STUDIES OF AN ALKALINE PROTEASE INHIBITOR FROM A *STREPTOMYCES SP*.

THESIS SUBMITTED TO
THE UNIVERSITY OF PUNE
FOR **THE DEGREE OF**
DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

BY
JUI PANDHARE

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE-411 008
INDIA

JUNE 2002

Acknowledgement

Words fail me, to express my feelings towards my all time friend CV for constantly encouraging and motivating me to excel. His enduring support, untiring help and inspiration will always be cherished. He has always been there all the time with me in my ups and downs .I would also like to thank my dear friend Swaroop for always being there for me.

I owe my deepest gratitude to my parents, my father for his inspiration and encouragement without which it would not have been possible to embark upon this journey in life, my mother for her endurance, teaching me to sustain and work untiringly, my brother and sister-in-law for all the support and encouragement and finally my nephew Ameya and my niece Ojasi for all the delightful moments.

Jui Pandhare

Dedicated to My Family…………..

TABLE OF CONTENTS

CHAPTER 2. SCREENING AND ISOLATION OF ALKALINE PROTEASE INHIBITOR PRODUCING MICROORGANISMS   **37-49**

<u>DECLARATION</u>

This is to certify that the work incorporated in the thesis entitled "**STUDIES OF AN ALKALINE PROTEASE INHIBITOR FROM *STREPTOMYCES* SP."** submitted by **Ms. Jui Pandhare** was carried out under my supervision at the Division of Biochemical Sciences, National Chemical Laboratory, Pune, India. Materials obtained from other sources have been duly acknowledged.

June 2002
Deshpande

Dr. (Mrs) Vasanti V.

Research Guide
Division of Biochemical Sciences

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**STUDIES OF AN ALKALINE PROTEASE INHIBITOR FROM *STREPTOMYCES* SP.**" submitted for the degree of Doctor of Philosophy in Biochemistry to the University of Pune has not been submitted by me for a degree to any other university or institution. The work was carried out at the Division of Biochemical Sciences, National Chemical Laboratory, Pune, India.

June 2002   **Jui Pandhare**

Research Student
Division of Biochemical Sciences

ABBREVIATIONS

5-IAF	5-iodoacetamidofluorescein
API	Alkaline protease Inhibitor
APS	Ammonium persulfate
BSA	Bovine serum albumin
CD	Circular dichroism
DEAE	Diethylaminoethyl
DTNB	Dithiobisnitrobenzene
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FAP	*Fusarium* alkaline protease
GdnHCl	Guanidine hydrochloride
GSH	Glutathione reduced
GSSG	Glutathione oxidized
HPLC	High performance liquid chromatography
IAN	isatoic anhydride
IC$_{50}$	50% inhibitory concentration
MID	Minimum inhibitory dose
NBS	N-bromosuccinimide
NCIM	National center for industrial microorganisms
PDI	Protein disulfide isomerase
PEG	Polyethylene glycol
pI	isoelectric point
PK	Proteinase K
PMSF	Phenyl methyl sulfonyl fluoride
rd-API	reduced-denatured API
sAAPF-pNA	N-succinyl-L-Ala-Ala-Pro-PhenyAla-p-nitroanilide
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TEMED	N,N,N',N' tetramethyl ethylenediamine

ABSTRACT

Protease inhibitors are an important class of proteins that are ubiquitously present in all life forms. They have evoked tremendous interest because of their pivotal role in the regulation of various physiological and pathological processes involving the mobilization of tissues proteins and in the processing of precursors of proteins. Proteases are responsible either, directly or indirectly for all bodily functions including cell growth, nutrition, differentiation, and protein turn over. Determination of the kinetic parameters of the inhibition of proteases will provide insight into the mechanism of the interaction between the enzyme and the inhibitor. Proteases are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer, and AIDS. The specific inhibition of proteases by the protease inhibitors can be used as a strategy for drug design for the prevention of propagation of the causative agents. Protease inhibitors regulate the action of proteases and play a significant role in the protection of plants from pest and pathogen invasion. In view of the importance of protease inhibitors from microbial sources in developing therapeutics and as biocontrol agents, the present work was initiated by screening for microorganims producing potent inhibitors. In order to gain insight into the mechanism of inhibition of alkaline proteases by the inhibitors, the present work has been undertaken with following main objectives.

1. Screening and Isolation of alkaline protease inhibitor producing microorganisms.
2. Purification and biochemical characterization of an alkaline protease inhibitor, API from a *Streptomyces* sp.
3. API as a novel antifungal protein: Protease inhibitory activity as the biochemical basis of antifungal activity
4. Protein disulfide isomerase accelerated oxidative refolding of API: effect of macromolecular crowding on refolding kinetics
5. Interaction of API with fungal alkaline proteases: kinetic parameters involved in the inactivation of the proteases
6. Studies on the inhibitor induced thermal stability of Proteinase K.

Chapter 1. General Introduction.

This chapter is an overview of the classes of serine protease and serine protease inhibitors, their occurrence, and mechanism of action. It covers the review of literature of

serine protease inhibitors, their families, with special reference to the microbial serine alkaline protease inhibitors.

Chapter 2. Screening and Isolation of alkaline protease inhibitor producing microorganisms

The essential nature of the alkaline proteases in numerous physiological and biotechnological applications has evoked tremendous interest towards isolating new inhibitors from various resources. After extensive screening of the vast diversity of soil samples, we have isolated four actinomycetes strains producing alkaline protease inhibitors (APIs) designated as API (265 U/ml), API-I (242 U/ml), API-II (116 U/ml) and API-III (186 U/ml). The time course for the production of APIs was determined and optimization of fermentation conditions was carried out by substituting an assortment of different carbon and nitrogen sources. The inhibitors possessed a unique specificity of inhibition confined only to alkaline proteases. They exhibited differences in their molecular nature and in their pH and temperature stabilities. API, API-I and API-II were high molecular weight (>10 kD) proteinaceous inhibitors whereas API-III was a low molecular weight inhibitor (<10 kD). API, API-I and API-II exhibited stability over a pH range of 5-12 whereas API-III displayed a wide pH stability from 2- 12. API was the most stable inhibitor. It was stable in a temperature range of 40-95°C and exhibited a half-life of 5 h at 85°C. API-I was stable at 60°C with a half-life of 1 h but API-II showed a half-life of 20-25 min at 45°C. API-III exhibited the least thermal stability with complete loss of activity at 37°C after 1 h. The stability of API-I, II and III at 65, 55 and 45°C, respectively, was enhanced by the addition of various additives. Glycine (1 M) offered complete protection to the three APIs. Polyethylene glycol 8000 (10 mM) prevented the thermoinactivation of API-I. In the presence of glycerol and sorbitol (10%) the stability of API-I and API-II was increased by 40-60%.

Chapter 3. Purification and biochemical characterization of an alkaline protease inhibitor, API from a *Streptomyces* sp. NCIM 5127

The alkaline protease inhibitor (API) producing actinomycete strain isolated from soil was identified to be a *Streptomyces sp*. by its colony characteristics and spore chain morphology. API has been purified to homogeneity by ammonium sulfate precipitation, preparative polyacrylamide gel electrophoresis and DEAE cellulose chromatography. Purified API moved as a single protein on native and SDS-PAGE. The homogeneity of the purified

protein was also confirmed by a single peak at a molecular mass of 28kD upon gel filtration on HPLC and a single peak upon isoelectric focusing with an isoelectric point of 3.8. API is a competitive type of inhibitor with a K_i value of 2.5x 10^{-9} M for subtilisin. Purified API is stable over a pH range of 6 to12 and a temperature range of 40 to 95°C. Amino acid composition of the purified API showed an abundance of Ala, Val, Gly and Asp accounting for about 50% of the total amino acid content. DTNB titration yielded two free sulfhydryl groups and five disulfide linkages per mole of inhibitor. The oxidation of Trp by NBS resulted in a progressive decrease in absorption at 280 nm. The number of Trp residues oxidized per mole of API was calculated to be 2. API was found to specifically inhibit the alkaline proteases such as subtilisin, proteinase K from *Triticharium album* Limber and the alkaline protease from *Conidiobolus* sp. The binding interaction of API with the alkaline protease from *Conidiobolus* sp revealed that the API and alkaline protease combine in a molar ratio of 1:2.

Chapter 4. API as a novel antifungal protein: Protease inhibitory activity as the biochemical basis of antifungal activity

Besides its antiproteolytic activity, API was found to exhibit antifungal activity (*in vitro)* against several phytopathogenic fungi such as *Fusarium*, *Conidiobolus, Alternaria, Rhizoctonia* and also against *Trichoderma,* a saprophytic fungus. Retardation in the rate of hyphal growth extension was observed in the presence of API. *Fusarium oxysporum* f. sp. *ciceri* was found to be the most sensitive to inhibition requiring 0.5 µg/disc as a minimum inhibitory dose (MID). The fungal strains *F. oxysporum* f. sp. *ciceri* and *Conidiobolus* sp. produce extracellular alkaline protease(s) when grown in a liquid medium. The serine alkaline protease(s) are known to be vital for the growth and development of the above fungal strains; therefore, the antiproteolytic activity of API could be conveniently correlated to its antifungal activity. Parallel enrichment of both the antiproteolytic and antifungal functions obtained during purification of API indicated its bifunctional behavior. The inactivation of API at 95°C for 30 min resulted in the concomitant loss of the two activities revealing their presence on a single molecule. Chemical modification of API with NBS resulted in the complete loss of antiproteolytic and antifungal activities, with no gross change in conformation implying the involvement of a Trp residue in the active site of the inhibitor and the presence of a single active site for the two activities. Treatment of API with DTT abolished both the activities

although the native structure of API remained virtually unaffected, indicating the catalytic role of the disulfide bonds. Inactivation of API either by active site modification or by conformational changes leads to the concurrent loss of both the antiproteolytic and antifungal activities. Based on the correlation between antiproteolytic and antifungal activities during co-purification, heat inactivation, chemical modification and its binding interaction with the fungal protease, we have demonstrated that the dual function of API is a consequence of its ability to inhibit the essential alkaline protease.

Chapter 5. Protein Disulfide Isomerase Accelerated Oxidative Refolding of API: Effect of Macromolecular Crowding on Refolding Kinetics

The spontaneous refolding and reactivation of API upon reduction and denaturation occurs to a limited extent mainly due to its propensity to aggregate and its dependence on the regeneration of the native disulfide linkages. Protein disulfide isomerase (PDI), a catalyst of oxidative protein folding accelerates the refolding rates and reactivation yields due to its isomerase activity and chaperone like properties. The oxidative refolding pathway of API has been investigated using PDI. At lower concentrations of API, catalytic amounts of PDI are effective in accelerating the reactivation rates and yields indicating its isomerase function. With higher concentrations of API, reactivation yield decreased and catalytic amounts of PDI failed to promote efficient reactivation. However, at a 10 fold molar excess of PDI, the yield is almost doubled and reactivation rates are also increased indicating that PDI functions as a chaperone preventing aggregate formation and as an isomerase, which promotes the correct formation of disulfide bonds in API. To simulate the intracellular environment, we have also studied the influence of macromolecular crowding agents on the protein folding kinetics of reduced and denatured API. Concentration dependent decrease in the refolding yields was obtained in the presence of crowding molecules (50-250 g/L). The addition of PDI under crowded conditions counteracted the decrease in reactivation significantly and improved yields and rates of API were obtained. Our results on the chemoaffinity labeling reinforced the role of PDI as a foldase displaying both its chaperone and isomerase activities in the oxidative refolding of API.

Chapter 6. Interaction of API with Fungal Alkaline Proteases: Kinetic Parameters Involved in the Inactivation of the Proteases

API exhibited a slow-tight binding inhibition mechanism towards the fungal alkaline proteases, Proteinase K from *Tritirachium album* limber and the alkaline protease from *Fusarium oxysporum* (FAP) with IC_{50} values of 5.5 ± 0.5 x 10^{-5} M and 6.5 ± 0.5 x 10^{-5} M respectively. The steady-state kinetics revealed time-dependent competitive inhibition of Proteinase K and FAP by API, consistent with a two-step inhibition mechanism: E + I $\leftrightarrows$ EI $\leftrightarrows$ EI*. The first step involved the rapid equilibrium towards the formation of a reversible enzyme-inhibitor complex (EI) with the K_i values of 5.2 ± 0.6 x 10^{-6} M for Proteinase K and 4.5 ± 0.5 x 10^{-6} M for FAP. Subsequently the EI complex isomerizes to a stable second enzyme-inhibitor complex (EI*). The kinetic parameters involved in the two-step inhibition mechanism of Proteinase K-API were evaluated in detail. The rate constant k_5 (9.2 ± 1 x 10^{-3} s^{-1}) associated with the isomerization of EI to EI*, revealed a faster induction of the localized conformational changes in the EI complex resulting in the clamping down of the enzyme onto the inhibitor. However, the second enzyme inhibitor complex, EI* dissociated at a very slow rate. The dissociation rate constant, k_6 depicted that API dissociated from the EI* in a much slower rate revealing its tight binding nature. The overall inhibition constant K_i* involved in the slow-tight binding inhibition of Proteinase K by API was 2.5 ± 0.3 x 10^{-7} M. The conformational changes induced in Proteinase K by API were monitored by fluorescence spectroscopy and the rate constants derived were in agreement with the kinetic data. Thus, the conformational changes are the consequences of the isomerization step of EI to EI*. A time dependent quenching of the fluorescence was obtained without any shift in the emission maximum, which represents no changes in the enzyme structure upon binding of API.

Chapter 7. Inhibitor Induced Thermal Stability of Proteinase K

Proteases find tremendous applications in various biotechnological industries. Higher thermostability is one of the essential features for the commercial exploitation of enzymes and often a prerequisite for the application of enzymes in several industrial processes. The thermal inactivation of industrially important enzymes can be circumvented by their stabilization through the formation of reversible enzyme-inhibitor complexes. We have selected Proteinase K, the alkaline protease from *Tritirachium album* Limber as the model enzyme to study the thermal stability of enzyme-inhibitor complexes. The thermal stability profile of Proteinase K revealed that it is stable at 65°C for 10 min followed by a rapid loss

of activity as a function of time. Interestingly, the stability of Proteinase K is enhanced by the binding of API to the enzyme. The incubation of uncomplexed Proteinase K at 70°C abolished its activity, however the inhibitor bound Proteinase K-API complex retained 50-55% activity. Thermal inactivation is often correlated to the structural and conformational changes in a protein. The structural changes induced in Proteinase K at 70°C were probed by circular dichroism and fluorescence spectroscopy. Our results implicated that the thermal inactivation of alkaline proteases can be prevented by API, and thus can have enormous applications in industries utilizing alkaline proteases at higher temperatures.

PUBLICATIONS

1. **Pandhare, J.** (Vernekar)**.**, Ghatge, M. S., and Deshpande, V. V. (1999). Alkaline Protease Inhibitor: A novel class of antifungal proteins against phytopathogenic fungi. ***Biochem. Biophys. Res. Commun.*** 262, 702–707.

2. Tanksale, A. M., **Pandhare, J.** (Vernekar)**.,** Ghatge, M. S., and Deshpande, V. V. (2000) Evidence for tryptophan in proximity to histidine and cysteine as essential to the active site of an alkaline protease. ***Biochem. Biophys. Res. Commun.*** 270, 910-917.

3. **Pandhare, J.** (Vernekar)**.**, Tanksale, A. M., Ghatge, M. S., and Deshpande, V. V. (2001) Novel Bifunctional Alkaline Protease Inhibitor: Protease inhibitory activity as the biochemical basis of antifungal activity. ***Biochem. Biophys. Res. Commun.*** 285, 1018-1024.

4. **Pandhare**, **J.,** Zog, K., and Deshpande, V. V. (2002) Differential stabilities of alkaline protease inhibitors from actinomycetes: Effect of various additives on thermostability. ***Biores. Technol.*** 84, 165-169.

5. **Pandhare, J.,** and Deshpande, V. V. Oxidative Refolding of Dimeric Alkaline Protease Inhibitor is Accelerated by Isomerase and Chaperone Functions of PDI (Communicated).

6. **Pandhare, J.,** Dash, C., Rao, M., and Deshpande, V. Mechanism based Inhibition of Proteinase K by a Novel Alkaline Protease Inhibitor: Kinetic parameters determining the mechanism of inhibition. (Manuscript under preparation).

7. **Pandhare, J.,** and Deshpande, V. V. Enhanced thermostability of Proteinase K through the formation of reversible enzyme-inhibitor complex. (Manuscript under preparation).

8. **Pandhare, J.,** Zog, K., and Deshpande, V. V. Kinetics of inhibition of alkaline protease from *Fusarium oxysporum*. (Manuscript under preparation).

