. udum* in the presence of VrAPI. The spores in the presence of the inhibitor remained ungerminated and swollen for 5 days (Figure 2 c and d) while the control spores in water germinated with substantial mycelial growth (Figure 2 a and b). This indicates that the inhibitor is effective in

stopping the germination of the spores and the subsequent formation of the mycelium.

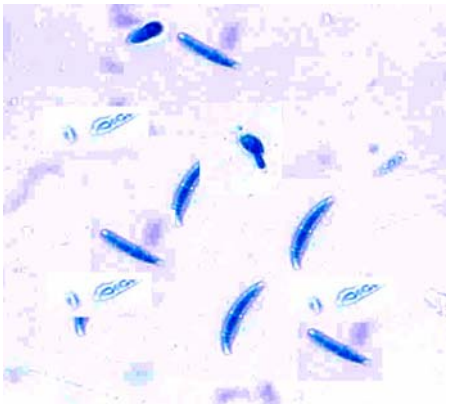
Figure 10: Effect of VrAPI on spore germination of *F. udum*



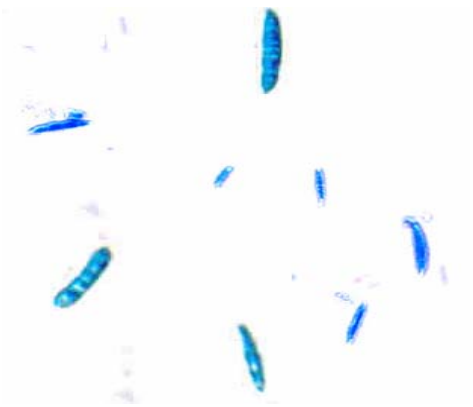
Water 0 day



Water 5th day



VrAPI 0 day



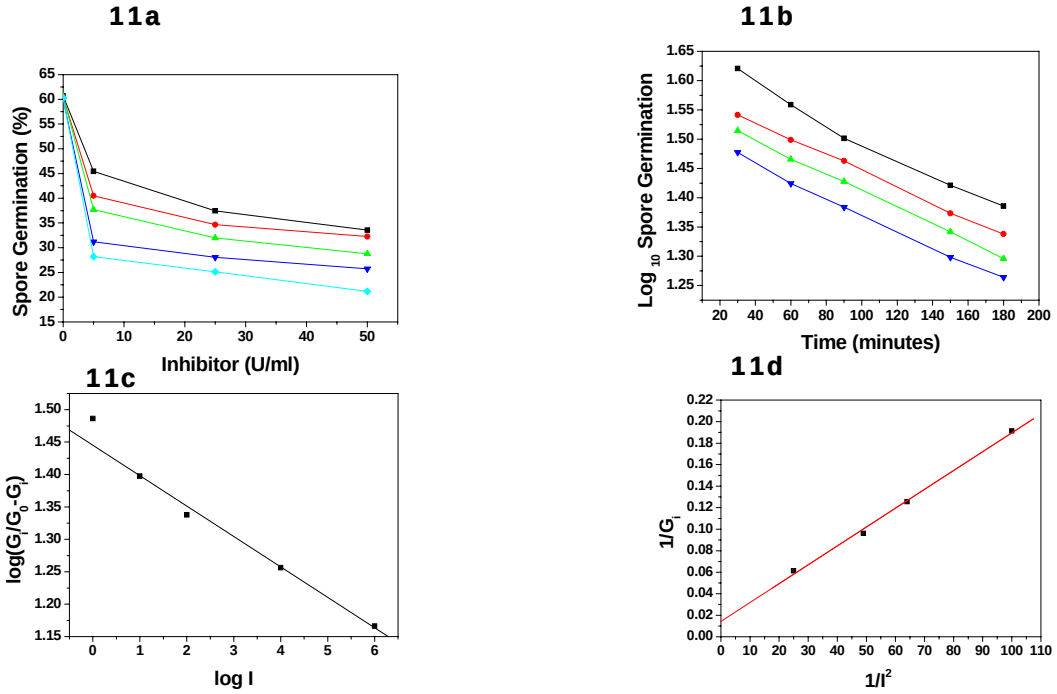
VrAPI 5th day

The spore germination was observed in the presence and absence of VrAPI at 400 X magnification. 10a: Spores of *F. udum* suspended in sterile water as observed on the 0 day of incubation. 10b: Spores suspended in water as observed on the 5th day of incubation at ambient temperature. 10c: Spores suspended in solution containing 0.1 inhibitor units of VrAPI as observed on the 0th day of incubation. 10d: Spores in the presence of VrAPI on the 5th day of incubation.

Kinetics of inhibition of germination of spores of F. udum

The effect time and concentration dependent inhibitory effect of VrAPI against germination of spores of *Fusarium udum* is shown in Figure 11a and 11b. The effect of VrAPI was not reversed even after 3 days of exposure as observed by continuous microscopic monitoring. The Hill plot of the data for *Fusarium udum* in Figure 11c gives a value of n=0.7542 and when a graph of reciprocal of percent inhibition (Figure 11d) is plotted against reciprocal of square of inhibitor concentration a straight line is observed.

Figure 11: Kinetics of inhibition of *F.udum* by VrAPI



The effect of VrAPI on inhibition of spore germination was assessed by carrying out kinetic experiments. 11a: The concentration dependent effect of increasing concentrations of VrAPI (5, 25 and 60 inhibitor units / ml) at 30 (■), 60 (•), 90 (▲), 150 (▼) and 180 (♦) minutes on the spores of *F. udum* is indicated. 11b: Time dependent effect of 0 (■), 40 (●), 80 (▲) and 160 (▼) inhibitor units / ml of VrAPI over a period of time are indicated 11c: The Hill plot for the characterization of the mode of inhibition of spore germination by 10, 100, 500 and 1000

inhibitor units of VrAPI is provided which indicates a complex mode of inhibition while the reciprocal plot (11d) for the inhibition of *F. udum* spore germination using VrAPI confirms it (11d). G_0 and G_i are percent germination in the absence and presence of inhibitor respectively and I is the inhibitor concentration.

Inhibition of hydrolytic enzymes of *F.udum*

Inhibition of the extracellular xylanase by VrAPI indicated that 0.08 unit/ml inhibitor units / ml inhibited 33% of the activity of the enzyme. 0.1 inhibitor units/ml of VrAPI inhibited 45% activity of 10 units of aspartic protease.