PATENTS/POSTERS/ABSTRACTS

J. Pandhare (Vernekar), M.S.Ghatge, M.B.Rao and V.V.Deshpande (1999). "Process for the preparation of a proteinaceous Alkaline Protease Inhibitor." **Indian patent** no. 442-DEL-99 dated 19-3-99

J. Pandhare (Vernekar), M.S.Ghatge, M.B.Rao and V.V.Deshpande. (1999). "Process for the preparation of a proteinaceous Alkaline Protease Inhibitor." **US Patent Filed**

J. Pandhare (Vernekar), M.S.Ghatge and V.V.Deshpande. "Production, purification and characterization of a novel alkaline protease inhibitor (API) exhibiting antifungal properties from *Streptomyces sp*. NCIM 5127. " Abstract for poster presentation at the **International Conference on "Life sciences in the Next Millennium**" held at the University of Hyderabad from December 11th to 14th 1999.

J. Pandhare (Vernekar), M.S.Ghatge and V.V.Deshpande. "Alkaline Protease Inhibitor Exhibiting Novel Antifungal Properties Against Phytopathogenic Fungi." Paper presented in the International Conference on "**Women: Biotechnology, Environment, and Non-conventional Energy**" at **Indian Institute of Chemical Technology**, Hyderabad, India, from October 17-21, 2000.

J. Pandhare (Vernekar), M.S.Ghatge and V.V.Deshpande. "Alkaline Protease Inhibitor a Novel Biocontrol Agent." Paper presented in the VIII National Conference of "**Women in Science**" at **Agharkar Research Institute,** Pune, India, on September 12-14, 2001.

J. Pandhare (Vernekar), and V.V.Deshpande. "A novel thermostable alkaline protease inhibitor from *Streptomyces sp.*" Poster presented in **Thermophiles 2001**, the International Conference on "**Biology and Biotechnology of Thermophilic Microbes**" at the **Department of Microbiology University of Delhi South Campus, New Delhi,** India, from 3-7 December, 2001.

Chapter 1

General Introduction

Introduction

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields (Poldermans, 1990). They are degradative enzymes that catalyze the cleavage of peptide bonds in other proteins. Proteases have a long history of application in various biotechnological industries. They are extensively used mainly in the detergent and food industries. In view of the recent trend of developing environment-friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance (Rao, *et al*., 1998). Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Their involvement in the life cycle of disease-causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. Proteases play a critical role in many complex physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. Besides being necessary from the physiological point of view, proteases are potentially hazardous to their proteinaceous environment and their activity must be precisely controlled by the respective cell or organism. When uncontrolled, proteases can be responsible for serious diseases. The control of proteases is generally achieved by regulated expression/ secretion and / or activation of proproteases, by degradation of mature enzymes, and by the inhibition of their proteolytic activity. Protease inhibitors exercise control of unwanted proteolysis and play an essential role in physiological and pathological regulation. Biological roles of some inhibitors have been suggested (Ryan, 1973) and many others are of

economic and social importance as they affect human or animal nutrition (Whitaker and Feeney, 1973). Therefore, the research interest in protease inhibitors has evoked tremendous attention in many disciplines.

Classification of proteases

Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960; Barrett, *et al*., 1998). There are a few miscellaneous proteases that do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Menon, and Goldberg, 1987). Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings and Barrett, 1993). Each family of peptidases have been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.

Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further subdivided into about six clans with common ancestors (Barrett, 1994). The primary structures of the members of four clans, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala–D-Ala pepti-dase A (SE) are totally unrelated, suggesting that there are at least four separate evolutionary origins for serine proteases. Clans SA, SB, and SC have a common reaction mechanism consisting of a common catalytic triad of the three amino acids, serine (nucleophile), aspartate (electrophile), and histidine (base). Although the geometric orientations of these residues are similar, the protein folds are quite different, forming a typical example of a convergent evolution. Another interesting feature of the serine proteases is the conservation of glycine residues in the vicinity of the catalytic serine residue to form the

motif Gly-Xaa-Ser-Yaa-Gly (Brenner, 1988). Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), di-isopropylfluorophosphate (DFP), phenylmethylsulfonyl fluo-ride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

Serine alkaline proteases

Several bacteria, molds, yeasts, and fungi produce serine alkaline proteases. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. Their substrate specificity is similar to but less stringent than that of chymotrypsin. They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9. Their molecular masses are in the range of 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacte*r, *Streptomyce*s, and *Flavobacterium* sps. (Boguslawski, *et al*., 1983), subtilisins produced by *Bacillus* sp. are the best known. Alkaline proteases are also produced by *S. cerevisiae* (Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* sp. (Tanksale, *et al*., 2000) and *Aspergillus* and *Neurospora* sp. (Lindberg, *et al*., 1981).

Subtilisins

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN'), have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang, and Ottesen at the Carlsberg laboratory. Subtilisin Novo or BPN' is produced by *Bacillus amyloliquefacien*s. Subtilisin Carlsberg is widely used in detergents. Its annual production amounts to about 500 tons of pure enzyme protein. Subtilisin BPN' is less commercially important. Both subtilisins have a molecular mass of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60°C and an optimal pH of 10. Both enzymes exhibit broad substrate specificity and have an active-site triad made up of Ser221, His64 and

Asp32. The active-site conformation of subtilisins is similar to that of trypsin and chymotrypsin despite the dissimilarity in their over-all molecular arrangements.

Mechanism of action of serine proteases

The catalytic site of proteases is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site S1 through Sn toward the N terminus of the structure and Sl' through Sn' toward the C terminus. The residues which they accommodate from the substrate are numbered Pl through Pn and P1' through Pn', respectively. Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment (Fastrez and Fersht, 1973). This acylation step is followed by a deacylation process which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide (Fig. 1).

The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Ser. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accepts the OH group of the reactive Ser. Serine endopeptidases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin- like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues. The Pl residue exclusively dictates the site of peptide bond cleavage. The primary specificity is affected only by the Pl residues while the residues at other positions affect the rate of cleavage. The subsite interactions are localized to specific amino acids around the Pl residue to a unique set of sequences on the enzyme.

Figure 1. Catalytic mechanism of serine proteases

Cysteine proteases

Cysteine proteases are widely distributed and include plant proteases such as papain, bromelain, various mammalian cathepsins, the cytosolic calpains as well as several parasitic proteases (eg., *Trypanosoma*, *Schistosoma*). About 20 families of cysteine proteases have been recognized. The cysteine proteases consist of a catalytic dyad of essential cysteine and histidine residues. The order of Cys and His (Cys-His or His-Cys) residues differs among the families (Barrett, 1994). The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps and hence a water molecule is not required. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain is the archetype and best-known member of this family.

Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, occur in a wide range of organisms ranging from vertebrates to plants, fungi, parasites, retroviruses and bacteria (James, 1998). They are directly dependent on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barrett, 1995). The members of families A1 and A2 are related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Crystallographic studies have shown that the enzymes of the pepsin family have a bilobal structure with the active-site cleft located between the lobes, and each lobe contributes one aspartate residue of the catalytically active dyad of aspartates (Sielecki, *et al*., 1991). The lobes are homologous to one another, having arisen by gene duplication. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. In contrast, to the serine and cysteine proteases, catalysis by aspartic proteases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the dyad of the two-carboxyl groups and a

second one from the dyad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Miller, *et al*., 1989; Holm, *et al*., 1984).

Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases found in bacteria, fungi as well as in higher organisms (Barrett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. Majority of enzymes contain a zinc atom, which is catalytically active. They include enzymes such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Weaver, *et al*., 1977; Okada, *et al*., 1986; Shannon, *et al*., 1989). Bacterial thermolysin has been well characterized and its crystallographic structure indicates that two histidines and one glutamic acid bind to zinc that is embedded in a cleft formed between two folded lobes of the protein. Many enzymes contain the sequence HEXXH, which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group. Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) *Myxobacter* I, and (iv) *Myxobacter* II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. *Myxobacter* protease I is specific for small amino acid residues on either side of the cleavage bond, whereas protease II is specific for lysine residue on the amino side of the peptide bond. Collagenase, another important metalloprotease, was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic products.

Protease inhibitors

Proteolytic processes are involved in the mobilization of tissue proteins, in the regulation of intracellular protein metabolism, in neuropeptide processing and other neural functions, and in the processing of precursors of proteins and polypeptides which are biologically and pharmacologically active, such as proenzymes, hormones and kinins. These

functions extend from the cellular level to the organ and organism level to produce cascade systems such as homeostasis and inflammation, and complex processes at all levels of physiology and pathophysiology (Birk, 1987). They are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer, and AIDS. The specific inhibition of these proteases by the protease inhibitors can be used as a strategy for drug design for the prevention of propagation of the causative agents. Thus, the studies on protease inhibitors the effective regulators of proteases are very important. An increased understanding of the enzymes specificity for the substrate and inhibitor binding enables a more rational design of potent inhibitors, selective for a particular enzyme. Determination of the kinetic parameters of the inhibition of proteases will provide insight into the mechanism of the interaction between the enzyme and the inhibitor. Protease inhibitors regulate the action of proteases and play a significant role in the protection of plants from pest and pathogen invasion (Ryan, 1990). 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. Naturally occurring protease inhibitors can be grouped under two categories, i) Low molecular weight inhibitors, and ii) Proteinaceous inhibitors.

Low molecular weight inhibitors

The inhibitors belonging to this class are generally of microbial origin and are low molecular weight peptides of unusual structures (Umezawa, 1982). Presence of protease inhibitors in microorganisms came into existence from the studies on antibiotics as they act as inhibitors of enzymes which are involved in growth and multiplication. Extracellular proteolytic enzymes hydrolyze organic nitrogen compounds in the medium and are thought to be harmful to cells. The production of inhibitors of the proteolytic enzymes by microorganisms has probably evolved as a mechanism to provide cell protection. Majority of the microbial protease inhibitors are produced extracellularly by various *Streptomyces* sps. The microbial serine protease inhibitors include leupeptin, inhibiting plasmin, trypsin, papain and cathepsin B; antipain, inhibiting, trypsin, papain and cathepsin B; chymostatin inhibiting chymotrypsins and papain; elastinal, that inhibits pancreatic elastase; and elasnin, a strong inhibitor of human granulocyte elastase. All of these inhibitors have an α-amino aldehyde group in the C-terminal part of the peptide. Several applications for medicinal purpose have

been found for these inhibitors. Specific inhibitors of microbial origin have been used as useful tools in biochemical analysis of biological functions and diseases.

Proteinaceous inhibitors

Protein inhibitors of proteases are ubiquitous and are present in numerous plants, animals and microorganisms (Birk, 1987). Since the protein inhibitors of proteolytic enzymes are, indeed, proteins, they should be substrates for proteolysis. The elucidation of this paradox remains a central focus for much of the work on the structure and function of protein inhibitors of proteases. Inhibitor proteins have therefore, been studied as model systems for elucidation of the mechanism of inhibition of proteases and also for studies of protein-protein interactions. The recognized importance of proteolytic processes in the regulation of post-translational processing of precursor proteins, and the involvement of proteases in intracellular protein metabolism and in various pathological processes has recently stimulated tremendous interest in naturally-occurring, target oriented, protease inhibitors. They are exploited as valuable tools in medical research by virtue of their unique pharmacological properties that suggest clinical application. The proteinaceous inhibitors are divided into the same four classes as proteases namely, serine, cysteine, aspartic and metalloprotease inhibitors. With the exception of the plasma macroglobulins, which inhibit proteases of all classes (Barrett, 1980), individual protein inhibitors inhibit only proteases belonging to a single mechanistic class. Of these inhibitors, the most extensively studied are the inhibitors of serine proteases.

Protein inhibitors of serine proteases

Serine proteases and their protein inhibitors have been the most intensively studied group of protein-protein complexes. The number of known and partially characterized inhibitors of serine proteases is enormous. They are classified into different families based on their sequence homologies, assignment of the reactive site or inhibitory site(s) of the inhibitors and their mechanism of action. Currently a large number of three-dimensional structures of serine protease inhibitors families from plants, animals and microorganisms are available (Table 1) (Otlewski, *et al*., 1999).

Serine protease inhibitors from plants

Proteinaceous protease inhibitors are widely distributed among several botanical families (Leiner and Kakade, 1980). Mostly they are located in the seeds and in some legumes

they are also found in the leaves. In tuberous plants they are present in the leaves and tubers. Among the cereals such as corn, barley, wheat and rye they are primarily present in the endosperm. The plant protease inhibitors differ in specificities and in their ability to inhibit one or more proteases at the same time. Majority of them inhibit trypsin and many inhibit chymotrypsin. Inhibitors of elastase, kallikrein, plasmin, subtilisin and thrombin have also been found. Several plant protease inhibitors have been purified and characterized. Their molecular weights range from 3000-25000. The first plant protease inhibitor to be isolated and well characterized was the trypsin inhibitor from soybean (Kunitz, 1947, 1947a). Its purification, crystallization, kinetics of interaction and complex formation with trypsin is considered a classic study of protease inhibitor chemistry. The inhibitors from soybean represent two dominant inhibitor families: the Kunitz soybean trypsin inhibitor (STI) and the Bowman-Birk trypsin and chymotrypsin inhibitor (BBI). Occurrence of inhibitors in nearly all leguminous plants is now well known (Vogel, *et al*., 1968). The presence of inhibitors has possibly evolved as a defense mechanism against predatory insects (Ryan, 1990).

Serine protease inhibitors from animals

The inhibitors of animal origin are found in tissues and in secretions of organs. Many of them are secretory proteins, such as the trypsin inhibitors of blood plasma, milk colostrums, seminal plasma, cervical mucus, mucous membrane of the respiratory passages, synovial fluids and sub mandibular glands. Preotease inhibitors are also isolated from animals such as *Ascaris,* sea anemones, leeches, snake venoms and snail slimes (Vogel, *et al*., 1968; Tschesche, 1974). The plasma protease inhibitors constitute another major group of the functional proteins of blood plasma. They react with proteases from a wide variety of sources. Generally they inhibit serine proteases but their mechanism of inhibition is different than the standard mechanism. These inhibitors have probably involved for the control of numerous proteolytic processes occurring in blood. The α_1-proteinase inhibitor (α_1-antitrypsin) is the well-characterized class of plasma protease inhibitors (Travis and Selvesen, (1983). The pancreas produces two distinct, well-defined inhibitor families: the bovine pancreatic trypsin inhibitor (Kunitz) family, also known as Kunitz BPTI family and the pancreatic secretory trypsin inhibitor (Kazal) family (Laskowski and Kato, 1980). Trypsin and chymotrypsin inhibitors have also been isolated from numerous avian species (Kassell, 1970). These inhibitors are glycoproteins present in bird egg whites. They comprise two widely studied

groups, the ovomucoids and the ovoinhibitors. These inhibitors show domains homologous to the inhibitors of the Kazal family (Laskowski, *et al*., 1978).

Mechanism of action of serine protease inhibitors

Majority of the protein inhibitors of serine proteases interact with the enzymes by a common, generally accepted mechanism, "The Standard Mechanism" (Laskowski and Kato, 1980). Inhibitors obeying the standard mechanism are highly specific substrates for limited proteolysis by their target enzymes. They bind to enzymes in the manner of a good substrate very tightly, and are cleaved very slowly. On the surface of each inhibitor molecule lies at least one peptide bond called the reactive site. The reactive site is defined as the part of the inhibitor molecule that enters into direct molecular contact with the active center of the protease upon formation of the protease-inhibitor complex. Inhibition occurs as a consequence of binding of the active site substrate-binding region of a protease to the corresponding substrate-like reactive site on the surface of the inhibitor. The reactive site contains P_1- P_1' peptide bond located in the most exposed region of the protease binding loop (P_1, P_2 and P_1', P_2' designate inhibitor residues amino- and carboxy-terminal to the scissile peptide bond; S_1, S_2 and S_1', S_2' denote the corresponding subsites on the protease (Schechter and Berger, 1967) which can be cleaved by a serine protease. The inhibitor is converted from a 'virgin" (peptide bond intact) to a 'modified' (peptide bond hydrolyzed) inhibitor. Generally inhibitors obeying the standard mechanism show the presence of at least one disulfide linkage near the reactive site peptide that ensures that during conversion of virgin to modified inhibitor the two peptide chains are unable to dissociate. Therefore, conformation of the cleaved inhibitor is very similar to that of its intact form. The k_{cat} /K_m value for the hydrolysis of the reactive site peptide bond at neutral pH is very high typical for normal substrates, however, the individual values of k_{cat} and K_m for the inhibitors are several orders of magnitude lower than those for normal substrates, leading to an extremely slow hydrolysis of the reactive site peptide bond.

Table 1: The three dimensional structures of different representative families of serine protease inhibitors and their enzyme complexes:

Family	Structure	Abbreviation	PDB code	Reference
Ecotin	Ecotin	Ecotin	1ECY	Shin, *et al*., 1996
	Ecotin: crab collagenase	Ecotin: cCOLL	1AZZ	Perona, *et al*., 1997
Ascaris inhibitor	*Ascaris* trypsin inhibitor	ATI	1ATA	Grasberger, *et al*., 1994
	Ascaris chymotrypsin/ elastase inhibitor: porcine pancreatic elastase	C/E-1:PPE		Huang, *et al.,* 1994
Hirustatin	Antistatin	Antistatin	1SKZ	Lapatto, *et al*., 1997
	Hirustatin	Hirustatin	1BX7	Uson, *et al.,* 1999
Cereal-inhibitor	Ragi bifunctional α-amylase /trypsin inhibitor	RBI	1BLU	Gourinath, *et al*., 1999
	Corn Hageman factor inhibitor	CHFI	1BEA	Behnke, *et al*., 1998
	Barley α-amylase/subtilisin inhibitor: barley α-amylase	BASI: AMY2	1AVA	Vallee, *et al*., 1998
STI	Soybean trypsin inhibitor	STI	1AVU	Song and Suh, 1998
	Erythrina caffra trypsin inhibitor	ETI	1TIE	Onesti, *et al*., 1991
	Bifunctional proteinase K/ α-amylase inhibitor	PKI3		Zemke, *et al*., 1991
	Winged bean chymotrypsin inhibitor	WCI	2WBC	Dattagupta, *et al*., 1999
BPTI	Bovine pancreatic trypsin inhibitor: bovine trypsin	BPTI BPTI:bTP		Berndt, *et al*., 1992 Huber *et al*., 1974
	Amyloid *b*-protein precursor	APPI: rTP		Hynes, *et al*., 1990

inhibitor domain: rTP

Family	Structure	Abbreviation	PDB code	Reference
BPTI *contd..*	Bikunin	Bikunin	1BIK	Xu, *et al.*, 1998
	Proteinase inhibitor from sea anemone	ShPI	1SHP	Antuch, *et al.*, 1993
Kazal	Porcine pancreatic secretory trypsin inhibitor (PSTI): bovine trypsinogen	pPSTI: pTG	1TGS	Bolognesi, *et al.*, 1982
	Turkey ovomucoid third domain	OMTKY3		Krezel, *et al.*, 1994
	Rhodniin: bovine thrombin	Rhodiin: BTHRO	1TBQ	Van de Locht, *et al.*, 1995
	Leech-derived tryptase inhibitor	LDTI-C		Muhlhahn, *et al.*, 1994
	Porcine PEC-60	PEC-60	1PCE	Liepinsh, *et al.*, 1994
Potato inhibitor 1	Barley proteinase inhibitor 2	CI-2	3CI2	Ludvigsen, *et al.*, 1991
	CI-2: subtilisin BPN'	CI-2: SBPN	2SNI	McPhalen and James, 1988
	Eglin c	eglin	1EGL	Hyberts, *et al.*, 1992
	Cucurbita maxima trypsin inhibitor-V	CMTI-V		Cai, *et al.*, 1995
Potato inhibitor 2	Polypeptide inhibitor: SGPB	PCI: SGPB	4SGB	Greenblatt, *et al.*, 1989
	Streptomyces griseus proteinase	Na-C1		Nielsen, *et al.*, 1994
	Nicotiana alata proteinase inhibitor C1			
SSI	*Streptomyces* subtilisin inhibitor (SSI)	SSI	3SSI	Mitsui, *et al.*, 1977

	SSI: SBPN	SSI: SBPN	2SIC	Takeuchi, *et al*., 1991
	SSI mutant: bovine trypsin	SSI (M70G, M73K): bTP	2TLD	Takeuchi, *et al*., 1992
Family	**Structure**	**Abbreviation**	**PDB code**	**Reference**
Bowman-Birk inhibitor	Soybean chymotrypsin / trypsin BBI	BBI-I	1BBI 2BBI	Werner and Wemmer, 1992
	Peanut proteinase inhibitor	A-II		Suzuki, *et al*., 1993
	Soybean proteinase inhibitor II	PI-II	1PI2	Chen, *et al*., 1992
	Winter pea trypsin/ chymotrypsin inhibitor	PsTI	1PBI	Li de la Sierra, *et al*., 1999
	Azuki bean protease inhibitor: bTP	AB-I: bTP	1TAB	Tsunogae, *et al*., 1986
	Mung bean trypsin inhibitor: pTP	MBTI: pTP		Lin, *et al*., 1993
Chelonia-nin	Mucous proteinase inhibitor: bovine chymotrypsin	MPI: bCHYM		Grutter, *et al*., 1988
	Elafin	Elafin	1REL	Francart, *et al*., 1997
	Elafin: porcine pancreatic elastase	Elafin: PPE	1FLE	Tsunemi, *et al*., 1996
Sqash seed inhibitor	*Cucurbita maxima* trypsin inhibitor I	CMTI	1CTI 3CTI	Holak, *et al*., 1989; 1991
	Cucurbita pepo trypsin inhibitor II: pTP	CPTI II: bTP		Helland, *et al*., 1999
	Ecbalium elaterium trypsin inhibitor II	EETI II	2ETI	Heitz, *et al*., 1989 Chiche, *et al*., 1989
	Trypsin carboxypeptidase peptide inhibitor	TCPI		Chiche, *et al*., 1993
Locusta haemoly-	*Locusta migratoria* inhibitor D2	PMP-D2		Mer, *et al*., 1994

| mph inhibitor | *Locusta migratoria* | inhibitor C | PMP-C | 1PMC | Mer, *et al.*, 1996 |

Global structures of proteins representing different inhibitor families are completely different and comprise *a*-helical proteins, *b*-sheet proteins, *a/b*-proteins and different folds of disulfide-rich proteins. Protease inhibitors adopt various structures ranging from mini-proteins to large macromolecular structures, much larger than the target enzyme. From the structural point of view blocking of the enzyme active site is almost always achieved by docking of exposed structural elements, like loops or protein termini, either independently or in combination of two or more such elements. Besides recognition of different surfaces in the active site area, some inhibitors directly utilize the mechanism of protease action to achieve inhibition. The majority of the known protease inhibitors were reported to be substrate-like-binding molecules directed towards serine proteases blocking the enzyme at the distorted Michaelis complex reaction stage (Bode and Huber, 1992). Based on the mechanism of inhibition the serine protease inhibitors can be distinguished into three different classes: canonical (standard mechanism) inhibitors, non-canonical inhibitors and serpins.

Canonical inhibitors

Canonical inhibitors inhibit proteases by the standard mechanism. The standard mechanism implies that inhibitors are peculiar protein substrates. The binding loop is in similar, so called canonical, conformation in inhibitor structures representing different inhibitor families (Bode and Huber, 1992; Apostoluk and Otlewski, 1998). Generally the standard mechanism inhibitors exhibit canonical conformation of the binding loop.

Non-canonical inhibitors

Inhibitors originating from blood sucking organisms are of the non-canonical type. They specifically block enzymes of the blood clotting cascade, particularly thrombin or factor Xa. The interaction is mediated mainly through inhibitor N-terminus, which is disordered in solution and rearranges upon binding in the active site of an enzyme (Szyperski, *et al.*, 1992). There are also extensive secondary interactions, which provide an additional buried area and contribute significantly to the strength and specificity of interaction. In the studied cases there is a two-step kinetics of association, the initial slow binding step occurs at the secondary binding site, then the N-terminus locks in the active site of protease. The classic example is recognition of thrombin by hirudin (Stubbs and Bode, 1995).

Serpins

Serpins (*ser*ine *p*rotease *in*hibitors) are single domain proteins of about 400 amino acid residues in variably glycosylated forms (Travis and Salvesen, 1983; Potempa, *et al.*, 1994). The complex structure of serpins enables regulation of their action through association with a variety of cofactors and receptors. Generally the serpins are plasma proteins which are targeted towards serine proteases and thus control critically important processes, such as phagocytosis, coagulation and fibrinolysis. Serpins contain three ***b***-sheets (A-C) and nine ***a***-helices (A-I). Like the canonical inhibitors, they interact with their target enzyme in a substrate-like manner through the exposed loop of poorly defined structure. The protease binding loop of serpins, located in the C-terminal part of the molecule, comprises about 30 residues, and due to inherent flexibility, can adopt a number of different conformations.

Microbial serine protease inhibitors

Although a plethora of low molecular weight non-protein inhibitors of various proteases from microorganisms have been reported there are few reports of proteinaceous protease inhibitors. Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli,* is a competitive inhibitor that strongly inhibits trypsin, chymotrypsin and elastase (Yang, *et al.*, 1998) Majority of the extracellular protein protease inhibitors produced by microorganims are from the genus *Streptomyces.* The filamentous bacteria of the genus *Streptomyces* are ubiquitous soil microorganisms characterized by a morphologically complex life cycle. These organisms possess the ability to synthesize a variety of secondary metabolites including many useful antibiotics. Unlike the Gram-negative bacteria, *e.g. Escherichia coli,* Gram-positive bacteria lack a cell wall and are therefore able to secret proteins directly into the external milieu. The widely distributed and well-characterized proteinaceous inhibitors from *Streptomyces* are the inhibitors of the bacterial serine alkaline protease subtilisin (Sato and Murao, 1973; Uyeda, *et al.*, 1976; Kourteva and Boteva, 1989; Tsuchiya, *et al.*, 1989) . They form a separate inhibitor family: the *Streptomyces* subtilisin inhibitor family (Taguchi, *et al.,* 1993). Besides the subtilisin inhibitors there are reports of other related inhibitors of trypsin and other serine proteases from *Streptomyces.* A potent plasmin inhibitor, plasminostreptin has been studied from *S. antiplasminolyticus* (Sugino, *et al.*, 1978). Two naturally occurring abundantly produced trypsin inhibitors have been purified from *S. lividans* and *S. longisporus* (Strickler, *et al.*, 1992). Recently, a novel double-headed

proteinaceous inhibitor for serine and metalloprotease has been reported from a *Streptomyces* sp. (Hiraga, *et al*., 2000). Kexstatin, a proteinaceous Kex 2 proteinase and subtilisin inhibitor was purified from the culture supernatent of *Streptomyces platensis* (Oda, *et al*., 1996).

Streptomyces **subtilisin inhibitor family**

The first inhibitor reported from *Streptomyces* was the *Streptomyces* subtilisin inhibitor (SSI) from *Streptomyces albogriseolus* (Sato and Murao, 1973). It was found that homologous proteins of SSI are widely distributed particularly in *Streptomyces*, at high frequency and have been named as 'SSI-like (SIL) proteins' (Taguchi, *et al.,* 1993a). SIL1 from *S. cacaoi* was the first of these proteins to be isolated and given a serial number (Kojima, *et al*., 1994). Since then several SIL proteins have been characterized from different *Streptomyces* sps and comparative studies on their primary structures and inhibitory properties have been carried out (Taguchi, *et al*., 1996). SIL2 from *S. parvulus*, SIL3 from *S. coelicolor* and SIL4 from *S. lavendulae,* were found to display strong inhibition towards subtilisin and also inhibited trypsin though with a reduced potency (Taguchi, *et al*., 1994). SIL5 fom *S. fradiae*, SIL7 from *S. ambofaciens,* SIL10 from *S. thermotolerans,* SIL12 from *S. hygroscopicus,* SIL13 from *S. galbus,* and SIL14 from *S. azureus* inhibit subtilisin as well as trypsin (Terabe, *et al*., 1994; 1996). A novel member of the SSI family, SIL8 isolated from *S. virginiae* was the first SIL member to show marked inhibitory activity towards alpha-chymotrypsin in addition to strong inhibitory activity towards subtilisin (Terabe, *et al*., 1994a). Such a high frequency occurrence of SIL inhibitors suggests that they might have an important common role in some physiological function, *e. g.*, as regulatory and/ or defensive molecules against proteases. It has been shown that there is a pronounced structural homology between SSI family and the Kazal (PSTI) family of inhibitors (Hirono, *et al*., 1984). This homology has been observed earlier in the amino acid sequences (Ikenaka, *et al*., 1974). SSI has been extensively studied and characterized.

Streptomyces **subtilisin inhibitor (SSI)**

Biochemical properties of SSI

Streptomyces subtilisin inhibitor (SSI) was isolated in crystalline form from the culture filtrate of *S. albogriseolous* (Sato and Murao, 1972, 1973). It is unique in that it

strongly inhibits microbial alkaline proteases, such as subtilisin and pronase, but not serine proteases from animal tissues, thiol proteases, metal proteases and acid proteases. SSI could be recovered easily under low pH conditions. The inhibitor exists as a dimer of two identical subunits of molecular weight 11,500 and is stable under extreme temperature and pH conditions (Akasaka, *et al*., 1975). One molecule of SSI stoichiometrically binds and inhibits two molecules of subtilisin with the inhibitor constant $K_i < 1$ nm (Inouye, *et al*., 1977). The complete primary structure of the subunit containing 113 amino acid residues has been sequenced by fragmentation of the protein with cyanogens bromide, proteolytic digestion of the fragment, the Edmann degradation procedure, and digestion with carboxypeptidases A and B (Ikenaka, *et al*., 1974). Each monomer unit contains one Trp (Trp86), three Tyr (Tyr7, 75 and 93) and two intrachain disulfide linkages (Cys35-Cys50, Cys71-Cys101). SSI exhibits an unusual fluorescence spectrum when excited at 280 nm with an emission maximum at 307 nm and a shoulder at 340 nm, a feature which has been recognized only for a handful of proteins containing both Trp and Tyr residues (Uehara, et al., 1976). When excited at 295 nm, at which Tyr scarcely absorbs, the inhibitor showed an emission at 340 nm characteristic of Trp residue with a low quantum yield, these results indicated that the Trp fluorescence of SSI is strongly quenched in the native state. Due to its unique specificity, unusual properties and high stability SSI has been a protein of interest and is extensively characterized from multiple points of views in many laboratories (Hiromi, *et al*., 1985).

Reactive site and chemical modification

The localization and characterization of the amino acids comprising the reactive center and their correlation with the inhibitory function are essential for understanding the mechanism of action of the inhibitor. The first attempts to determine the amino acid residues involved in the reactive site of SSI were carried out by Aoshima (Aoshima, 1976). They established that the photooxidation of SSI resulted in the destruction of three Met and one His residues with concomitant loss of inhibitory activity. Also the modification of Met by chemical oxidation by H_2O_2 or Cl_2 led to the loss of inhibition, there fore it was concluded that at least one Met residue is involved in the reactive site. The hydrophobic amino acids such as Tyr, Trp and Lys were not essential for inhibition was confirmed by modification of SSI by diazonium-1-H-tetrazole. Based on the high susceptibility of a peptide bond to proteases and the sequence homology of SSI with bovine secretory trypsin inhibitor (Kazal) it

was proposed that the reactive site of SSI was Met73-Val74 (Ikenaka, *et al*., 1974). This view was supported by chemical modification and by the chymotryptic cleveage of the reactive site peptide bond of SSI and its resynthesis by subtilisin-SSI complex formation (Omichi, *et al*., 1980). The carboxy-terminal four amino acid residues were shown to be important for maintaining the tertiary structure and the inhibitory activity of SSI (Sakai, *et al*., 1980). Specific fluorescent-labeling of Tyr and Lys residues of SSI was carried out by dansyl chloride and fluorescein isothoicyanate respectively (Tanizawa, *et al*., 1990). The modified SSI retained complete activity and its interaction with subtilisin was studied.

Protein-protein interactions

The interactions between serine protease inhibitors and their target enzymes have received increased attention since their complexes represent excellent model systems for investigating the fundamental biochemical and biophysical principles of protein-protein recognition. There are several reports on the interaction of SSI with its target protease. The binding and kinetics of the interaction between SSI and subtilisin has been studied by single photon counting technique and stopped-flow fluorescence spectroscopy (Uehara, *et al*., 1978, 1980). An increase in protein fluorescence was obtained upon complex formation of SSI and subtilisin. The changes in the fluorescence has been suggested due to Trp106 of subtilisin BPN' and was influenced by the ionization of Tyr104 of the enzyme (Masuda-Momma, *et al*., 1993). The mechanism of binding involves two steps, in which a fast bimolecular association is followed by a slow unimolecular isomerization step. The increase of Trp fluorescence due to the complex formation occurs solely in the rate-determining unimolecular process. A similar increase in the fluorescence intensity was shown during the complexation of fluorescent-labeled SSI and the *Streptomyces griseus* proteases SGPA and SGPB (Tanizawa, *et al*., 1990a). Although SGPA and SGPB belong to a different family, a computer simulation study suggested that the structure of the contact regions of SGPA and SGPB with SSI are highly complementary to the surface structure of SSI (Christensen, *et al*., 1985). Structure of the complex of SSI with subtilisin BPN' was also studied by examining the thermal denaturation and reducibility of disulfide bonds (Komiyama, *et al*., 1986). The denaturation temperature of the complex was reported to be significantly higher than that of the enzyme. Two disulfide bonds localized in the inhibitor side were completely reduced in the complex, whereas only one of them was reduced in the free SSI. The interaction of SSI and subtilisin

BPN' has also been studied by chemical modification, isothermal calorimetry and SDS-PAGE (Inouye, *et al*., 1979a; Takahashi and Fukada, 1985; Narhi, *et al*., 1991).