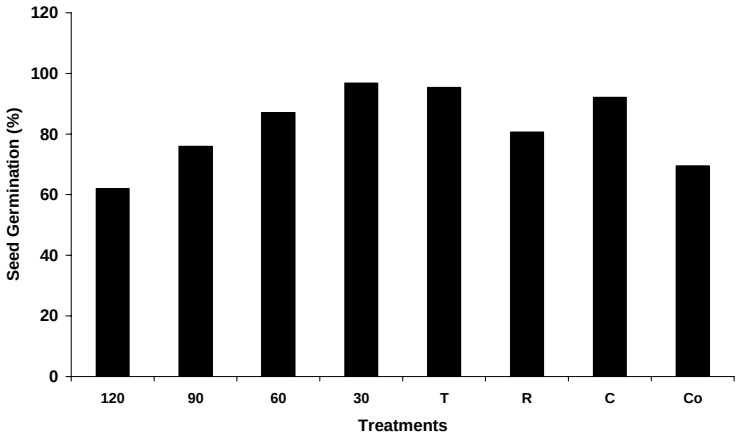
Assay for pathogen levels

The pathogen levels monitored in the wilt sick plot were in the range of 6-8 x 10^6 spores / ml. This data was obtained by random sampling of the soil at different time intervals.

Pot Trials

A maximum inhibition of 78% of wilt of pigeon pea ICP 2376 and ICPL 87 was observed with 120 inhibitor units of VrAPI which showed minimum effect of 37% on seed germination (Figure 12). Therefore further field trials were conducted with 120 inhibitor units of VrAPI.

Figure 12: Effect of VrAPI on seed germination in pot trials



Dose dependent drenching of soil containing spores of *F. udum* by VrAPI (range used: 120, 90, 60, 30 inhibitor units per liter of inhibitor) in pots containing eight pigeon pea seeds per pot was conducted to see the efficacy of the inhibitor in controlling infection. Control treatments used were *Trichoderma* (T), Carbendazim (C), *Rhizobium* (R) and untreated pigeon pea (Co). The efficacy was evaluated by checking the seed germination in percent and the overall health of the plants.

Field trials

Table 7 shows the dose dependent effect of the partially purified VrAPI on wilt incidence in a highly wilt sick plot. A maximum disease intensity of 50% was observed in the absence of any control measures. The disease was controlled to an extent of 78% by 120 inhibitor units of VrAPI as observed in Table 10. Carbendazim was effective in controlling 71% of the disease while the biocontrol agents *Trichoderma* and *Rhizobium* were successful in managing 53 and 61% of wilt respectively.

Table 7: Effect of VrAPI on wilt of pigeon pea

Sr No	Treatment	Number of Plants			Number of plants wilted			Percent Wilt			Mean % Wilt	Percent Disease Control
		RI	RII	RIII	RI	RII	RIII	RI	RII	RIII		
1	100 % PP VrAPI	35	44	61	3	10	1	8.57	22.72	1.64	10.97	77.91
2	75% PP VrAPI	47	46	90	7	10	18	14.89	21.73	20	18.87	62.00
3	50% PP VrAPI	57	28	79	10	11	18	17.54	39.28	22.78	26.53	46.58
4	25% PP VrAPI	65	45	59	18	20	17	27.69	44.44	28.81	33.64	32.27
5	*Trichoderma viride*	61	72	68	15	15	16	24.59	20.83	23.52	22.98	53.73
6	Carbendazim	47	75	47	10	8	5	21.27	10.66	10.63	14.19	71.43
7	*Rhizobium*	51	50	74	8	14	10	15.68	28	13.51	19.06	61.62

8	**Control**	**46**	**53**	**31**	**23**	**20**	**19**	**50.00**	**37.73**	**61.29**	**49.67**	**-------**

100% PP VrAPI corresponds to the Partially Purified *Vigna radiata* Protease Inhibitor containing 120 inhibitor units of VrAPI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, *RII* and *RIII* signify the replications in the randomized block design

A dosage of 120 inhibitor units per liter of the inhibitor was found to be optimum for the treatment of the fungus in the field. The healthy pigeon pea plants bore flowers and subsequently the amount of seeds obtained i.e. yield was also substantially increased. The health and vigor of the plants can be observed in the following photographs

Figure 13: Effect of VrAPI on pigeon pea wilt

A

B

Field trials were conducted to evaluate the efficacy of VrAPI on the pigeon pea variety ICP 2376 in field trials. A: Untreated Control ICP 2376; B: ICP 2376 treated with 100% VrAPI. The disease intensity was as high as 55% and an effective control of 78% was achieved with the inhibitor.

The same field trials were conducted with ICPL 87, one of the most popular varieties of pigeon pea and which is widely cultivated. Similar results were obtained with 77% control of wilt disease.

Figure 14: Effect of VrAPI on ICPL 87

The statistical analysis of the field trial data performed by the Chi test indicated a significant deviation from the expected value with 2 degrees of freedom. The data was subjected to the Yates correction and the degrees of freedom were therefore corrected to 1. The expected value of chi under the experimental conditions with a single degree of freedom is 10.83 with a confidence interval of 0.001 while the value obtained is 53.67. This deviation from the expected indicates that the null hypothesis that there is no effect of the inhibitor concentration on the wilting of plants is not true. Therefore we conclude that there is a significant effect of VrAPI on the wilting of plants. The non linear regression analysis indicated a negative correlation signifying that as the inhibitor concentration increases the wilting percentage

decreases. The t test is a popular test for the comparison of means of two groups. The groups used here were the treatments and the control. In the present study the value obtained 1.3265 is less than the expected value of 2.57 for the 2 sided t test as well as 2.01 for the 1 sided t test. This indicates that there is a significant difference in the means of the treated and the untreated samples and that this could be attributed to the inhibitor.

Effect of formulation studies of VrAPI on wilt

The results obtained with VrAPI indicated that 120 inhibitor units per liter were sufficient not only to control wilt occurrence but also in enhancing plant growth and vigor. The application of seed coating and booster doses of drenching was found to be better than any of the other applications in achieving a disease control of 64 and 70% in varieties ICP 2376 and ICPL 87 (Table 8). The increased vigor of the plants is evident in terms of the nodulation, fresh and dry weight, and root and shoots length data as also in the enhanced plant height data (Table 9, 10).