Denaturation studies

Delineating the mechanism of protein denaturation and renaturation has been an interesting field of research. Kinetic investigations on denaturation of proteins whose three-dimensional structures have been elucidated by X-ray crystallography are especially useful for understanding the detailed mechanisms of conformational transitions of proteins. Denaturation in proteins can be triggered by various methods. Several studies on the denaturation of SSI have been carried out. A kinetic study with the stopped flow method on the pH induced denaturation and renaturation of SSI with intact disulfide linkages was carried out by monitoring the change in Trp fluorescence (Uehara, *et al*., 1983). These studies revealed that both the acid denaturation and renaturation proceeded in two phases. A two-step sequential mechanism involving a common intermediate for both the processes was shown. Acid denaturation of SSI was carried out to probe the microenvironment of the indiviual His residues and the denaturation transitions of SSI by proton magnetic resonance (Fujh, *et al*., 1980). The thermal unfolding of SSI and its complex with subtilisin has been studied by circular dichroism (CD), differential scanning calorimetry (DSC), SDS-PAGE and 1H NMR spectroscopy (Takahashi and Sturtevant, 1981; Komiyama, *et al*., 1984; Arakawa and Horan, 1990; Tamura, *et al*., 1991, 1991a). The thermal denaturation of SSI was reversible and cooperative, proceeding in a two-state transition and leads to the dissociation of the dimers. SSI can exist in two denatured states; the cold denatured and heat denatured states in the temperature range of –10 to 60 °C in the acidic pH range. The two denatured states were not perfect random coiled and differed from each other, indicating the existence of three states in this temperature range, the cold denatured, native and heat denatured state. An increased transition temperature of subtilisin was observed in the presence of SSI. The solution X-ray scattering analysis of the cold-, heat-, and urea-denatured states of SSI has been reported (Konno, *et al*., 1995). The conformational variations of the distinctly different cold and heat denatured states of SSI as a function of urea concentration have been investigated (Konno, *et al*., 1997). The effect of sodium dodecyl sulfate on the structure and function of SSI has also been studied (Inouye, *et al*., 1979)

X-ray crystallography

The crystal structure of free SSI has been refined and solved at 2.3, 2.6 and 4.0 Å resolution (Mitsui, *et al.*, 1977; Satow, *et al.*, 1978; Mitsui, *et al.*, 1979). The SSI molecule is described as an ellipsoid of about 30 x 40 x 65 Å composed of two identical subunits each having dimensions of about 35 x 25 x 40 Å. Each subunit has a unique fold of polypeptide chain with a five-fold extensive anti-parallel *b*-sheet structure and two small *a*-helices. The P1 residue, Met73, of the reactive site is at the protruding edge of the subunit. The region around the reactive site, Met73-Val74, is held tight by a combination of various structural features. At the subunit-subunit interface, a *b*-sheet of one unit is stacked on top of the corresponding *b*-sheet of the other subunit. The crystal structure of free SSI at 2.6 Å is illustrated in Fig. 2A. The crystal structure of subtilisin BPN' complexed with SSI has been resolved at 4.3, 2.6 and 1.8 Å (Hirono, *et al.*, 1979; Mitsui, *et al.*, 1979; Hirono, *et al.*, 1984; Takeuchi, *et al.*, 1991). These studies have

(A)   **(B)**
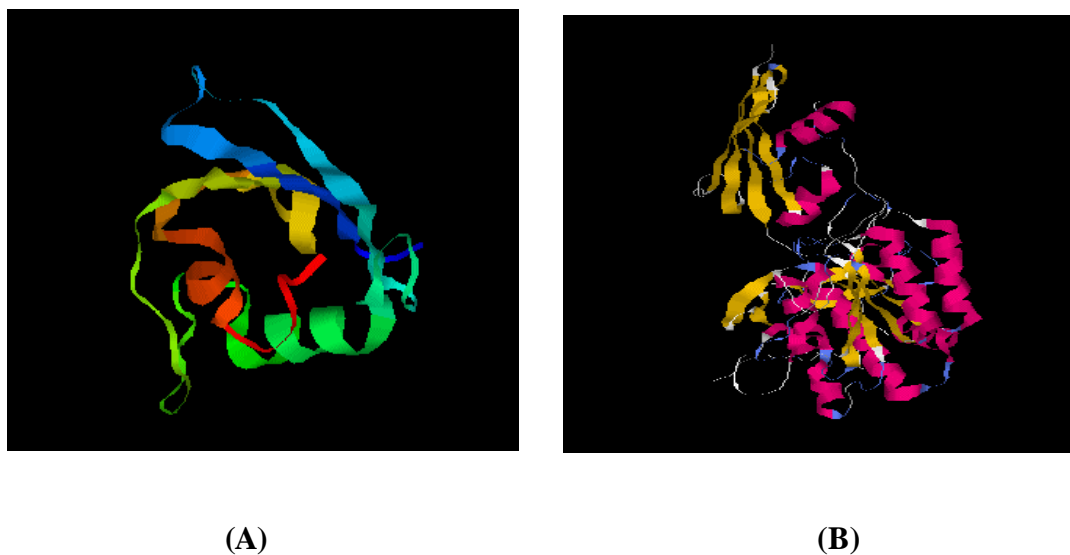
Figure 2: Ribbon diagram of the three-dimensional structure of SSI and its complex with Subtilisin
        BPN'.
        (A) The crystal structure of SSI at 2.3 Å resolution as determined by X-diffraction (Mitsui, *et al.*, 1977 and http:// www. rcsb. org/ pdb/ cgi/ explore. cgi?pid=156991023091910& pdbId=3SSI). **(B)** The crystal structure of SSI in complex with Subtilisin BPN' at 1.8 Å resolution (Takeuchi, *et al.*, 1991 and http://www. rcsb. org /pdb/ cgi/ explore.cgi?pid=156991023091910 &pdbId=2SIC).

established that the SSI-subtilisin complex is a Michaelis complex with a 2.7 Å distance between the O$^{\gamma}$ of active Ser221 and the carbonyl carbon of the scissile peptide bond. The

enzyme-inhibitor **b**-sheet interaction is composed of two separate parts: that between the P1-P3 residues of SSI and the 125-127 chain segment (the S1-S3 site) of subtilisin and that between the P4- P6 residues of SSI and the 102-104 chain segment (the S4-S6 site) of subtilisin. The latter interaction is unique to subtilisin and does not exist in serine proteases of the trypsin family. The complex of SSI-subtilisin BPN' at 2.6 Å is shown in Fig. 2B. The global induced-fit movement occurs on SSI upon complexing with subtilisin in which a channel-like structure in SSI where hydrophobic chains are sandwiched between two lobes becomes about 2 Å wider. The main role of the "secondary contact region" of SSI is to support the reactive site loop ("primary contact region"). The crystal structure of engineered SSI (P1 Met converted to Lys and P4 Met converted to Gly, alters the specificity of SSI to strongly inhibit trypsin) complexed with bovine trypsin has been resolved at 2.6 Å (Takeuchi, *et al*., 1992). The structure and function of subtilisin BPN' was studied through crystallographic studies on a series of its complexes with genetically engineered SSI (Nonaka, *et al*., 1996).

Genetic *engineering*

Molecular cloning and recombinant DNA technology have been instrumental in improving our understanding of the structure-function relationship of genetic systems. Since SSI is secreted in a large amount from *S. albogriseolus* cells, the gene encoding SSI is expected to have efficient transcriptional and translational machinery, as well as an effective leader peptide sequence, which is involved in secretion of the protein. Therefore, analysis of the gene encoding SSI is essential to elucidate its structure and function of transcriptional and translational signals for a structure gene from *Streptomyces*. SSI gene localized on a 1.8 kbp DNA fragment was cloned into *E. coli* (Obata, *et al*., 1989). The nucleotide sequence analysis showed the presence of a putative signal peptide comprising 31 amino acids preceding the mature SSI region. The main transcriptional start point was identified to be 60 nucleotides upstream from the putative initiation codon for translation by the primer extension method. A secretory expression system for the 1.8 kbp fragment of the SSI gene was established by introducing it in *Streptomyces lividans* 66 by using a *Streptomyces* multicopy vector, pIJ 702 (Obata, *et al*., 1989a). The expressed SSI in *S. lividans* was secreted in the culture medium in a large amount, as observed with the original strain of *S. albogriseolus.* Amino acid sequence analysis of secreted SSI showed the presence of three additional amino acids in the N-

terminal region however they were shown to have no effect on the inhibitory activity towards subtilisin. A high-level production of SSI in *E. coli* was also achieved by replacing the SSI signal sequence with the OmpA signal sequence using inducible pIN-III-ompA vector (Taguchi, *et al*., 1993b). Comparison of secretory expression in *E. coli* and *Streptomyces* of the SSI gene has been studied (Taguchi, *et al*., 1990).

Protein engineering

Protein engineering through site directed mutagenesis allows the introduction of presdeigned changes into the gene for the synthesis of a protein with an altered function. Generally the properties of an amino acid residue at the reactive site (especially its center, the P1 site) of a protease inhibitor correspond to the specificity of the cognate protease. SSI is known to specifically inhibit bacterial subtilisins, it has been demonstrated that a functional change in SSI was possible by replacing the amino acids at the reactive site (Met73) of SSI (Kojima, *et al*., 1990). Replacement by Lys or Arg resulted in trypsin inhibition, replacement only by Lys gave inhibition of lysyl endopeptidase, and replacement by Tyr or Trp resulted in inhibition of alpha-chymotrypsin. The four mutant SSIs retained their native activity against subtilisin. Additional effects of replacing the Met70 at the P4 site of mutated SSI (Lys73) by Gly or Ala resulted in increased inhibitory activity towards trypsin and lysyl endopeptidase, while replacement with Phe weakened the inhibitory activity towards trypsin (Kojima, *et al*., 1990a). The influence of replacements of several others amino acids at the reactive site of SSI has also been reported (Kojima, *et al*., 1991). The tertiary structure of SSI is maintained by various interactions and by disulfide linkages. The role of amino acid residues involved in these interactions can be conveniently studied by protein engineering. The salt bridge between Arg29 and the carboxyl group of carboxy-terminal Phe113 is essential for maintaining the tertiary structure and biological activity was shown by replacing Arg29 with Ala, Met or Lys (Kojima, *et al*., 1994a). The inhibitory activity of each mutated SSI was found to decrease with increasing incubation time, indicating that it was converted to a temporary inhibitor upon mutation. The requirement for a disulfide bridge near the reactive site of SSI was demonstrated by replacing the disulfide bridge between Cys71 and Cys101 near the reactive site with two Ser residues (Kojima, *et al*., 1993). The mutated SSI was converted to a temporary inhibitor indicating the conformational rigidity around the reactive site to be

crucial for inhibition. Similarly, the introduction of an intersubunit disulfide linkage into the dimeric SSI was shown to enhance its stabilization (Tamura, *et al*., 1994).

Applications of serine protease inhibitors

Defense tools for plant protection

Protease inhibitors regulate the action of proteases and play a significant role in the protection of plants from pest and pathogen invasion. Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material and therefore protease inhibitors by virtue of their antinutritional interaction can be employed effectively as defense tools (Ryan, 1990). Some inhibitors are constitutively expressed in seeds and storage organs while others are induced on wounding in leaves (Green and Ryan, 1972; Jongsma, *et al*., 1994). Overexpression of heterologous inhibitors in transgenic plants has been shown to reduce the growth rates of several insect larvae (Hilder, *et al*., 1987; Johnson *et al*., 1989; Gatehouse, *et al*., 1997; Jouanin, *et al*., 1998; Schuler, *et al*., 1998). Proteases are also shown to be indispensable for the growth of fungi and a few plant protease inhibitors have been shown to possess fungicidal activity (Lorito, *et al*., 1994; Valueva, *et al*., 1998; Joshi, *et al*., 1998; Chen, *et al*., 1999).

Therapeutic agents

Proteases are responsible either, directly or indirectly for all bodily functions including cell growth, nutrition, differentiation, and protein turn over. They are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer and AIDS. The specific inhibition of these proteases can be used as a strategy for drug design for the prevention of propagation of the causative agents thus their effective regulators, i.e., protease inhibitors, are very essential, because of their pivotal role in the regulation of various physiological and pathological processes involving the mobilization of tissues proteins and in the processing of precursors of proteins. Fungal infections are one of the important causes of morbidity and mortality of immunocompromised patients. *Aspergillus fumigatus* is the most common human pathogen causing invasive aspergillosis. The fungal strain secretes a protease belonging to the subtilisin family that is involved in pathogenesis, therefore its specific inhibitors are potential prophylactic therapeutic agents against this disease. SSI is known to inhibit microbial alkaline

proteases and therefore its potency was tested against the fungal protease. It was shown that SSI strongly inhibits this protease and can therefore be used in the control of invasive aspergillosis (Markaryan, *et al*., 1996).

Miscellaneous applications

Besides their applications in therapeutics and as potential bicontrol agents, protease inhibitors play an important role in basic research. Protease inhibitors and their enzymes have been excellent model systems to study protein-protein interactions. The properties of interfaces between polypeptide chains during interaction have been carried out using SSI as a model protein (Valdar and Thornton, 2001).

Recently, large-scale production of useful proteins has become possible by use of recombinant DNA techniques. In the application of these techniques, the target protein is often accumulated in the host cells as inactive precipitates, also referred to as inclusion bodies. The refolding or renaturation of the protein molecule from such precipitates into its intrinsic bioactive structure is a significant step in the downstream processes of protein engineering. The central theme in the refolding procedure is the selection of environmental conditions for optimum refolding after solubilization of the precipitates. In the case of proteases, vigorous autoproteolysis is known to occur during refolding resulting in low recovery of activity. The presence of *Streptomyces* subtilisin inhibitor (SSI) during the refolding of subtilisin was shown to induce quantitative renaturation by prevention of the autoproteolysis (Matsubara, *et al*., 1994; Hayashi, *et al*., 1994)). Furthermore, application of a digestible mutant SSI engineered specially as a temporary inhibitor allowed the complete recovery of fully active subtilisin BPN'. The application of a temporary inhibitor may be greatly effective in not only improvement of yield but also selection of media for the refolding of protease. Refolding of reduced and denatured *Streptomyces griseus* trypsin (SGT) was also achieved by SSI (Nohara, *et al*., 1999). Other than protecting the denatured subtilisin during refolding SSI is known to enhance the thermal stability of subtilisin (Arakawa, *et al*., 1991).

The efficient secretory production system for SSI is well established (Obata, *et al*., 1989). The *ssi* gene region has been demonstrated to possess unique structures; there are two tandemly arranged promoters and two translation initiation signals closely related to protein processing (Taguchi, *et al*., 1989a; 1991). The expression and secretion signal of SSI has been potentially exploited for high-level expression of several heterologous proteins (Taguchi, *et*

al., 1989). The heterologous production and extracellular secretion of an antibacterial peptide apidaecin found in the lymph fluid of honeybee *Apis mellifera* has been reported (Taguchi, *et al*., 1992; Maeno, *et al*., 1993). The fusion protein produced showed bifunctional activity: inhibitory activity of SSI and antibacterial activity of the peptide. In another study a secretory production system for the Fv domain of a monoclonal antibody against hen egg-white lysozyme was established using the SSI secretory system (Ueda, *et al*., 1993). The extracellular production of biologically active human transforming growth factor α (TGFα) as fusion protein with SSI has been demonstrated (Taguchi, *et al* ., 1995). The hybrid protein was shown to possess bifunctional activity, the TGFα activity for cell growth promotion and the antiproteolytic activity of SSI. The secretory system of another subtilisin inhibitor-like protein from *Streptomyces venezuelae* (VSI) has been used effectively for the expression of mouse tumor necrosis factor alpha (Lammertyn, et al., 1997).

Future prospects

Proteases inhibitors are a unique class of proteins, since they are of immense physiological as well as biotechnological importance. Since protease inhibitors are physiologically necessary, they occur ubiquitously in animals, plants, and microbes. The majority of the protease inhibitors reported as defense tools for protection are from plants (Ryan 1990). However, many insects have shown to be adapted to plant protease inhibitors by the induction of proteases insensitive to inhibition (Jongsma, *et al*., 1995; 1996). This problem can be combated by the administration of a cocktail of protease inhibitors. Exploration of novel protease inhibitors and the genes encoding them from plants as well as other sources such as microorganisms can lead to the combinatorial expression of several defense proteins, which could be a promising strategy to engineer plants with enhanced and broad-spectrum resistance. Since, microbes are a gold-mine of protease inhibitors they represent a preferred source of proteins in view of their rapid growth, limited space required for cultivation, and ready accessibility to genetic manipulation. Advances in genetic manipulation of microorganisms by site-directed mutagenesis of the cloned gene open new possibilities for the introduction of predesigned changes, resulting in the production of tailor-made protease inhibitors with novel and desirable properties. A detailed understanding of the various interactions that exists in protease-inhibitor complexes will allow the engineering of improved inhibitors within the basic molecular frameworks of the inhibitor using computer

modeling. These approaches will benefit the use of protease inhibitors not only in biocontrol but also for several other applications.