Table 8: Effect of formulations of VrAPI on pigeon pea wilt

Treatments	Disease Intensity (%) ICP 2376	Disease Control (%) ICP 2376	Disease Intensity (%) ICPL 87	Disease Control (%) ICPL 87
Seed Coating	29.42	44.47	27.18	24.70
Drenching	21.66	58.38	17.66	51.72
Seed Coating & Drenching	20.72	63.65	12.33	69.68
Control	55.79	-	35.24	-

Table 9: Effect of different formulations on nodulation, plant height, root and shoot length of pigeon pea after 150 days of treatment

Variety ICP 2376

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	5.7	89.50	24.00	105.00
Drenching	6.33	139.70	40.50	99.00
Seed coating and Drenching	7.33	149.00	34.00	135.00
Control	5.66	133.70	35.00	115.00

Variety ICPL 87

Treatment	Nodulation	Plant Height	Root Length	Shoot Length
Seed Coating	9.33	117.00	23.77	60.00
Drenching	7.66	88.40	35.91	57.00
Seed coating and Drenching	9	84.00	33.23	53.00
Control	5.66	133.70	31.06	59.00

Table 10: Effect of VrAPI on fresh and dry weight of pigeon pea

Variety: ICP 2376

Treatment	**Fresh weight**			**Mean**	**Dry weight**			Mean
	I	**II**	**III**		**I**	**II**	**III**	
Seed coating	44.8	44.2	49	46	22.5	20.5	19.2	20.73
Drenching	57.2	56.4	58.1	57.23	37.8	32.5	33.6	34.63
Seed coating + drenching	58	60	58.5	58.83	35.9	32	31.5	33.13
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

Variety: ICPL 87

Treatment	**Fresh weight**			**Mean**	**Dry weight**			**Mean**
	I	**II**	**III**		**I**	**II**	**III**	
Seed coating	86.2	89.1	86.4	87.23	44.2	41.0	42.0	42.73
Drenching	35.4	32.4	39.2	35.66	18.8	17	16.5	17.43
Seed coating + drenching	62	62.1	62.3	62.13	36.2	35.2	31.0	34.13
control	54.2	59.2	50.5	54.63	31.2	30.5	31	30.90

DISCUSSION

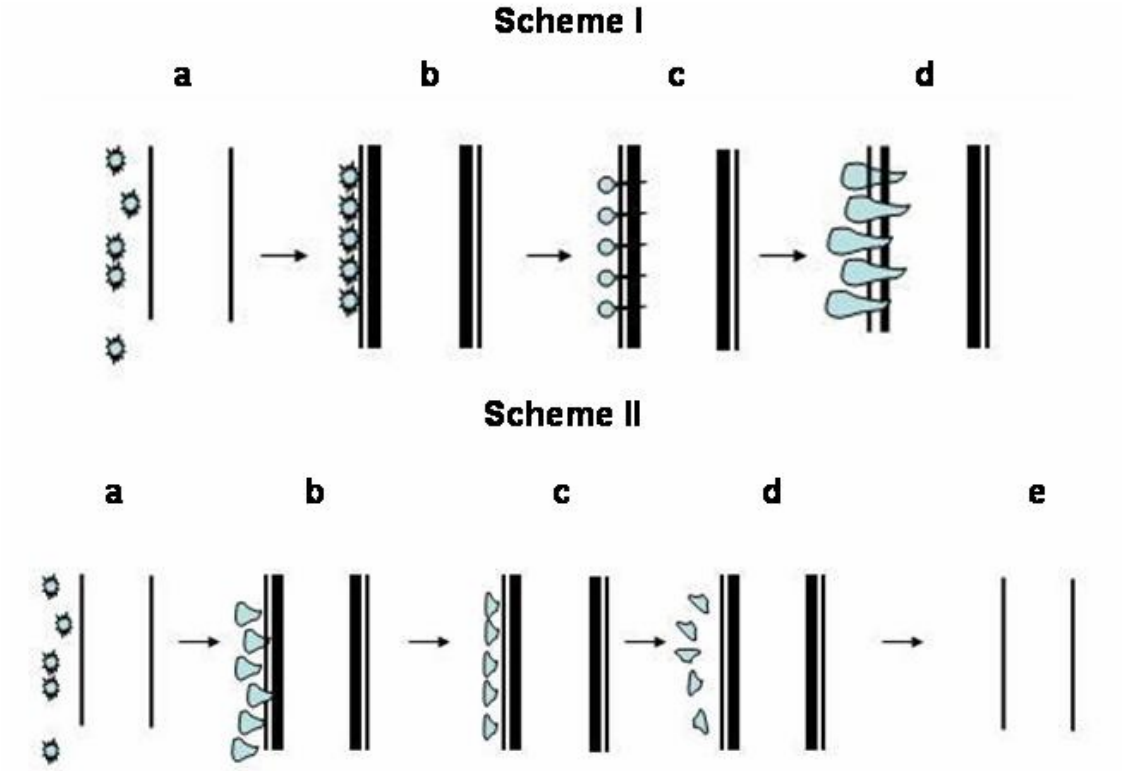
The biochemistry of mechanisms underlying host-pathogen interactions warrants study of the pathogen at the laboratory level. The inhibition of the growing phytopathogenic fungus and its enzymes b bioactive molecules may shed light on the strategies which could be employed for disease control. Xylanases, proteases and cellulases are some of the enzymes implicated in the invasion process of the pathogenic organisms (Whistler and Richards., 1970; Blake and Richards., 1971; Darvill et al., 1980., Cooper, R.M., 1983; Holden, F.R and Walton, J.D., 1992., Movahedi and Heale, 1990 a, b). Any successful pathogen produces a mixture of these enzymes for successful infection making them potential targets for the development of antifungal agents.

Protease inhibitors are an important class of defense proteins which have been implicated in a number of plant protection strategies. ATBI, a bifunctional inhibitor of xylanase and aspartic protease and VrAPI, an inhibitor of aspartic proteases were analyzed for their efficiency as an antifungal agent against *F. udum*, a pathogen of pigeon pea (Dash et al., 2001; Dash et al., 2001). The laboratory experiments included its effect on the growth of fungus at spore and mycelium stage and on the hydrolytic enzymes secreted by *Fusarium udum.* Both the inhibitors were efficient in stopping the growth of the fungus in plate assays thus proving its inhibitory action on the mycelia stage of *F. udum*. In addition the assays on germination of spores demonstrated their inhibitory effect of on the formation of germ tubes of the fungus. This indicated that the inhibitors were effective at both the mycelia as well as the spore stage. The complex multidimensional mode of inhibition could be visualized by the Hill and the reciprocal plots.

The inhibitors were found to inhibit the extracellular xylanase and aspartic protease produced by the fungus. These enzymes are critical in the germination of fungal spores and in the proliferation on the fungal mycelium. Their inhibition leads us to hypothesize that these critical processes are affected in the field also during pathogenesis of the host plant by the fungus. Thus the proposed mechanism of inhibition of *F. udum* by the inhibitors in the field is depicted in figure 15. In the soil, the spores of the fungus come into contact with the roots of the plant and adhere to it (Figure 15a, Scheme I). Upon adhesion the spores germinate forming the primary germ tubes which later form the penetrating mycelium, which proliferates inside the host tissues (Figure 15b-c, Scheme I). This leads to successful pathogenesis of the host plant leading subsequently to the occurrence of wilt (Figure 15d, Scheme I). This entire process is diagrammatically explained in Figure 15, Scheme I. In the presence of the inhibitor, the adhesion of the spores is unaffected. So, the spores succeed in adhering to the plant roots but they fail to form the germ tube (Figure 15a-b, Scheme II). Therefore invasion of host tissues is prevented (Figure 15c-d, Scheme II) leading to a healthy plant bereft of pathogen (Figure 15e, Scheme II). This mechanism is outlined in figure 15 Scheme II. The inhibition of growing mycelium by the inhibitors signifies that even if some of the spores germinate in soil their further proliferation is stopped. The reversibility of the inhibitory effect on the spores of *F. udum* indicates that it will not be killed in the soil but will be maintained in the environment in the spore form ensuring natural balance. The mechanisms outlined till date involve various modes of action, the most studied being mycoparasitism, competition and antibiosis (Elad, 1996). This bifunctional mode of action and its significance in the field provides a new area wherein further study will pinpoint the accuracy of the mechanism and will aid in the development of more novel molecules with potential as biocontrol agents.

The statistical analysis helped in scrutinizing the validity of the field experiments. The Chi test performed on the field trial data rejects the null hypothesis that there is no difference in disease occurrence between the control and the treatments by the inhibitors. The results indicate that there is a significant difference in the treatments and the control due to the use of the inhibitor. The non linear regression analysis evaluated the efficiency of the inhibitors in wilt control. The negative correlation obtained indicated that as the concentration of the inhibitor increased the intensity of wilt decreased. The t test was used to compare whether the groups indicated in the study i.e. the control and the treated plants group differ in their outcomes due to inhibitor effect and whether the difference is statistically valid. The null hypothesis was rejected and the alternative hypothesis that the groups do differ significantly was accepted at the 0.05 confidence level. This means that some of the difference between the control and the treated plants could be attributed to the treatment with the inhibitors.

Figure 15: Schematic mechanism of effect of inhibitors during infection by the pathogen in the field



In all schemes the plant roots are depicted by vertical lines and deposition of additional physical barriers by additional bold vertical lines. Scheme I depicts the normal method of progress of infection by spores of *F. udum*. a: The spores of *F. udum* are present in surrounding rhizosphere of the roots. b: They come in contact with the roots inducing additional physical barriers to be deposited. c: The spores form the primary germ tubes which invade the host tissues. d: Further mycelium formation and infection of host roots occurs.

Scheme II depicts the mechanism of the inhibitors in affecting the process of infection of roots of pigeon pea by *F. udum*. a: The spores are present in the vicinity of pigeon pea roots. b: The spores come in contact with the roots and try to form the primary germ tubes. c: Spores fail to form the germ tubes and are unable to initiate the infection process. d: The spores loose contact with the roots. e: The plant roots remain healthy and vigor increases.

Since the fungus is not killed it is maintained in the environment ensuring natural balance. The mechanisms outlined till date involve various modes of action, the most studied being mycoparasitism, competition and antibiosis (Elad, 1996). The present study outlines the control of wilt of pigeon pea by *F.udum* by aspartic protease inhibitors with a new mechanism providing a paradigm shift in biocontrol strategies wherein active biomolecules can be developed as biocontrol agents precluding the use of live organisms.

SECTION II

BIOLOGICAL CONTROL OF POWDERY MILDEW OF MUNG BEAN

Mung Bean

The genus *Vigna*, which is closely related to *Phaseolus* belongs to the family Fabaceae formerly known as Leguminosae. It is composed of more than 200 species that are native to the warm regions of the old and the new world. The genus contains several species that are of considerable economic importance especially in the developing countries. The species *V. radiata* was recognised as a separate species from *V. mungo* by Fery in 1980.

Vigna radiata

Scientific Classification:

Kingdom:	**Plantae**
Division:	**Magnoliophyta**
Class:	**Magnoliopsida**
Order:	**Fabales**
Family:	**Fabaceae**
Genus:	***Vigna***
Species:	***radiata***
Common Name:	**Mung Bean**
Former nomenclature:	***Phaseolus aureus***

Other names: nong taao, pua sha, moyashi-mame, lu tou, look dou, balatung, dau-xanh

Benefits

In India, *Vigna radiata* (mung bean) is one of the most important pulse crops from the agro-economic and nutritional point of view. India is the largest producer of mung bean accounting for 45% of the world's share of the area under cultivation and production. Area, production and productivity were 3.09 m ha, 1.12 m tones and 364 kg/ha respectively. It provides high quality protein, vitamins, carbohydrates and minerals especially for the vegetarian diet. Apart from human consumption it is used as a feed for the livestock and contributes substantially to soil nitrogen and phosphate solubilization. It is extensively grown in the Indian subcontinent, America and Africa. The overall significance of the crop especially the seeds and their importance in ayurveda makes mung bean a primary target for crop and yield improvement. This pulse is still today used as an opportunity crop in rotation with cereals. The plants are erect with few branches carrying pods borne in clusters near the top of the plant. Pods contain 8-15 green seeds. Its main advantages are that being a legume it does not require nitrogen fertilizer application and it has a short (75-90 days) growth duration which means that it requires less water and can be easily fitted into rotation programs. The seeds which are the edible part of the plant are small, cylindrical bean with a bright green skin.

Nutritional significance of mung bean

Nutrient	Units / kg
Water	734g
Energy	1060 kcal
Protein	75 g
Total lipid (fat)	4 g
Carbohydrate (by difference)	195 g
Fiber, total dietary	75 g
Sugars, total	20 g
Minerals	2.6 g – 0.05 mg
Vitamins	28 – 0.5 mg
Lipids	1 g
Cholesterol	0 mg
Amino acids	0.5 – 13 g
Beta carotene	140 mcg

USDA National Nutrient Database for standard Reference, Release 18 (2005)

Diseases

The productivity of the crop is restricted due to several abiotic and biotic stresses. In the recent past a large number of varieties having high yield potential, short maturity duration and resistant/tolerant to major diseases have been developed but their spread is slow due to non availability of seeds of improved varieties and poor knowledge of farmers about them.