Present investigation

The present work details various aspects of the mechanism of inhibition of alkaline proteases and their interactions with an alkaline protease inhibitor. The findings of the investigations have been presented in the following six chapters:

1. Screening and Isolation of alkaline protease inhibitor producing microorganisms.

2. Purification and biochemical characterization of an alkaline protease inhibitor, API from a *Streptomyces* sp. NCIM 5127.

3. API as a novel antifungal protein: Protease inhibitory activity as the biochemical basis of antifungal activity

4. PDI-accelerated refolding of API: effect of macromolecular crowding on *in vitro* refolding.

5. Interaction of API with fungal alkaline proteases: kinetic parameters involved in the inactivation of the proteases.

6. Inhibitor induced thermal stability of Proteinase K.

References

Akasaka, K., Aoshima, H., Hatano, H., Sato, S., and Murao, S. (1975) *Biochim. Biophys. Acta* **412,** 120-126.

Antuch, W., Berndt, K. D., Chavez, M. A., Delfin, J., and Wuthrich, K. (1993) *Eur. J. Biochem.* **212,** 675-684.

Aoshima, H. (1976) *Biochim. Biophys. Acta* **453,** 139-150.

Apostoluk, W., and Otlewski, J. (1998) *Proteins: Struct. Funct. Genet.* **32,** 459-474.

Arakawa, T., and Horan, T. P. (1990) *Agric. Biol. Chem.* **54,** 563-565.

Arakawa, T., Lazenby, K., Kolvenbach, C. G., Horan, T. P., McGinley, M., Rohde, M. F., and Narhi, L. O. (1991) *Agric. Biol. Chem.* **55,** 903-910.

Argos, P. (1987) *J. Mol. Biol.* **193,** 385–396.

Barrett, A. J. (1980) *Fed. Proc.* **39,** 9-14.

Barrett, A. J. (1994) *Methods Enzymol.* **244,** 1–15.

Barrett, A. J. (1995) *Methods Enzymol.* **248,** 183.

Barett, A. J., Rawlings, N. D., and Woessner, J. F. (1998) *Handbook of Proteolytic Enzymes*, Academic Press Inc., London

Behnke, C. A., Yee, V. C., Trong, I. L., Pedersen, L. C., Stenkamp, R. E., Kim, S. S., Reeck, G. R., and Teller, D. C. (1998) *Biochemistry* **37,** 15277-15288.

Berndt, K. D., Guntert, P., Orbons, L. P., and Wuthrich, K. (1992) *J. Mol. Biol.* **227,** 757-775.

Birk, Y. (1987) *Hydrolytic enzymes,* Elsevier Sci. Publishers, A. Neuberger and K. Brocklehurst (Eds.) 257-305.

Bode, W., and Huber, R. (1992) *Eur. J. Biochem.* **204,** 433-451.

Boguslawski, G., Shultz, J. L., and Yehle, C. O. (1983) *Anal. Biochem.* **132,** 41-49.

Bolognesi, M., Gatti, G., Menegatti, E., Guarneri, M., Marquart, M., Papamokas, E., and Huber, R. (1982) *J. Mol. Biol.* **162,** 839-868.

Brenner, S. (1988) *Nature* **334,** 528-530.

Cai, M., Gong, Y., Kao, J. L. –F., and Krishnamoorthi, R. (1995) *Biochemistry* **34,** 5201-5211.

Chen, P., Rose, J., Love, R., Wei, C. H., and Wang, B. C. (1992) *J. Mol. Biol.* **267,** 1990-1994.

Chen, Z. Y., Brown, R. L., Lax, A. R., Cleveland, T. E., and Russin, J. S. (1999) *Appl. Environ. Microbiol*. **65**, 1320-1324.

Chiche, L., Gaboriaud, C., Heitz, A., Mornon, J. –P., Castro, B., and Kollman, P. A. (1989) *Proteins: Struct. Funct. Genet.* **6,** 405-417.

Chiche, L., Heitz, A., Padilla, A., Le-Nguyen, D., and Castro, B. (1993) *Protein Engng.* **6,** 675-682.

Christensen, U., Ishida, S., Ishii, S., Mitsui, Y., Iitaka, Y., McClarin, J., and Langridge, R. (1985) *J. Biochem. (Tokyo)* **98,** 1263-1274.

Dattagupta, J. K., Podder, A., Chakrabarti, C., Sen , U., Mukhopadhyay, D., Dutta, S. K., and Singh, M. (1999) *Proteins* **35,** 321-331.

Fastrez, J., and Fersht. A. R. (1973) *Biochemistry* **12,** 2025–2034.

Francart, C., Dauchez, M., Alix, A. J. P., and Lippens, G. (1997) *J. Mol. Biol.* **268,** 666-677.

Fujh, S., Akasaka, K., and Hatano, H. (1980) *J. Biochem. (Tokyo)* **88,** 789-796.

Gatehouse, A. M. R., Davison, G. M., Newell, C. A., Merryweather, A., Hamilton, W. D. O., Burgess, E. P. J., Gilbert, R. J. C., and Gatehouse, J. A. (1997) *Mol. Breed.* **3,** 49-63.

Gourinath, S., Srinivasan, A., and Singh, T. P. (1999) *Acta Cryst.* **D55,** 25-30.

Grasberger, B. L., Clore, G. M., and Gronenborn, A. M. (1994) *Structure* **2,** 669-678.

Green, T. R., and Ryan, C. A. (1972) *Science* **175**, 776-777

Greenblatt, H. M., Ryan, C. A., James, M. N. G. (1989) *J. Mol. Biol.* **205,** 201-225.

Grutter, M. G., Fendrich, G., Huber, R., and Bode, W. (1988) *EMBO J.* **7,** 345-351.

Hartley, B. S. (1960) *Annu. Rev. Biochem.* **29,** 45-72.

Hayashi, T., Matsubara, M., Nohara, D., Kojima, S., Miura, K., and Sakai, T. (1994) *FEBS Lett.* **350,** 109-112.

Heitz, A., Chiche, L., Le-Nguyen, D., and Castro, B. (1989) *Biochemistry* **28,** 2392-2398.

Helland, R., Otlewski, J., Sundheim, O., Dadlez, M., and Smalas, A. O. (1999) *J. Mol. Biol.* **287,** 923-942.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F., and Boulter, D. (1987) *Nature* **300**, 161-163.

Hiraga, K., Suzuki, T., and Oda, K. (2000) *J. Biol. Chem.* **275,** 25173-25179.

Hiromi, K., Akasaka, K., Mitsui, Y., Tonomura, B., and Murao, S. (1985) *Protein Protease Inhibitors- The Case of Streptomyces Subtilisin Inhibitor (SSI),* Elsevier Science Publishers, B. V. Amsterdam.

Hirono, S., Nakamura K. T., Iitaka, Y., Mitsui, Y. (1979) *J. Mol. Biol.* **131,** 855-869.

Hirono, S., Akagawa, H., Mitsui, Y., and Iitaka, Y. (1984) *J. Mol. Biol.* **178,** 389-413.

Holak, T. A., Gondol, D., Otlewski, J., and Wilusz, T. (1989) *J. Mol. Biol.* **210,** 635-648.

Holak, T. A., Habazettl, J., Oschkinat, H., and Otlewski, J (1991) *J. Am. Chem. Soc.* **113,** 3196-3198.

Holm, I., Ollo, R., Panthier, J. J., and Rougeon, F. (1984) *EMBO J.* **3**, 557-562.

Huang, K., Strynadka, N. C. J., Bernard, V. D., and James, M. N. G. (1994) *Structure* **2,** 669-678.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W. (1974) *J. Mol. Biol.* **89,** 73-101.

Hyberts, S. G., Goldberg, M. S., Havel, T. F., and Wagner, G. (1992) *Protein Sci.* **1,** 736-751.

Hynes, T. R., Randal, M., Kennedy, L. A., Eigenbrot, C., and Kossiakoff, A. A. (1990) *Biochemistry* **29,** 10018-10022.

Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., sato, S., and Murao, S. (1974) *J. Biochem. (Tokyo)* **76,** 1191-1209.

Inouye, K., Tonomura, B., Hiromi, K., Sato, S., and Murao, S. (1977) *J. Biochem. (Tokyo)* **82,** 961-967.

Inouye, K., Tonomura, B., and Hiromi, K. (1979) *Arch. Biochem. Biophys.* **192,** 260-269.

Inouye, K., Tonomura, B., and Hiromi, K. (1979a) *J. Biochem. (Tokyo)* **85,** 1115-1126.

James, M. N. G. (1998) *Aspartic Proteinases*. Plenum Press. New York and London.

Johnson, R., Narvaez, J., An, G., and Ryan, C. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9871-9875.

Jongsma, M. A., Bakker, P. L., Visser, B., and Stiekema, W. J. (1994) *Planta* **195,** 29-35.

Jongsma. M. A., Bakker, P. L., Peters, J., Bosch, D., and Stiekema, W. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8041-8045.

Jongsma, M. A., Stiekema, W. J., and Bosch, D. (1996) *Trends Biotechnol.* **14,** 331-333.s

Joshi, B. N., Sainani, M. N., Bastawade, K. B., Gupta, V. S., and Ranjekar, P. K. (1998) *Biochem. Biophys. Res. Commun*. **246**, 382-387.

Jouanin, L., Bonade-Bottino, M., Girard, C., Morrot, G., and Giband, M. (1998) *Plant Sci.* **131,** 1-11.

Kassell, B. (1970) *Methods Enzymol.* **19,** 839-906.

Kojima, S., Obata, S., Kumagai, I., and Miura, K. (1990) *Bio/Techol.* **8,** 449-452.

Kojima, S., Kumagai, I., and Miura, K. (1990a) *Protein Eng.* **3,** 527-530.

Kojima, S., Nishiyama, Y., Kumagai, I., and Miura, K. (1991) *J. Biochem.(Tokyo)* **109,** 377-382.

Kojima, S., Kumagai, I., and Miura, K. (1993) *J.Mol. Biol.* **230,** 395-399.

Kojima, S., Terabe, M., Taguchi, S., Momose, H., and Miura, K. (1994) *Biochim. Biophys. Acta* **1207,** 120-125.

Kojima, S., Fujimura, K., Kumagai, I., and Miura, K. (1994a) *FEBS Lett.* **337,** 195-199.

Komiyama, T., Miwa, M., Yatabe, T., and Ikeda, H. (1984) *J. Biochem. (Tokyo)* **95,** 1569-1575.

Komiyama, T., Oomori, A., Fukuyo, K., Kanno, H., and Miwa, M. (1986) *Int. J. Pept. Protein Res.* **28,** 325-333.

Konno, T., Kataoka, M., Kamatari, Y. O., Kanaori, K., Nosaka, A., and Akasaka, K. (1995) *J. Mol. Biol.* **251,** 95-101.

Konno, T., Kamatari, Y. O., Kataoka, M., and Akasaka, K. (1997) *Protein Science* **6,** 2242-2249.

Kourteva, Y., and Boteva, R. (1989) *FEBS Lett.* **247,** 468-470.

Krezel, A. M., Darba, P., Robertson, A. D., Fejzo, J., Macura, S., and Markley, J. L. 1(994) *J. Mol. Biol.* **242,** 203-214.

Kunitz, M. (1947) *J. Gen. Physiol.* **30,** 291-310.

Kunitz, M. (1947a) *J. Gen. Physiol.* **30,** 311-320.

Lammertyn, E., Mellaert, L. V., Schacht, S., Dillen, C., Sablon, E., Broekhoven, A. V., and Anne, J. (1997) *Appl. Environ. Microbiol.* **63,** 1808-1813.

Lapatto, R., Krengel, U., Schreuder, H. A., Arkema, A., de Boer, B., Kalk, K. H., Hol, W. G. J., Grootenhuis, P. D. J., Mulders, J. W. M., Dijkema, R., Theunissen, H. J. M., and Dijkstra, B. W. (1997) *EMBO J.* **16,** 5151-5161.

Laskowski, M. Jr., Kato, I., and Kohr, W. J. (1978) *Versatility of Proteins* (Li, C. H., Ed.) Academic Press, New York, 307-318.

Laskowski, M., Jr. and Kato, I. (1980) *Annu. Rev. Biochem.* **49,** 593-626.

Leiner. I. E., and Kakade, M. (1980) *Toxic Constituents of Plant Foodstuffs* (Leiner, I. E., Ed.) 2nd Edn. Academic Press, NY. 7-71.

Li de la Sierra, I., Quillien, L., Flecker, P., Gueguen, J., and Brunie, S. (1999) *J. Mol. Biol.* **285,** 1195-1207.

Liepinsh, E., Berndt, K. D., Sillard, R., Mutt, V., and Otting, G. (1994) *J. Mol. Biol.* **239,** 137-153.

Lin, G., Bode, W., Huber, R., Chi, C., and Engh, R. A. (1993) *Eur. J. Biochem.* **212,** 549-555.

Lindberg, R. A., Eirich, L. D., Price, J. S., Wolfinbarger, L. Jr., and Drucker, H. (1981) *J. Biol. Chem.* **256,** 811-814.

Lorito, M., Broadway, R. M., Hayes, C. K., Woo, S. L., Noviello, C., Williams, D. L., and Harman, G. E. (1994) *Mol. Plant. Microbe Interact.* **7**, 525-527.

Ludvigsen, S., Shen, H., Kjaer, M., Madsen, J. C., and Poulsen, F. M. (1991) *J. Mol. Biol.* **222,** 621-635.

Maeno, M., Taguchi, S., and Momose, H. (1993) *Biosci. Biotech. Biochem.* **57,** 1206-1207.

Markaryan, A., Beall, C. J., and Kolattukudy, P. E. (1996) *Biochem. Biophys. Res. Commun.* **220,** 372-376.

Masuda-Momma, K., Shimakawa, T., Inouye, K., Hiromi, K., Kojima, S., Kumagai, I., Miura, K., and Tonomura, B. (1993) *J. Biochem. (Tokyo)* **114,** 906-911.

McPhalen, C. A., and James, M. N. G. (1988) *Biochemistry* **27,** 6582-6598.

Menon, A. S., and Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 14929–14934.

Mer, G., Kellenberger, C., Koehl, P., Stote, R., Sorokine, O., Van Dorsselaer, A. M., Luu, B., Hietter, H., and Lefvere, J. –F. (1994) *Biochemistry* **33,** 15397-15407.

Mer, G., Hietter, H., Kellenberger, C., Renatus, M., Luu, B., and Lefvere, J. –F. (1996) *J. Mol. Biol.* **258,** 158-171.

Miller, M., Jaskolski, M., Rao, J. K. M., Leis, J., and Wlodawer, A. (1989) *Nature (London)* **337**, 576-578.

Mitsui, Y., Satow, Y., Sakamaki, T., and Iitaka, Y. (1977) *J. Biochem. (Tokyo)* **82,** 295-298.

Mitsui, Y., Satow, Y., Watanabe, Y., and Iitaka, Y. (1979) *J.Mol. Biol.* **131,** 697-724.

Mitsui, Y., Satow, Y., Watanabe, Y., Hirono. S., and Iitaka, Y. (1979a) *Nature* **277,** 447-452.

Mizuno, K., and Matsuo, H. (1984) *Nature* **309,** 558-560.

Muhlhahn, P., Czisch, M., Morenweiser, R., Habermann, B., Engh, R. A., Sommerhoff, C. P., Auerswald, E. A., and Holak, T. A. (1994) *FEBS Lett.* **355,** 290-296.

Narhi, L. O., Connor, J., Rohde, M. F., McGinley, M. G., and Arakawa, T. (1991) *J. Protein Chem.* **10,** 385-389.

Nielsen, K. J., Heath, R. L., Anderson, M. A., and Craik, D. J. (1994) *J. Mol. Biol.* **242,** 231-243.

Nohara, D., Sugiura, H., Sakakibara, H., Matsubara, M., Kojima, S., Miura, K., and Sakai, T. (1999) *J. Biochem.* **125,** 343-347

Nonaka, T., Suzuki, T., Tanaka, N., Saito, S., Senda, T., Miura, K., and Mitsui, Y. (1996) *Adv. Exp. Med. Biol.* **379,** 43-47.

Obata, S., Taguchi, S., Kumagai, I., and Miura, K. (1989) *J. Biochem.* **105,** 367-371.

Obata, S., Furukubo, S., Kumagai, I., takahashi, H., and Miura, K. (1989a) *J. Biochem.* **105,** 372-376.

Oda, K., Takahashi, S., Kikuchi, N., and Shibano, Y. (1996) *Biosci. Biotechnol. Biochem.* **60,** 1388-1389.