The major diseases of mung bean are the yellow vein mosaic disease caused by a virus and the powdery mildew disease due to an obligate fungal pathogen. Insect pests especially bruchids are a persistent threat to the plants.

Powdery mildew

Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world. The name "powdery mildew" reflects the powdery tarnish on the plant surface and the tricking spores that are the result of the non-sexual reproductive phase of the fungus. Powdery mildew is a common and widespread plant disease that is caused by obligate biotrophic ecto-parasite. Powdery mildew fungi are classified in Kingdom, *Fungi*; Phylum, *Ascomycota;* Class, *Plectomycetes;* Order, *Erysiphales* and Family, *Erysiphaceae*. The family Erysiphaceae is divided into six main genera that are identifiable from one another by their differing clesothecial appendages and varying numbers of asci per cleistothecium. The genus *Erysiphe* is widely spread and contains many phytopathogens. It is identified by the cleistothecia which are dark brown, globose, having many unbranched appendages. There are multiple asci within each cleistothecium. This genus causes powdery mildew disease on plants such as clover, cucurbits, beans, soybeans, delphinium, hydrangea, zinnia, phlox, aster, crepe myrtle, and tulip poplar. The representative species *Erysiphe polygoni* ***c***auses powdery mildew disease of beans, soybeans, clovers, and other legumes, cabbage, beets, cucumber, canaloupe, delphinium, and hydrangea.

Disease Control

Fungicidal products containing dithiocarbamate, quinomethianate and sulphur have long been known as active against powdery mildew. However they are predominantly protective and their persistence and activity are often relatively small, thus requiring frequent applications. In the early 1970s, the situation changed dramatically with the

introduction of systemic products that established new standards of control for powdery mildews. The mode of action of these systemic fungicides involved specific inhibition of physiological processes and they were effective at lower concentrations. The first systemic fungicides used against powdery mildew were benomyl, mopholines, tridemorph and dodemorph, and the 2- aminophyrimidines, dimethirimol and ethirimol. Intesive research throughout the agrochemical industry expanded options for powdery mildew control in the 1980s through introduction of several triazoles (sterol demethylation inhibitors, DMIs) and two additional members of the morpholines group, fenpropimorph and fenpropidin. Recent new chemistry has improved standards of disease control still further through the development of strobilurins (Clough and Godfrey, 1998), azoxystrobin, kresoxim methyl, and trifloxystrobin (Margot et al., 1994), a "morpholine" type compound, spiroxamine (Dutzmann et al., 1996), and a phenoxyquinoline, quinoxyfen (Longhurst et al., 1996). One consequence of using of systemic fungicides was to reduce dose rates. This allowed chemistry to seriously challenge plant breeding as a cost effective way to control powdery mildews in cereals.

The impact of chemical control has been tempered by the ease of powdery mildews developing resistance, quickly rendering many systemic fungicides ineffective (Holloman and Wheeler, 1999; Limpert et al. 1996; Lyr et al., 1999). Indeed, powdery mildew has a number of characteristics, which favor a rapid adaptation rate, such as its relatively short generation time, with sexual recombination throughout the year, and the nature of its spread, as newly adapted pathotypes can be carried relatives quickly by wind over a wide area. For instance, sterol demethylation inhibitors (DMIs) are not used as single compounds against powdery mildew anymore. Likewise, inhibitors of sterol reductase and isomerase, the morpholines, have shown some sensitivity shifts (spiroketalmines are also affected due to cross

resistance). Isolates of wheat powdery mildew resistant to both strobilurins and the quinoline fungicides, quinoxyfen were detected at a low frequency in some parts of Europe. Many different resistance mechanisms are possible:

The modes of action of different fungicides within each of the groups are very similar or the same. Any pathogen population that is resistant to one fungicide within a group will almost certainly be resistant to other members of that same group. The issue of cross-resistance adds a dimension that limits the flexibility for managing resistance. Once resistance develops to one fungicide, others in that group are likely to also become less effective or useless.

In contrast to cross-resistance, pathogen populations have been shown to develop resistance to fungicides in more than one chemical group. The intensive use of at-risk fungicides in different chemical groups without following resistance management principles can result in the development of multiple resistances. Couple multiple resistances across groups with cross-resistance within groups, and the loss of efficacy for a large number of fungicides is possible.

The hazards of chemical control agents are evident in the form of environmental damage and accumulation of toxic residues within the body tissues of animals and humans. This has warranted the need to search for less hazardous and more eco-friendly alternatives in management of diseases of mung bean.

Current work

This section reports the development of aspartic protease as biocontrol agents in the disease management of powdery mildew of mung bean. There are no reports of control of this disease on mung bean by a biological agent. The obligate nature of

the pathogen inhibits the development of biological controls since laboratory studies cannot be performed. However, this study is based on the assumption that all fungi require more or less aspartic proteases and xylanases for their development and successful pathogenesis. Therefore the inhibitors of these enzymes were thought to be effective in controlling the growth of the fungi in field. Since laboratory studies could not be carried out, the inhibitors were directly analysed at the pot and the field stage. Another dimension was that if any agent could be found to be even partially successful in controlling the disease then it could be used in the partial substitution of the hazardous chemical agents especially sulphur which are used on a large scale.

MATERIAL AND METHODS

Production of inhibitors

The alkalothermophilic *Bacillus* species was grown for 48 hours as described in Dash *et al*, 2001. Extracellular culture filtrate obtained by centrifugation of the growth after 48 hours at 5000 rpm for 20 minutes was used for pot and the large-scale field trials. The seed of Kopergaon-1 variety were ground in 4 volumes of Gly HCl buffer (0.05M, pH 3.0) and used for the preparation of partially purified inhibitor as described in the section on pigeon pea.

Pot Trials

For the pot trials of 21 pots, a randomized block design with two plants of the susceptible variety Kopergaon-1 of mung bean per pot was used. For all the trials conducted sulfur and carbendazim were used as controls. Treatments began 1 week after the first observation of mildew lesions and continued for 8 weeks. Treatments were carried out with a 5-liter backpack sprayer. Per plant 50ml liquid was sprayed so that there was no drip off as indicated in the diagram below:

Figure 16: Spraying of inhibitors on mung bean crop

Field trials

For the large scale field trial for analyzing dose dependent inhibition of powdery mildew of mung bean a randomized block design experiment in a plot of size 4 x 2.4 metres with three replications was performed as given in Table 11.

Table11: Experimental Design

Coding of Treatments

Treatments	Code
100% PIS	T1
75% PIS	T2
50% PIS	T3
25% PIS	T4
Wettable Sulphur (0.2%)	T5
Carbendazim	T6
Control	T7

PIS: Protease Inhibitor solution

Randomized block design

RIII	T3	T5	T1	T7	T2	T6	T4
RII	T7	T6	T5	T4	T3	T2	T1
RI	T1	T2	T3	T4	T7	T6	T5

Variety used is Kopergaon-1; RI, RII and RIII represents the number of replicate (T1-T7); one bed (ex. T1) consist of five line i.e. 15-20 seed with plot size of 2 X 2 (4 sqm) with spacing of 10cm between plants and 30 cm between rows.

Plant distance was 10cm in the rows and 30cm between the rows. Each experimental unit included 75 plants of Kopergaon-1, the highly susceptible variety of mung bean. 3 sprayings were performed every 15 days with a low-pressure sprayer in a dose dependent manner. The plants were observed for the powdery mildew disease

symptoms. The inhibitor sprayed plants were compared with the suitable controls and the entire data was subjected to statistical analysis.

In each treatment 10 plants of mungbean were selected and 5 leaves were observed for mildew observations on 30 DAS and there after at 5 days interval upto 60 Days by following 0-9 scale. Thus the percentage of leaf area covered by mildew was assessed using a key with 9 disease classes (0= 0% mildew; 3= 6-10%; 5= 21-30%; 7= 41-60% and 9= 81-75%. The key provided most detail in the severity range 1-40%, the more relevant part of the total range 0-75%. The assessments were made weekly on a "middle" leaf level (about leaf 15) and a "high" leaf level (about leaf 28). From each experimental unit, mung bean was harvested and weighed to give the yield per plot. An average value of the replications is reported.

For statistical analysis the mildew assessments (means of scored classes) were averaged per plot. Observed responses (severities) were rearranged in derived responses (Campbell and Madden, 1990): AUDPC, EGR and MML. The percent disease intensity of powdery mildew was calculated by the formula given below –

$$\text{Percent Disease intensity (P.D.I.)} = \frac{\text{Sum of all Numerical Ratings}}{\text{No. of plants observed} \times \text{Highest Rating}} \times 100$$

The figures indicate that out of a population of 100 plants what it the proportion of the wilted ones.

The AUDPC (Area Under Disease Progress Curve) was calculated by the formula given below.

AUDPC = K [(Si + Si-1) / 2] x D

Where, Si. = Disease intensity at i^{th} day of evaluation

K = No. of successive evaluation

D = Interval between i and i=1 evaluation of disease.

MMLs were determined by taking the means of the last three assessments per experiment. The EGRs were calculated by linear regression through three data points per plot. An √EGR + 0.5) transformation was carried out before analysis of variance. Analysis of variance and the Tukey test were carried out with arc sine square root transformed data. Treatment effects on the cumulative yield of mung bean heavier than 150g per plot per experimental unit were tested by ANOVA and the Tukey test.

RESULTS

Area under disease progress curve

The AUDPC of mung bean summarize mildew progress during the 8 weeks of experimentation (Table 12). 75% dilution of the culture filtrate gave significant reduction of mildew. The AUDPC values for the other dilutions were significantly smaller than those of 75% dilution. There was significant reduction (79 and 86%) in the incidence of mildew with carbendazim and sulfur respectively as compared to control (Table 12).

Table 12: Effect of ATBI on powdery mildew of mung bean

Treatments (inhibitor units per liter)	**Disease Intensity (%)**			**Mean**	**Disease Control (%)**	**AUDPC**
	RI	RII	RIII			
75%ECF T2	19.95	15.53	22.84	19.44	69.59	273.83
50%ECF T3	19.95	15.53	24.84	20.10	68.55	353.60
25%ECF T4	27.08	31	28.44	28.84	54.88	424.6
Wettable Sulphur (0.2%) T5	7.55	8.88	9.99	8.77	86.26	135.94
Carbendazim T6	11.11	13.33	16.4	13.61	78.71	168.05
Control T7	66.8	60.50	64.50	63.93	-	649.13

75% ECF corresponds to the extracellular culture filtrate of Bacillus sp. containing 112 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

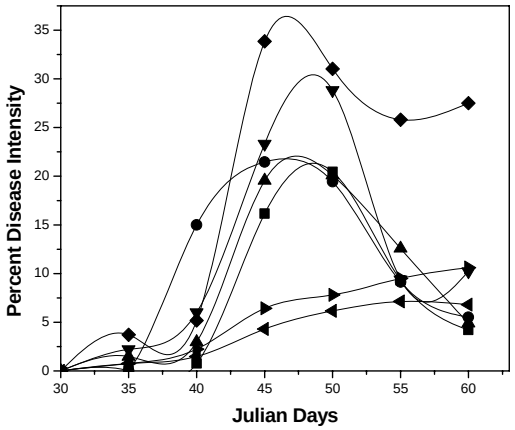
Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and RIII signify the replications in the randomized block design

Mildew Progress Curves (MPC's)

Figure 13 shows the MPC's of the field trial on the susceptible mung bean cv Kopergaon-1. Effective control of PM was obtained with 75% bacterial culture filtrate containing 112 inhibitor units per liter of the inhibitor. Although treatments with sulphur and carbendazim were more effective than with any dosage of the culture filtrate, the study indicated that biological control agents could be developed. After 40 days after sowing when the effect of the culture filtrate was reduced one booster dose of antifungal agent could be included to give a combined strategy for inhibition and for reducing the application of harmful pesticides. Similar results were obtained with the MML levels attained in the presence of the inhibitor and the chemical control agents. It is thought that an earlier start wherein the biocontrol agent is applied right from day 1 would have been a better alternative. Also it would have been better to use increased concentration of the bioactive principles in the culture filtrate by effectively concentrating the culture filtrate.

Figure 13: Mildew Progress Curves for powdery mildew of mung bean



The mildew progress curves for powdery mildew of mung bean indicated that substantial disease control was achieved with 100% ECF (▼), 75% ECF (●), 50% ECF (▲), 25% ECF (■), sulphur (▶) and carbendazim (◀)as compared to the control (◆).