Okada, Y., Nagase, H., and Harris. E. D. (1986) *J. Biol. Chem*. **261,** 14245– 14255.

Omichi, K., Nagura, N., and Ikenaka, T. (1980) *J. Biochem. (Tokyo)* **87,** 483-489.

Onesti, S., Brick, P., and Blow, D. M. (1991) *J. Mol. Biol.* **217,** 153-176.

Otlewski, J., Krowarsch, D., and Apostoluk, W. (1999) *Acta Biochimica Polonica* **3,** 531-565.

Perona, J. J., Tsu, C. A., Craik, C. S., and Fletterick, R. J. (1997) *Biochemistry* **36,** 5381-5392.

Poldermans, B. (1990) *Proteolytic enzymes, In* W. Gerhartz (ed.), VCH Publishers, Weinheim, Germany, 108-123.

Potempa, J., Korzus, E., and Travis, J. (1994) *J. Biol. Chem.* **269,** 15957-15960.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., and Deshpande, V. V. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 597-635.

Rawlings, N. D., and Barrett. A. J. (1993) *Biochem. J*. **290,** 205–218.

Ryan, C. A. (1973) *Annu. Rev. Plant Physiol.* **24,** 173.

Ryan, C. A. (1990) *Annu. Rev. Phytopathol.* **28,** 425-449.

Sakai, M., Odani, S., and Ikenaka, T. (1980) *J. Biochem.(Tokyo)* **87,** 891-898.

Sato, S., and Murao, S. (1972) *Agric. Biol. Chem.* **36,** 160-163.

Sato, S., and Murao, S. (1973) *Agric. Biol. Chem.* **37,** 1067-1074.

Satow, Y., Mitsui, Y., and Iitaka, Y. (1978) *J. Biochem. (Tokyo)* **84,** 897-906.

Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27,** 157-162.

Schuler, T. H., Poppy, G. M., Kerry, B. R., and Denholm, I. (1998) *Trends Biotechnol.* **16,** 168-175.

Shannon, J. D., Baramova, E. N., Bjarnason, J. B., and Fox. J. W. (1989) *J. Biol. Chem.* **264,** 11575–11583.

Shin, D. H., Song, H. K., Seong, I. S., Lee, C. S., Chung, C. H., and Suh, S. W. (1996) *Protein Sci.* **5,** 2236-2247.

Sielecki, A. R., Fujinaga, M., Read, R. J., and James, M. N. G. (1991) *J. Mol. Biol.* **219,** 671–692.

Song, H. K., and Suh, S. W. (1998) *J. Mol. Biol.* **275,** 347-363.

Strickler, J. E., Berka, T. R., Gorniak, J., Fornwald, J., Keys, R., Rowland, J. J., Rosenberg, M., and Taylor, D. P. (1992) *J. Biol. Chem.* **267,** 3236-3241.

Stubbs, M. T., and Bode, W. (1995) *Trends Biochem. Sci.* **20,** 23-28.

Sugino, H., Kakinuma, A., and Iwanaga, S., (1978) *J. Biol. Chem.* **253,** 1546-1555.

Suzuki, A., Yamane, T., Ashida, T., Norioka, S., Hara, S., and Ikenaka, T. (1993) *J. Mol. Biol.* **234,** 722-734.

Szyperski, T., Guntert, P., stone, S. R., and Wuthrich, K. (1992) *J. Mol. Biol.* **228,** 1193-1205.

Taguchi, S., Kumagai, I., Nakayama, J., Suzuki, A., and Miura, K. (1989) *Bio/ Technology* **7,** 1063-1066.

Taguchi, S., Nishiyama, K., Kumagai, I., and Miura, K. (1989a) *Gcne* **84,** 279-286.

Taguchi, S., Kumagai. I., and Miura, K. (1990) *Biochim. Biophys. Acta* **1049,** 278-285.

Taguchi, S., Nishiyama, K., Kumagai, I., Momose, H., and Miura, K. (1991) *Mol. Gcn. Genet.* **226,** 328-331.

Taguchi, S., Maeno, M., and Momose, H. (1992) *Appl. Microbiol. Biotechnol.* **36,** 749-753.

Taguchi, S., Kikuchi, H., Suzuki, M., Kojima, S., Terabe, M., Miura, K., Nakase, T., and Momose, H. (1993) *Appl. Environ.Micorbiol.* **59,** 4338-4341.

Taguchi, S., Kikuchi, H., Kojima, S., Kumagai, I., Nakase, T., Miura, K and Momose, H. (1993a) *Biosci. Biotech. Biochem.* **57,** 522-524.

Taguchi, S., Yoshida, Y., Matsumoto, K., Momose, H. (1993b) *Appl. Microbiol. Biotechnol.* **39,** 732-737.

Taguchi, S., Kojima, S., Terabe, M., Miura, K., and Momose, H. (1994) *Eur, J. Biochem.* **220,** 911-918.

Taguchi, S., Misawa, S., Yoshida, Y., and Momose, H. (1995) *Gene* **159,** 239-243.

Taguchi, S., M., Kojima, S., Miura, K., and Momose, H. (1996) *FEMS. Micorbiol. Lett.* **135,** 169-173.

Takahashi, K., and Sturtevant. J. M. (1981) *Biochemistry* **20,** 6185-6190.

Takahashi, K., and Fukada, H. (1985) *Biochemistry* **24,** 297-300.

Takeuchi, Y., Satow, Y., Nakamura, K. T., Mitsui, Y. (1991) *J. Mol. Biol.* **221,** 309-325.

Takeuchi, Y., Nonaka, T., Nakamura, K. T., Kojima, S., Miura, K. –I., and Mitsui, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4407-4411.

Tamura, A., Kimura, K., Takahara, H., and Akasaka, K. (1991) *Biochemistry* **30,** 11307-11313.

Tamura, A., Kimura, K., and Akasaka, K. (1991a) *Biochemistry* **30,** 11313-11320.

Tamura, A., Kojima, S., Miura, K., and Sturtevant, J. M. (1994) *Biochemistry* **33,** 14512-14520.

Tanizawa, K., Moriya, K., and Kanaoka, Y. (1990) *Chem. Pharm. Bull.* **38,** 2606-2609.

Tanizawa, K., Watanabe, S., and Kanaoka, Y. (1990a) *Bioorganic Chem.* **18,** 318-329.

Tanksale, A. M., Vernekar, J. V., Ghatge, M. S., and Deshpande, V. V. (2000) *Biochem. Biophys.Res. Commun.* **270,** 910-917.

Terabe, M., Kojima, S., Taguchi, S., Momose, H., and Miura, K. (1994) *J. Biochem. (Tokyo)* **116,** 1153-1163.

Terabe, M., Kojima, S., Taguchi, S., Momose, H., and Miura, K. (1994a) *Eur. J. Biochem.* **226,** 627-632.

Terabe, M., Kojima, S., Taguchi, S., Momose, H., and Miura, K. (1996) *Biochim. Biophys. Acta* **1292,** 233-240.

Travis, J., and Salvesen, G. S. (1983) *Annu. Rev. Biochem.* **52,** 655-709.

Tschesche, H. (1974) *Angew. Chem. Int. Ed. Engl.* **13,** 10-28.

Tsuchiya, K., Asami, Y., Sakuta, K., and Kimura, T. (1989) *Agric. Biol. Chem.* **53,** 841-842.

Tsunemi, M., Matsuura, Y., Sakakibara, S., and Katsube, Y. (1996) *Biochemistry* **35,** 11570-11576.

Tsunogae, Y., Tanaka, I., Yamane, T., Kikkawa, J., Achida, J. T., Ishikawa, C., Watanbe, K., Nakamura, S., and Takahashi, K. (1986) *J. Biochem. (Tokyo)* **100,** 1637-1646.

Ueda, Y., Tsumoto, K., Watanabe, K., and Kumagai, I. (1993) *Gene* **129,** 129-134.

Uehara, Y., Tonomura, B., Hiromi, K., Sato, S., and Murao, S. (1976) *Biochim.Biophys. Acta* **453,** 513-520.

Uehara, Y., Tonomura, B., and Hiromi, K. (1978) *J. Biochem. (Tokyo)* **84, 11**95-1202.

Uehara, Y., Tonomura, B., and Hiromi, K. (1980) *Arch. Biochem.Biophys.* **202,** 250-258.

Uehara, Y., Tonomura, B., and Hiromi, K. (1983) *J. Biochem. (Tokyo)* **94,** 903-915.

Umezawa, H. (1982) *Annu. Rev. Microbiol.* **36,** 75-99.

Uson, I*,* Sheldrick, G. M., de La Fortelle, E., Bricogne, G., Di Marco, S., Priestle, J. P., and Grutter, M. G. (1999) *Structure* **7,** 55-63.

Uyeda, M., Yoshida, S., Suzuki, K., and Shibata, M. (1976) *Agric. Biol. Chem.* **40,** 1237-1238.

Valdar, W. S. J., and Thornton, J. M. (2001) *Proteins: Struc. Funct. Genet.* **42,** 108-124.

Vallee, F., Kadziola, A., Bourne, Y., Juy, M., Rodenburg, K. W., Svensson, B., and Haser, R. (1998) *Structure* **6,** 649-659.

Valueva, T. A., Revina, T. A., Kladnitskaya, G. V., and Mosolov, V. V. (1998) *FEBS Lett*. **426**, 131-134.

Van de Locht, A., Lamba, D., Bauer, M., Huber, R., Friedrich, T., Kroger, B., Hoffken, W., and Bode, W. (1995) *EMBO J.* **14,** 5149-5157.

Vogel, R., Trautschold, I., and Werle, E. (1968) *Natural proteinase Inhibitors* New york, Academic Press, 159.

Weaver, L. H., Kester, W. R., and Matthews. B. W. (1977) *J. Mol. Biol*. **114,** 119–132.

Werner, M. H., and Wemmer, D. E. (1992) *Biochemistry* **31,** 999-1010.

Whitaker, J. R., and Feeney, R. E. (1973) *Toxicants occurring naturally in foods.* Natl. Acad. Sci. Washington, D. C. 276-298.

Xu, Y., Carr, P. D., Guss, J. M., and Ollis, D. L. (1998) *J. Mol. Biol.* **276,** 955-966.

Yang, S. Q., Wang, C-I., Gillmor, S. A., Fletterick, R. J., and Craik, C. S. (1998) *J. Mol. Biol.* **279,** 945-957.

Zemke, K. J., Muller-Farhnow, A., Jany, K. –D., Pal, G. P., and Saenger, G. W. (1991) *FEBS Lett.* **279,** 240-242.

Chapter 2

Screening and Isolation of Alkaline Protease Inhibitor Producing Microorganisms

Summary

The essential nature of the alkaline proteases in numerous physiological and biotechnological applications has evoked tremendous interest towards isolating new inhibitors from various resources. After extensive screening of the vast diversity of soil samples, we have isolated four actinomycetes strains producing alkaline protease inhibitors (APIs) designated as API (265 U/ml), API-I (242 U/ml), API-II (116 U/ml) and API-III (186 U/ml). The time course for the production of APIs was determined and optimization of fermentation conditions was carried out by substituting an assortment of different carbon and nitrogen sources. The inhibitors possessed a unique specificity of inhibition confined only to alkaline proteases. They exhibited differences in their molecular nature and in their pH and temperature stabilities. API, API-I and API-II were high molecular weight (>10 kD) proteinaceous inhibitors whereas API-III was a low molecular weight inhibitor (<10 kD). API, API-I and API-II exhibited stability over a pH range of 5-12 whereas API-III displayed a wide pH stability from 2- 12. API was the most stable inhibitor. It was stable in a temperature range of 40-95°C and exhibited a half-life of 5 h at 85°C. API-I was stable at 60°C with a half-life of 1 h but API-II showed a half-life of 20 min at 45°C. API-III exhibited the least thermal stability with complete loss of activity at 37°C after 1 h. The stability of API-I, II and III at 65, 55 and 45°C, respectively, was enhanced by the addition of various additives. Glycine (1 M) offered complete protection to the three APIs. Polyethylene glycol 8000 (10 mM) prevented the thermoinactivation of API-I. In the presence of glycerol and sorbitol (10%) the stability of API-I and API-II was increased by 40-60%.

Introduction

Protease inhibitors are an important class of proteins that are ubiquitously present in all life forms (Kassel, 1970; Umezawa, 1982)). They have evoked tremendous interest because of their pivotal role in the regulation of various physiological and pathological processes involving the mobilization of tissues proteins and in the processing of precursors of proteins. Due to the essential nature of proteases, protease inhibitors play a crucial role in developing therapeutics and as biocontrol agents. Proteases are essential in the life cycles of organisms that cause lethal diseases such as malaria, cancer and AIDS (Billings *et al.* 1987, Seelmeir *et al.* 1988). Therefore, specific inhibition of these proteases can be used as a strategy for drug design for the prevention of propagation of these causative agents. Protease inhibitors regulate the action of proteases and play a significant role in the protection of plants from pest and pathogen invasion by virtue of their antinutritional interaction. Serine protease inhibitors from plants have been shown to have potential usefulness as defense tools to protect the plants from invading pests (Green and Ryan, 1972; Ryan, 1990). Alkaline protease inhibitors will be more efficient as biopesticides, since the midguts of major insect pests are highly alkaline. A few plant protease inhibitors have been shown to possess fungicidal activity (Lorito *et al.,* 1994; Joshi *et al.,* 1999). Although there is profound literature on numerous protease inhibitors from plants, there is a paucity of reports on inhibitors from microbial sources. Microorganisms represent an efficient and inexpensive source of protease inhibitors due to their rapid growth, limited space required for cultivation and ready accessibility for genetic manipulation to facilitate their expression in transgenic plants for obtaining improved resistance against pests and pathogens. Therefore, there is an urgent need to screen and isolate microbial strains producing potent alkaline protease inhibitors by exploring the biodiversity prevalent in soil, water, insects and tropical plants.

Alkaline proteases constitute one of the most important classes of proteolytic enzymes that have been maximally exploited in various industries such as detergent and leather industries (Tanksale *et al.,* 2001). Higher thermal stability is one of the crucial properties for their biotechnological application in many industrial processes (Gupta, 1991). Various methods are employed to enhance the thermostability of proteases. A novel cost-effective strategy of stabilization of proteases through the formation of reversible enzyme-inhibitor complexes for enhancing the thermostability can be explored. Their interaction with their

target proteases serves as a model for studying protein-protein interactions and opens up several avenues for their applications.

The present chapter deals with the screening and isolation of microorganisms producing alkaline protease inhibitors. We have isolated four actinomycetes strains, which produce alkaline protease inhibitors and have studied their production and properties. The pH and temperature stability of the alkaline protease inhibitors and the influence of various additives on their thermostability are investigated.

Materials and Methods

Materials

Starch, casein, yeast extract, malt extract, beef extract, peptone and other media components were purchased from Himedia, India. Sucrose, glucose, potassium nitrate, sodium chloride, magnesium sulfate and dipotassium hydrogen phosphate were from Qualigens, Glaxo India. UM-10 membrane was from Amicon Inc. USA. Casein hammerstein for enzyme assay was from E-Merck, Germany. All other chemicals were of analytical grade.

Screening and isolation of actinomycetes producing API

Humus rich soil was collected from different locations in Pune, India. Appropriate soil dilutions were plated out on modified enrichment medium for actinomycetes (Kuster and Williams, 1964) containing casein (0.1%) and starch (1%) and incubated at 28°C for 5-7 days. Isolated colonies typical of actinomycetes were picked and purified by single colony plating technique. The isolates were then grown in liquid starch-casein medium for 96h at 28°C on a rotary shaker at 200 rpm. The cells were separated by centrifugation and the cell free supernatant was checked for the presence of APIs against subtilisin, the alkaline protease from *Bacillus subtilus*. The organisms that showed a high production of API were selected for further studies. The actinomycete isolates showing anti-protease activity were routinely maintained on starch-casein agar slants and preserved at 4°C. For long-term storage, 25% glycerol spore suspensions were kept frozen at -70°C.

Optimization of fermentation conditions for production of APIs

Time profile for the production of APIs

The production of the inhibitors in starch-casein medium at various time intervals was monitored by removing samples at different time intervals and assaying for their anti-proteolytic activity.

Medium optimization

Optimization of medium conditions for maximum production of the alkaline protease inhibitor was carried out by replacement of the casein and starch medium with different carbon and nitrogen sources. The various carbon sources (1%) used were glucose, sucrose, mannose, lactose fructose and sorbitol. The nitrogen sources (0.1%) tested were skimmed milk, soyabean meal, yeast extract, malt extract, beef extract, peptone, casamino acids and urea. The alkaline protease inhibitor production was estimated by the caseinolytic assay.