Early Growth Rates and MMRs

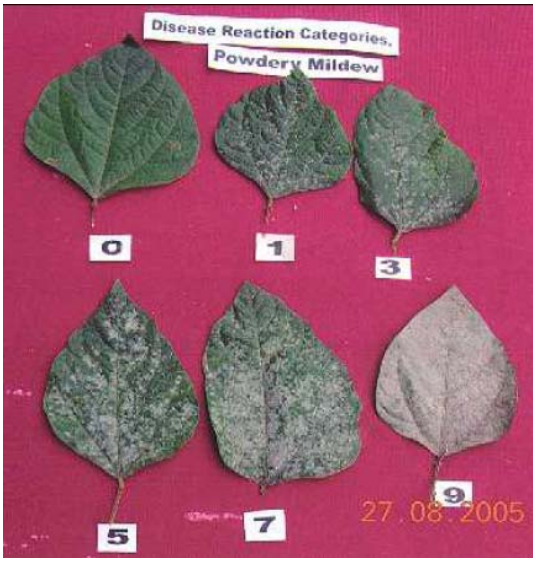
As observed in Figure 13, the early growth rate of mildew fungus was slow in case of all the treatments. After the 35th day of observation, mildew levels were seen to rise. As compared to the control the growth rate was less in the treatments with the biocontrol agent especially with higher doses of the agent. Maximum mildew levels attained in the control were 35-40% which increased to 70% by the end of the season. The maximum mildew level attained with sulphur remained low at 10% while that with the highest dosage of the biocontrol agent remained at 35%.

Mildew Observations

Figure 14 illustrates the intensity of the powdery mildew disease under the local climatic conditions indicating more than 51% leaf area to be infected by the

pathogen. This percentage figure was obtained by using the disease scale for the disease reaction categories.

Figure 14: Disease Reaction Categories for powdery mildew of mung bean



The disease intensity of powdery mildew was scaled on a scale of 0-9 as

0 : No powdery growth on leaves.

1 : Small scattered powdery speaks covering 1% or less area of leaf.

3 : Small scattered powdery speaks covering 1-10% of the leaf area.

5 : Powdery leaf scions enlarging with gray coloured powdery mass.

7 : Gray coloured powdery growth covering 26-50% of the leaf area.

9 : Gray coloured pathogen of powdery growth covering 51% or more leaf area.

Field trial

Figure 14 denotes the effectiveness of ATBI in controlling the infection of powdery mildew in the pot trials. The dose dependent field trials indicate that 84 inhibitor units of the inhibitor were able to control 70.00% of the disease (Figure 14, Table 13). Chemical control with carbendazim and wettable sulphur showed that 79% and

86% of the disease could be managed. Also, the inhibitor being extracellular, the organism can be effectively separated from the bio-formulation which becomes eco- and user friendly.

Figure 14: Effect of the extracellular culture filtrate of *Bacillus* on powdery mildew of mung bean

A: A disease intensity of 64% was observed in the absence of any control measures.

B: The disease was controlled to an extent of 70% using the extracellular culture filtrate of *Bacillus* containing 112 inhibitor units of ATBI.

Yield

The mean yield showed a two fold increase with 75% of the culture filtrate as compared to the yield in control. Carbendazim and Sulfur showed almost equal increase in the yield. The mean increase in yield for the biocontrol agent was comparable to the chemical control agents used as seen in Table 14.

Table 14: Effect of application of extracellular culture filtrate on yield of mung bean infected with powdery mildew

Treatments (inhibitor units per liter)		Yield of mung bean (g)			Mean
		RI	RII	RIII	
75%ECF	T2	317	295.18	317.01	309.73
50%ECF	T3	291.86	321.05	294.08	302.33
25%ECF	T4	257	249	238	248
Wettable Sulphur (0.2%)	T5	361.95	346.08	379.17	326.4
Carbendazim	T6	340.2	325.6	337	334.26
Control	T7	178	166.80	160.02	168

75% ECF corresponds to the extracellular culture filtrate of Bacillus sp. containing 112 inhibitor units of ATBI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

RI, RII and RIII signify the replications in the randomized block design

Aspartic protease from plant source

Area under disease progress curve

The AUDPC of mung bean summarizes mildew progress during the 8 weeks of experimentation (Table 15). 100% of the partially purified VrAPI solution gave 60% reduction in mildew. There was significant reduction (79 and 86%) in the incidence

of mildew with carbendazim and sulfur respectively as compared to control (Table 15).

Table 15: Effect of VrAPI on powdery mildew of mung bean

SN	Aspartic Protease Inhibitors	Percent disease intensity (%)	% Disease control	AUDPC
1	**100 % PP VrAPI**	25.62 (30.36)	59.92	338.39
2	**75% PP VrAPI**	32.09 (34.48)	52.00	405.15
3	**50% PP VrAPI**	32.63 (34.20)	48.95	436.71
4	**25% PP VrAPI**	34.37 (35.89)	46.26	435.17
5	**Wett sulphur (0.25%)**	8.77 (17.17)	86.26	135.94
6	**Carbendazim (0.1 %)**	13.61 (21.59)	78.71	168.05
7	**Unsprayed control**	63.93 (53.10)	---	649.13
	SE +/- CD at 5 % CV (%)	1.25 3.68 7.05		

100% PP VrAPI corresponds to the Partially Purified *Vigna radiata* Protease Inhibitor containing 120 inhibitor units of VrAPI per liter.

20 liters of the extracellular culture filtrate were applied as a single dose in the field.

Trichoderma viride and *Rhizobium* were applied as recommended by the commercial manufacturers.

Carbendazim was applied at the rate of 2g/kg of seed.

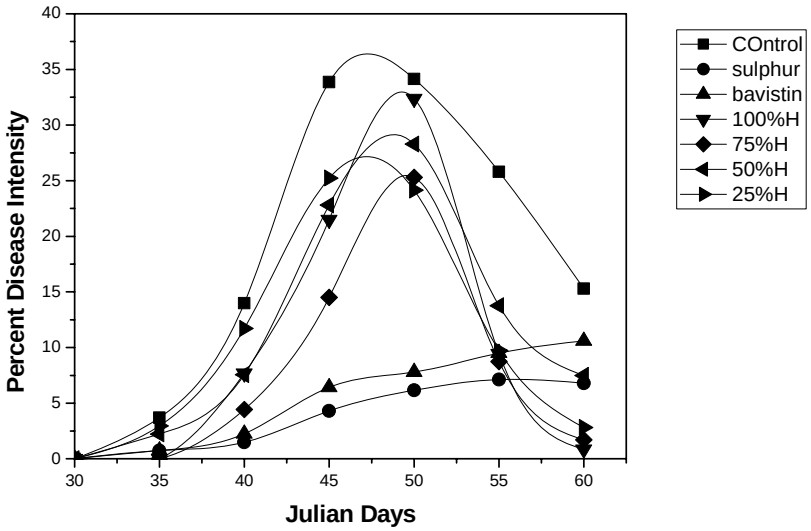
RI, *RII* and *RIII* signify the replications in the randomized block design

Figures in parentheses are arc sin values.