Assay for protease inhibitory activity

The plate assay for the detection of API was performed in a petriplate containing skimmed milk agar with wells made in the center and on the periphery at a distance of 1 cm from the central well. 20 µl of subtilisin (1 mg/ml) was added in the central well. An appropriate dilution of the culture filtrate was added in one of the peripheral wells and sterile distilled water in the other to serve as a negative control. The plate was incubated at 37°C. The inhibition of hydrolysis of casein by subtilisin was indicated by the absence of clearance zone around the well containing the inhibitor.

The Kunitz caseinolytic assay was used with slight modifications for determining protease inhibitory activity (Kunitz, 1947). The reaction mixture (2 ml) contained subtilisin (7.5 µg) in 0.1 M carbonate- bicarbonate buffer, pH 10, casein (1%) and a suitable dilution of inhibitor. After incubation at 37°C for 20 min the reaction was terminated by addition of TCA (5%). The tubes were kept for 30 min at room temperature and filtered through Whatmann no.1 filter paper. Absorbance of the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that causes an increase in optical density of 0.001 at 280 nm per ml of reaction mixture per min at 37°C. One protease inhibitor unit was defined as the amount of inhibitor that inhibits one unit of protease activity. The inhibition of alkaline protease from *Conidiobolus macrosporus* and proteinase K from *Tritirachium album* Limber was also assayed in a similar manner. Trypsin, chymotrypsin, papain and pepsin were tested under their standard assay conditions.

Properties of API

pH stability studies

The pH stability was determined by incubating the culture filtrates (0.1 ml) of APIs in various buffer solutions (pH range 2-12) at room temperature for 1 h followed by the measurement of residual inhibitory activity.

Temperature stability studies

Culture filtrate (0.1 ml) of API was incubated at different temperatures for 1 h and the residual inhibitory activity was determined. The effect of various additives on the thermal stability was determined by incubating the inhibitor in presence of an additive at the desired temperature for a stipulated period of time. At the end of incubation, the inhibitor was further incubated on ice for 15 min and the residual activity was determined.

Charcoal treatment

Culture filtrates of APIs (1 ml) were treated with activated charcoal (0.5 g) and incubated at 4°C overnight. The mixture was centrifuged at 10,000 rpm for 10 min and the residual inhibitory activity of the supernatant was estimated.

Ammonium sulfate precipitation

Salting out of the proteins in the culture supernatants of APIs was carried out by ammonium sulfate precipitation (0.9 saturation). The precipitate was centrifuged at 10,000 rpm, resuspended in potassium phosphate buffer (0.05 M), dialyzed extensively against same buffer (5 mM) and the protease inhibitory activity was evaluated.

Results

On the basis of colony characteristics, 30 actinomycetes strains were isolated by single colony plating technique, out of which 12 isolates showed secretion of alkaline protease inhibitors inhibiting subtilisin by plate assay. Four isolates producing more than 100 U/ml of extracellular inhibitors were characterized further. The four alkaline protease inhibitors were designated as API, API-I, API-II and API-III. The plate assay for the anti-protease activity using skimmed milk (1%) agar by the zone of inhibition of hydrolysis of casein is shown in Fig. 1.
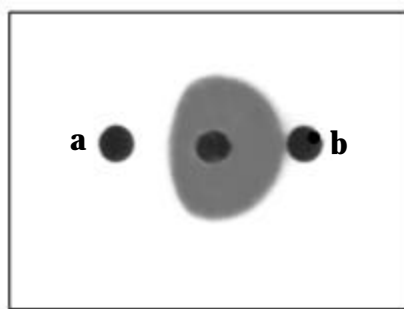
Figure 1: Inhibition of subtilisin, the alkaline protease from *Bacillus subtilus* by API:
The zone of inhibition of the hydrolysis of casein by API was observed. Central well: subtilisin 20 μl (1 mg/ml); Peripheral wells: (a) culture filtrate of API and (b) sterile distilled water as control.

Production of APIs

The time course of production of APIs during fermentation and the influence of various nitrogen and carbon sources on their overall production were investigated. The maximum production of API (265 U/ml), API-I (242 U/ml) and API-II (116 U/ml) was obtained after 96 h of growth while that of API-III (186 U/ml) was obtained after 48 h of growth. Thereafter, a rapid decrease of production was obtained for all the APIs (Fig. 2). To increase the production of the alkaline protease inhibitors, the starch-casein medium was optimized by substituting an assortment of various carbon and nitrogen sources. Starch-casein medium was found to be the most suitable medium for maximum production of API (265 U/ml) and API-I (242 U/ml). The production of API-II increased to 183 and 190 U/ml, respectively, by replacing starch with glucose or mannose. An increase of API-III to 219 U/ml

was observed in the presence of fructose. Addition of yeast extract and casamino acids resulted in an increase in the production of API-II from 116 U/ ml to 189 U/ml and 166 U/ml, respectively, while malt extract and yeast extract enhanced production of API-III to 224 U/ml and 206 U/ml respectively. In contrast, there was no effect of changing the carbon or nitrogen sources on the production of API and API-I.
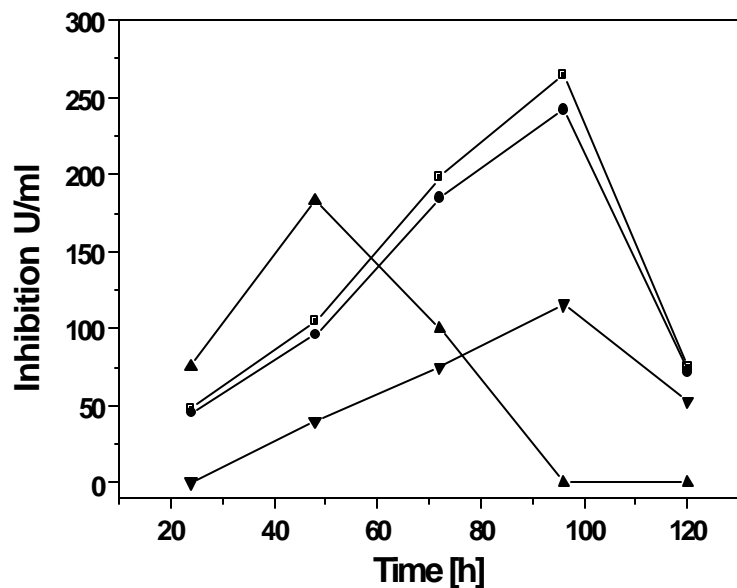
Figure 2: Time profile for the production of the APIs.
API (■); API-I (●); API-II (▲) and API-III (▼) were grown in the starch-casein medium and course for production of inhibitor as a function of time was monitored.

Properties of APIs

Potency against different proteolytic enzymes

APIs failed to inhibit neutral serine proteases such as trypsin and chymotrypsin and proteases from other mechanistic classes such as papain and pepsin. However, the ability of the APIs to inhibit subtilisin, proteinase K and alkaline protease from *Conidiobolus*, revealed their absolute specificity towards alkaline proteases.

Molecular nature of APIs

Charcoal is known to adsorb high molecular weight molecules and therefore, provides a basis to ascertain the molecular nature of the compounds. The charcoal-treated API, API-I and API-II showed absence of inhibitory activity in the supernatant liquid indicating their high molecular weight nature. The high molecular proteinaceous nature was confirmed by salting out with ammonium sulfate (0.9 saturation) that resulted in the retention of the inhibitory activity in the precipitate. These results were also corroborated by retention of their inhibitory activity in the retentate after membrane filtration using amicon UM10 membrane that allows only the passage of molecules lower than molecular weights of 10 kD. However, API-III was found to be low molecular weight as deduced by the retention of the inhibitory activity in the supernatant liquid after charcoal treatment. This was also confirmed by its passage through the amicon UM10 membrane and its inability to be precipitated by ammonium sulfate.

Stability of APIs

pH stability

As shown in figure 3, API-III exhibited pH stability in an expansive pH range from 2 to 12. However, the other three inhibitors were stable at pH 2 and in the pH range of 5-12, with a loss in activity at around pH 3 to 4.
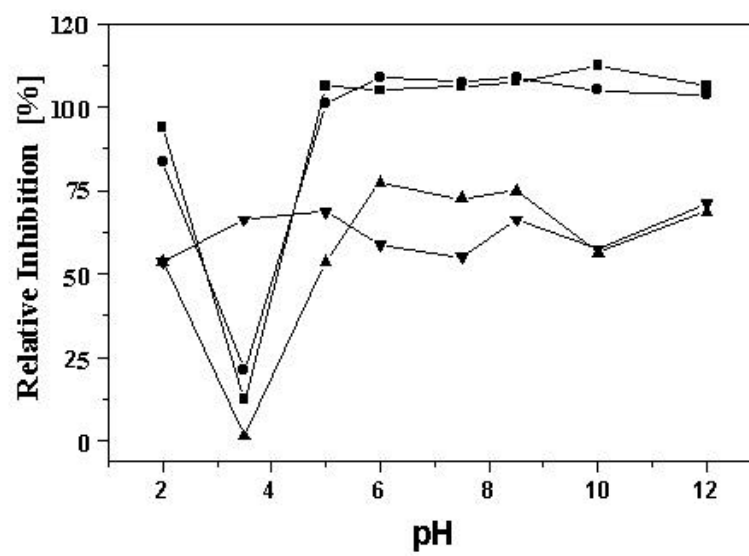
Figure 3. pH stability of APIs.

      API was incubated with various buffers of different pH and its anti-proteolytic activity was determined by the caseinolytic assay. API (■); API-I (●); API-II (▲) and API-III (▼)

Temperature stability

The inhibitors exhibited varying degrees of thermal stabilities. Among the four inhibitors, API was the most stable inhibitor with a half-life of 5 h at 85°C and stable in a temperature range of 40-95°C. At 95 °C, API lost its antiproteolytic activity after 30 min. API-I showed a half-life of 1 h at 60°C while API-II was stable only upto 45°C with a half-life of 20 min. API-III was very labile as compared to other inhibitors and lost its activity at 37°C after 1 h.
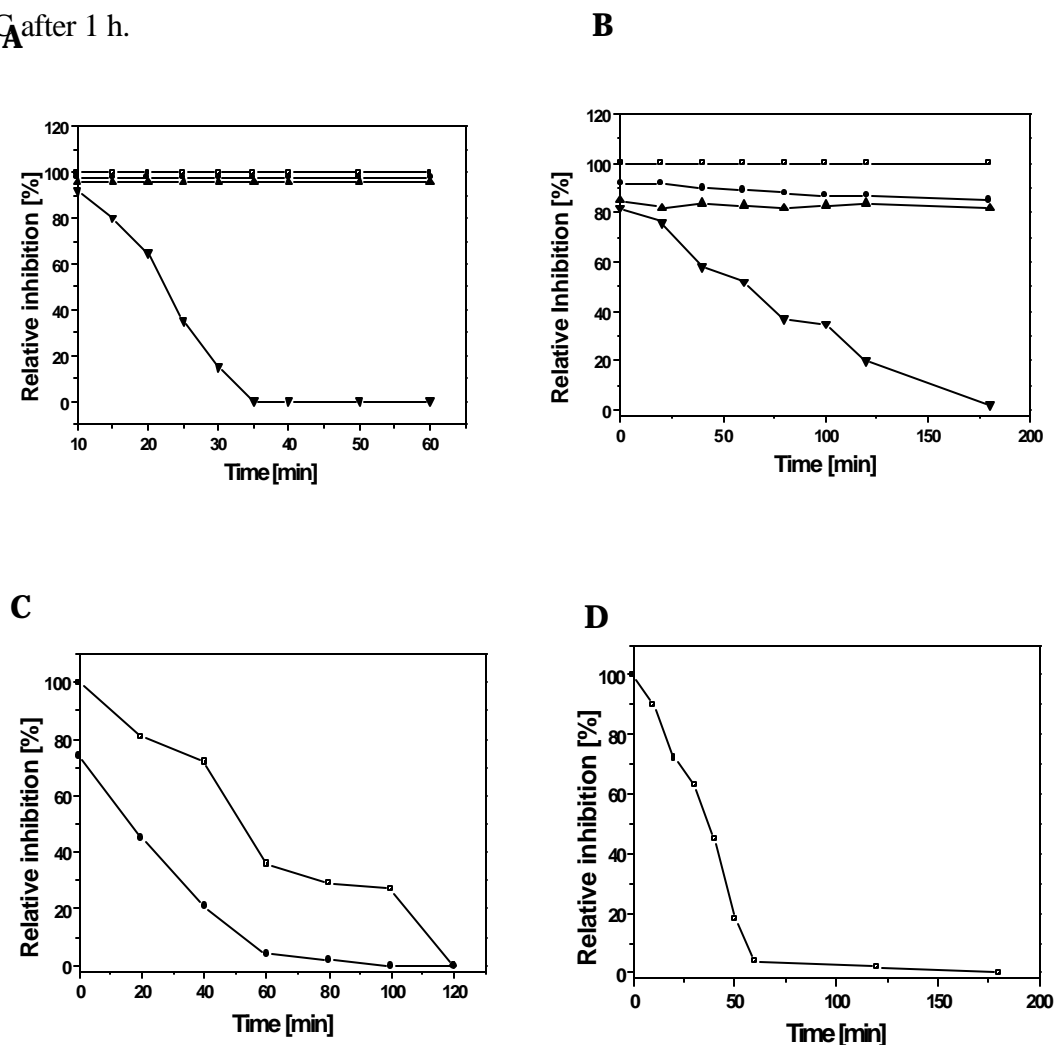
Figure 4. Temperature stability of APIs:

(A), API was incubated at 37 °C (■), 50 °C (●), 85 °C (▲), and 95 °C (▼) (B), API-I was incubated at 37 °C (■), 45 °C (●), 55 °C (▲), and 60 °C (▼), (C) API-II was incubated at 37 °C (■), and 45 °C (●), (D) API-III at 37 °C (■) for the stipulated time period and the inhibitory activity was determined.

Effect of additives on the thermostability

Effect of additives on API-I, II, and III against thermoinactivation was determined at 65, 55 and 45°C, respectively, at which they were found to lose activity. Glycine (1 M) offered complete protection with a 10% increase in the activity of API-I. However, cysteine hydrochloride (10 mM) showed no effect on thermostability. Polyethylene glycol 8000 (10 mM) conferred complete stability to API-I and a 10% increase in activity at 65°C. Glycerol and sorbitol at a concentration of 10% enhanced the stability of API-I by 45 and 60%, respectively. The two polyols, glycerol and sorbitol offered 55 to 60% protection to API-II at 55°C but failed to protect API-III at 45°C. Casein (1%), the substrate for the protease, also stabilized the inhibitors substantially by 50 - 65%. The stability of the inhibitors was also investigated in the presence of detergents. SDS provided 60% protection only to API-I and Tween-80 conferred 45 and 30 % protection to API-I and API-II, respectively; whereas both the detergents failed to protect API-III against thermal inactivation. Addition of divalent cations such as Ca^{2+} (10 mM) stabilized API-I upto 40% while the addition of urea (10 mM) enhanced the activities of API-I, II and III by 40%, 50%, and 30%, respectively.
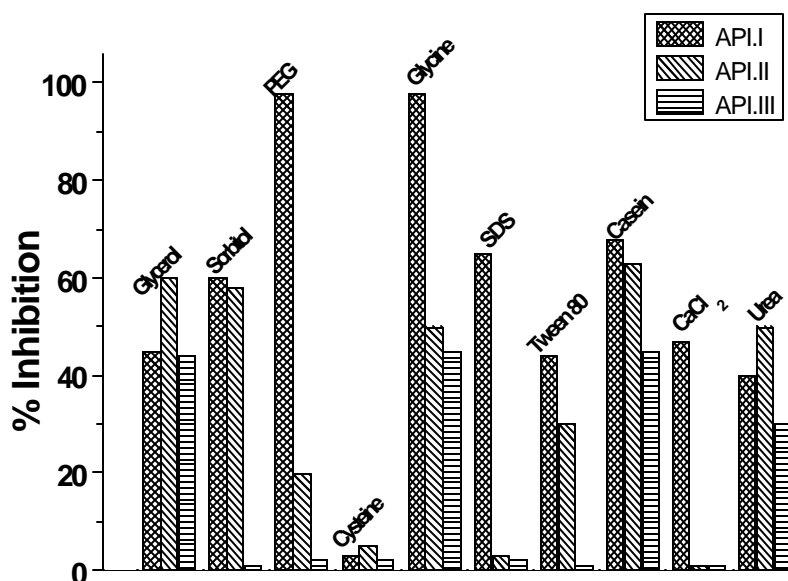
Figure 4: Effect of various additives on thermostabilty of APIs:
API-I at 65°C, API-II at 55°C and API-III at 45°C were incubated in the presence of glycerol (10%), sorbitol (10%), polyethylene glycol 8000 (10 mM), cysteine HCl (10 mM), glycine (10

mM), SDS (1%), tween-80 (1%), casein (1%), $CaCl_2$ (10 mM) and urea (10 mM) and the residual inhibitory activity was monitored.