Mildew Progress Curves (MPC's)

Figure 15 shows the MPC's of the field trial on the susceptible mung bean cv Kopergaon-1. Effective control of PM was obtained with 100% partially purified VrAPI containing 120 inhibitor units per liter of the inhibitor. Although treatments with sulphur and carbendazim were more effective than with any dosage of the culture filtrate, the study indicated that biological control agents could be developed. After 40 days after sowing when the effect of the culture filtrate was reduced one booster dose of antifungal agent could be included to give a combined strategy for inhibition and for reducing the application of harmful pesticides. Similar results were obtained with the MML levels attained in the presence of the inhibitor and the chemical control agents.

Figure 15: Mildew progress curves for VrAPI



The mildew progress curves for powdery mildew of mung bean indicated that substantial disease control was achieved with the control agents as compared to the untreated control.

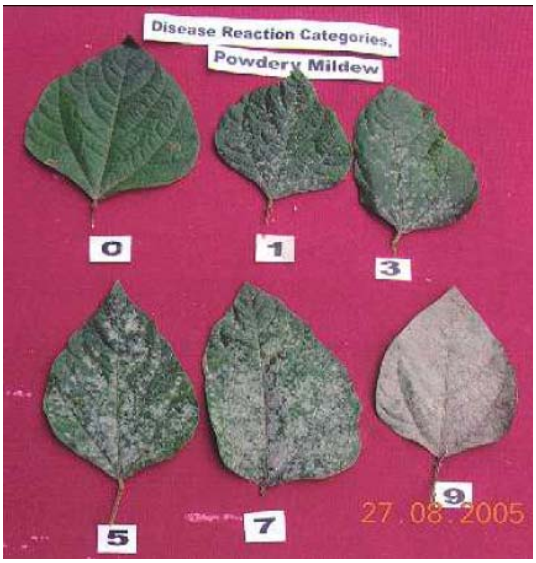
Early Growth Rates and MMRs

As observed in Figure 15, the early growth rate of mildew fungus was slow in case of all the treatments. After the 35th day of observation, mildew levels were seen to rise. As compared to the control the growth rate was less in the treatments with the biocontrol agent especially with higher doses of the agent. Maximum mildew levels attained in the control were 35-40% which increased to 70% by the end of the season. The maximum mildew level attained with sulphur remained low at 10% while that with the highest dosage of the biocontrol agent remained at 35%.

Mildew Observations

Figure 16 illustrates the intensity of the powdery mildew disease under the local climatic conditions indicating more than 51% leaf area to be infected by the pathogen. This percentage figure was obtained by using the disease scale for the disease reaction categories.

Figure 16: Disease Reaction Categories for powdery mildew of mung bean



The disease intensity of powdery mildew was scaled on a scale of 0-9 as

0 : No powdery growth on leaves.

1 : Small scattered powdery speaks covering 1% or less area of leaf.

3 : Small scattered powdery speaks covering 1-10% of the leaf area.

5 : Powdery leaf scions enlarging with gray coloured powdery mass.

7 : Gray coloured powdery growth covering 26-50% of the leaf area.

9 : Gray coloured pathogen of powdery growth covering 51% or more leaf area.

Field trial

Figure 17 denotes the effectiveness of VrAPI in controlling the infection of powdery mildew in the field trials. The dose dependent field trials indicate that 120 inhibitor units per liter of the inhibitor were able to control 60% of the disease (Figure 3, Table 4). Chemical control with carbendazim and wettable sulphur showed that 79% and 86% of the disease could be managed.

Figure 17: Effect of VrAPI on powdery mildew

The figure indicates the positive control obtained with 100% partially purified VrAPI (right). All the healthy plants bore flowers and fruits. The control remained the same as with ATBI (left).

More yields were obtained in the presence of inhibitor. This indicates the positive effect of VrAPI on health of the plants.

DISCUSSION

Phytopathogens are responsible for enormous losses ($30-$50 billion annually) in cultivated and stored crops and are a major impediment to effective food distribution worldwide[1]. Countervailing measures in the form of antimicrobials and pesticides not only significantly increase production costs and are regarded as serious environmental contaminants, they contribute to the increase in antimicrobial-resistant species. Consequently, there has been considerable activity in developing disease resistance strategies; however, most of these have been fairly narrow with respect to the microbial spectrum of protection.

Antimicrobial peptides play a primary role in host defense. To most researchers, they immediately call to mind protein fragments as antigens, or signaling molecules between the cellular components of innate and/or adaptive immunity. However, all life forms also produce peptides with a direct capacity to inactivate microorganisms, as a central component of innate defense (Fossom; 1970). These antimicrobial peptides (AMPs) have an ancient lineage and have likely participated in the relentless battle between host and pathogen throughout evolution, so helping to shape the relationship between host and microbe, be it commensal or pathogen (Broekaert et al., 1997). Plant protection has seen significant progress in the use of antimicrobial peptides (Weindling and Emerson., 1936; Weindling., 1937; Weindling., 1941; Hanson and O'Leary., 1981; Papavizas., 1985., Ignoffo and Anderson., 1979). However the nature of these antimicrobial peptides has not been outlined and studied in detail. Even though biocontrol agents have been developed against many other crop pathogens, there are no biocontrol agents reported for powdery mildew of mung bean.

Powdery mildew is a devastating disease on a major crop plants. The obligate nature of the pathogen has however hampered progress in the development of biocontrol agents against the fungus, *Erysiphe polygoni*. Since no laboratory studies can be carried out for the development of specific agents against the fungus, the control of powdery mildew has lagged behind. The development of the fungus on the host plant occurs via the formation of haustoria or the sucking roots which penetrate the host tissues and form the mycelium which further leads to proliferation inside the plant ultimately ending in disease. During the formation of haustoria from the spores of the fungus, xylanases and proteases are required. Therefore the inhibitors of these enzymes which do not affect the plant enzymes could be developed as effective biocontrol agents against the fungus. This is all the more possible since the process of haustoria formation occurs only after the spores sit on the host tissues and get an ambient condition for germination. If at this stage inhibitors of the critical enzymes are applied then the spores are unable to germinate and form haustoria. Therefore they cannot invade the plant tissues. This leads to effective disease control.

Both ATBI and VrAPI were found to be effective against xylanases and proteases of phytopathogenic fungi such as *F. udum*. Therefore these could be responsible for the prevention of haustoria formation leading to effective powdery mildew management. Although partial control could be achieved which was less as compared to the chemical control agent, sulphur; it was thought that these inhibitors could be developed further such that the use of sulphur could be reduced by combinatorial treatments.

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