Discussion

Actinomycetes are known to produce a wide range of antibiotics, enzymes, enzyme inhibitors and other secondary metabolites extracellularly. In view of the promising role of protease inhibitors in various physiological and biotechnological areas, the isolation of actinomycetes strains secreting protease inhibitory activity from soil was undertaken. Four actinomycetes strains exhibiting specificity for alkaline protease inhibitors were isolated from soil. They showed different properties in their molecular nature. The inhibitors could be explored for their potential applications in biocontrol and therapeutics. Based on the ability to inhibit proteases of insect digestive tracts, protease inhibitors have been shown to have potential usefulness as antifeedent agents. Insects that feed on plant material possess alkaline guts and depend predominantly on serine proteases for digestion of food material. The majority of the protease inhibitors exhibiting anti-feedent properties reported so far are active against the neutral serine proteases such as trypsin and chymotrypsin (Ryan 1990). However, they are little effective as biocontrol agents since the midguts of major pests such as lepidopteran insects are highly alkaline (Harsulkar *et.al.* 1999). Therefore, the use of protease inhibitors reported here having specificity for alkaline proteases would be more efficacious against serious pests and fungal pathogens.

Enhancement of thermal stability is beneficial for most of the biotechnological applications of proteins. Naturally occurring osmolytes such as amino acids, polyols and salts are known to protect proteins against thermal inactivation by stabilizing the thermally unfolded proteins (Yancey *et al.,* 1982). Thermal stability increases the efficiency of proteins and is one of the essential features for their commercial exploitation. API was very stable while the thermal inactivation of API-I, II and III could be overcome by the additions of different additives.

In summary, although the four alkaline protease inhibitors shared a similar property of having specificity of inhibition against alkaline proteases, they showed differential pH and temperature stabilities. The proteinaceous inhibitors, exhibited stability over a wide pH range along with higher temperature stability and would be ideal candidates for developing environment-friendly biocontrol agents and in transgenic research for conferring resistance against pests and pathogens. The distinctly different non-proteinaceous inhibitor, API-III,

with its small molecular weight and stability in the physiological range could be explored for its application in developing therapeutic agents.

References

Billings, P. C., Carew, J. A., Keller-McGandy, C. E., Goldberg, A. L., and Kennedy, A. R. (1987) *Proc. Natl. Acad. Sci.* **84**, 4801-4805.

Green, T. R., and Ryan, C. A. (1972) *Science* **175**, 776-777.

Gupta, M. N. (1991) *Biotechnol. Appl. Biochem.* 14, 1-11.

Joshi, B. N., Sainani, M. N., Bastawade, K. B., Deshpande, V. V. Gupta, V. S., and Ranjekar, P. K. (1999) *Eur. J. Biochem.* **265**, 1-9.

Kassel, B. (1970) *Methods Enzymol.* **19**, 839-932.

Kunitz, M. (1947) *J. Gen. Physiol.* **30**, 291-310.

Kuster, E., and Williams, S.T. (1964) *Nature* **202**, 928-929.

Lorito, M., Broadway, R. M., Hayes, C. K., Woo, S. L., Noviello, C., Williams, D. L., and Harman, G. E. (1994) *Mol. Plant. Microbe. Interact.* **7**, 525-527.

Harsulkar, A.M., Giri, A.P., Patankar, A.G., Gupta, V.S., Sainani, M.N., Ranjekar, P.K. and Deshpande V.V. (1999) *Plant Physiology* **121**, 497-506,

Ryan, C. A. (1990) *Ann. Rev. Phytopathol.* **28**, 425-449.

Seelmeir, S., Schmidt, H., Turk, V., and vonder Helm, K. (1988) *Proc. Natl. Acad. Sci.* 85, 6612-6616.

Tanksale, A.M., Chandra., M.P., Rao, M.B., and Deshpande, V.V. (2001) *Biotech. Lett.* **23**, 51-54.

Umezawa, H. (1982) *Ann. Rev. Microbiol.* **36**, 75-99.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) *Science* 217, 1214-1222.

Chapter 3

Purification and Biochemical Characterization of an Alkaline Protease Inhibitor, API, from a *Streptomyces* sp. NCIM 5127

Summary

The alkaline protease inhibitor (API) producing actinomycete strain isolated from soil was identified to be a *Streptomyces sp*. by its colony characteristics and spore chain morphology. API has been purified to homogeneity by ammonium sulfate precipitation, preparative polyacrylamide gel electrophoresis and DEAE cellulose chromatography. Purified API moved as a single protein on native and SDS-PAGE. The homogeneity of the purified protein was also confirmed by a single peak at a molecular mass of 28kD upon gel filtration on HPLC and a single peak upon isoelectric focusing with an isoelectric point of 3.8. API is a competitive type of inhibitor with a K_i value of 2.5x 10^{-9} M for subtilisin. Purified API is stable over a pH range of 6 to12 and a temperature range of 40 to 95°C. Amino acid composition of the purified API showed an abundance of Ala, Val, Gly and Asp accounting for about 50% of the total amino acid content. DTNB titration yielded two free sulfhydryl groups and five disulfide linkages per mole of inhibitor. The oxidation of Trp by NBS resulted in a progressive decrease in absorption at 280 nm. The number of Trp residues oxidized per mole of API was calculated to be 2. API was found to specifically inhibit the alkaline proteases such as subtilisin, proteinase K from *Triticharium album* Limber and the alkaline protease from *Conidiobolus* sp. The binding interaction of API with the alkaline protease from *Conidiobolus* sp as probed using activity measurements and fluorescence titration revealed that the API and alkaline protease combine in a molar ratio of 1:2.

Introduction

Elucidating the structure and mechanism of inhibition of protease inhibitors is essential in view of their promising physiological and biotechnological applications. Proteinaceous protease inhibitors are widely distributed in the plant kingdom (Laskowaski and Kato, 1980). Some of them are strictly specific, inactivating only one class of proteolytic enzyme, while others have broad specificity, inhibiting different mechanistic classes. Leguminous seed inhibitors of trypsin and chymotrypsin classified in the 'Bowman-Birk and Kunitz plant inhibitor' families are among the best characterized proteinaceous inhibitors from plants. There have been several reports of isolation and purification of inhibitors of the alkaline protease subtilisin from plants and microorganisms. The isolation of specific subtilisin inhibitor and trypsin inhibitor from *Vigna catjang* has been reported (Vartak *et al.*, 1980). There have been also reports on the purification and characterization of subtilisin inhibitors from barley (Yoshikawa, *et al.*, 1976), black bean (Seidl, *et al.*, 1978, 1982), broad bean (Svendsen, *et al.*, 1984), Adzuki beans (Yoshikawa, *et al.*, 1985) and foxtail millet (Tashiro, *et al.*, 1991). The inhibitors have been purified by conventional protein purification techniques employing a combination of chromatographic procedures. The homogeneity of the purified protein is checked by polyacrylamide gel electrophoresis or by gel filtration or by IEF and the pure protein is used for further characterization.

A number of extracellular proteinaceous inhibitors of microbial alkaline proteases have been isolated from different streptomycetes (Taguchi, *et al.,* 1993). The first inhibitor reported from *Streptomyces* was the *Streptomyces* subtilisin inhibitor (SSI) from *Streptomyces albogriseolus* (Sato and Murao, 1973). Based on the structural homologies and protease inhibition specificities, the inhibitors produced by *Streptomyces* have been named as SSI-like (SIL) proteins and classified as members of the SSI family (Taguchi, *et al.,* 1993a). SSI from the culture filtrate of *S. albogriseolus* has been purified to homogeneity by salting out with ammonium sulfate, column chromatographies on DEAE-cellulose and Sephadex G-100. SSI was also isolated and purified by pH adjustment of the effluent from DEAE-cellulose column (Sato and Murao, 1973).

Amongst the four alkaline protease inhibitors isolated (Chapter 2), API was selected for further characterization based on its high stability and proteinaceous nature, which could be exploited for various biotechnological applications. For a comprehensive understanding of

the mechanism of inhibition of API and delineating its structure-functional relationship, the purification of API is a prerequisite. The present chapter deals with the purification and biochemical characterization of the inhibitor.

Materials and Methods

Materials

Casein, acrylamide, bisacrylamide, was purchased from Sisco Research Laboratories (SRL), India. Tris, trichloroacetic acid, glacial acetic acid, and glycerol were from Qualigens, Glaxo India.   N,N,N',N' tetramethyl ethylenediamine (TEMED), ammonium persulfate (APS), N-bromosuccinimide (NBS), dithiobisnitrobenzene (DTNB), diethylaminoethyl (DEAE) cellulose, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sAAPF-pNA), sodium dodecyl sulfate (SDS), β-mercaptoethanol, coomassie blue G-250 and R-250, molecular weight markers were from Sigma Chem. Co. USA. Ampholines were from Pharmacia, Sweden. All other chemicals used were of analytical grade. The alkaline protease from the *Conidiobolus* sp. was purified as described (Tanksale *et al.,* 2000).

Scanning Electron Microscopy

The actinomycete isolate was grown in a cover slip culture on casein-starch agar by a modified method (Williams and Davies, 1967). The cover slip was fixed overnight in 2% glutaraldehyde at 4°C and then gently washed with several changes of distilled water to remove the excess of glutaraldehyde. Further, it was dehydrated by successive passages through increasing concentrations of ethanol (10 - 95% v/v) and air-dried. The specimen was coated with gold using a sputter coater unit (Bio-Rad, UK) and examined under a scanning electron microscope S-120 Cambridge Instruments, UK.

Assay for Protease Inhibitory Activity

The antiproteolytic activity of API was determined by the caseinolytic assay (Kuntiz, 1947) as described in Chapter 2. The proteolytic assay of subtilisin was estimated using the synthetic substrate sAAPF-pNA (DelMar, 1979). The reaction was carried out in 0.1 M Tris-Cl buffer, pH 7.8 at 28 °C containing subtilisin (7.5 µg), appropriately diluted inhibitor solution and sAAPF-pNA (60 µg). One unit of enzyme activity is defined as the amount of the enzyme required to cause an increase of one absorbance unit at 410 nm per ml of reaction mixture per minute. The reaction mixture was incubated for 5 min and the absorbance was measured at 410 nm. The protein content was determined by Bradford method using BSA as a standard (Bradford, 1976).

Production and Purification of API

API was produced in 500 ml Erlenmeyer flask containing 100 ml of casein-starch medium. The flasks were incubated at 200 rpm at 28°C for 96 h. Bacterial cells were removed by centrifugation and the culture supernatant was used as a source of API. All purification steps were carried out at 4°C unless otherwise mentioned. The culture filtrate (Step I) was precipitated with ammonium sulfate (0.9 saturation). The precipitate was dissolved and dialysed against 5 mM potassium phosphate buffer pH 7.5 (Step II). API was further subjected to electrophoresis on preparative polyacrylamide gel (PAGE) in a column (15 x 4.8 cm) using a 7.5 % gel and bromophenol blue as the tracking dye. Electrophoresis was carried out at 200 V and 20 mA for 28 h, following which the band corresponding to the API was detected by gel-X-ray film contact print technique (Pichare and Kachole, 1994). Briefly, a vertical strip of the gel was cut and incubated in the assay buffer (0.1 M carbonate-bicarbonate, pH 10.0) containing, 0.5 mg/ ml subtilisin for 10 min. The gel strip equilibrated with the enzyme solution was overlaid on an equal sized X-ray film and the hydrolysis of gelatin was followed for 20 min at 37°C. The X-ray film was removed and washed with warm water and the zone where the hydrolysis was inhibited was detected. The band corresponding to the antiproteolytic activity was excised and eluted by homogenization and vacuum filtration in 50 mM potassium phosphate buffer pH 7.5. The eluted protein was concentrated by lyophilisation (Step III) and further purified by anion exchange chromatography on a DEAE cellulose column equilibrated with 50 mM potassium phosphate buffer pH 7.5. The adsorbed protein was eluted with 0.3 M NaCl, dialyzed and concentrated by lyophilization (Step IV).

Biochemical Properties of API

The purity and subunit M_r were checked by analytical SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide gel using standard molecular weight marker proteins containing Bovine serum albumin (BSA), 66,000; Pepsin, 34,700; Carbonic anhydrase, 29,000; Trypsinogen, 24,000; β-lactoglobulin 18,000 and Lysozyme, 14,400

The M_r of the inhibitor was also determined by gel filtration on HPLC using a Protein Pak 300SW column (7.8 mm x 300 mm) and Water's liquid chromatograph. The column was

pre-equilibrated with potassium phosphate buffer (0.05 M), pH 7.5 and calibrated using the above marker proteins.

Isoelectric focusing was carried out as described by Vesterberg (1972) over the pH range of 3 to 10.

The inhibition constant K_i was determined by the Dixon's method (Dixon, 1953), where the proteolytic activity of subtilisin was measured at two different concentrations of substrate as a function of inhibitor concentration.

Amino acid analysis of the API was carried out on Pharmacia LKB alpha plus amino acid analyzer, by standard acid hydrolysis conditions using 6 N HCl at 110°C for 22 h. Number of Trp residues were determined by the spectrophotometric method (Spande and Witkop, 1967). Free sulfhydryl groups and disulfide linkages were determined by the DTNB method (Habeeb, 1972).

pH and thermal stability of API

The pH and temperature stability of API was determined by pre-incubating API (15 μg) in various buffer solutions (pH range 2 - 12) at room temperature for 1h and at temperatures ranging from 40 to 95°C at pH 7.5 for different time periods respectively followed by the measurement of residual enzyme inhibitory activity.

Fluorescence analysis

The fluorescence measurements were performed with a Perkin-Elmer LS-50 spectrofluorimeter at 25 °C using a slit width of 7.5 mm in a 1 cm path length quartz cuvette, with an excitation wavelength of 295 nm. Measurements were performed in triplicate. In the enzyme-inhibitor interaction studies, the fluorescence spectrum of only API was subtracted from the corresponding spectrum of enzyme plus inhibitor.

Results

Characterization of the Actinomycete Strain Producing API

The actinomycete isolate producing extracellular API is shown in Fig.1A. Pure culture was obtained by single colony plating technique using the starch-casein agar medium. Typical colonies with firm, leathery substrate mycelium were formed in the early stages of the development followed by the formation of loose, cottony aerial mycelium bearing spores of gray color. The colonies possess strong odor of damp earth on the laboratory media. The scanning electron micrographs of the organism (Fig. 1B) revealed that it has a branching mycelium with conidia produced in chains on top of the aerial hyphae. Spores bear a spiny surface, mature spore chains with approximately 10 to 50 spores per chain, were found arranged in spirals. These observations are characteristic of the organism belonging to the order *Actinomycetales*, genus *Streptomyces*. The strain has been deposited at our in house
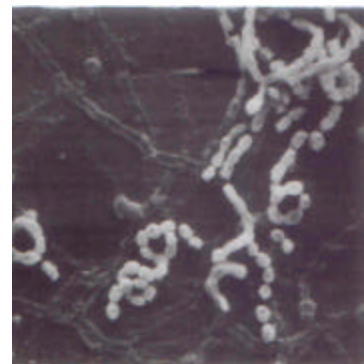
culture collection unit National Collection for Industrial Microorganisms (NCIM), with the serial number NCIM 5127.
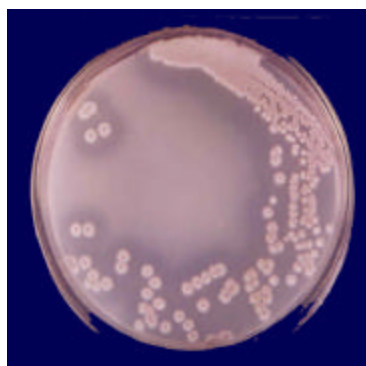
A   **B**

Figure 1. Characterization of the actinomycete strain producing API

A) The actinomycete isolate was grown on a starch-casein agar plate for 5-7 days at 28 °C and the colony characteristics were observed. **B)** Scanning electron micrograph showing the arrangement and morphology of spore chains.

Purification of API

The crude extracellular culture filtrate of the *Streptomyces* sp. NCIM 5127 showed the presence of only one protease inhibitor band as detected by the gel-X-ray film contact print technique which has been purified to homogeneity. The flow diagram for purification of API is illustrated in Fig. 2. The purified API showed a single band on SDS-PAGE (Fig. 3) indicating its purity. It eluted as a single peak upon gel filtration on HPLC (Fig. 4) confirming the homogeneity of the preparation. The specific activity of API increased from 4 U/mg to 164 U/mg with a 37 fold purification over the culture filtrate. Table 1 summarizes the results of the purification of API.

Culture filtrate

↓

Ammonium sulfate precipitation

↓

Preparative polyacrylamide gel electrophoresis

Detection by gel X-ray film contact print technique
Elution, concentration by lyophilisation

↓

DEAE-cellulose chromatography

Purity SDS PAGE,
Gel filtration on HPLC

↓

Homogeneous API
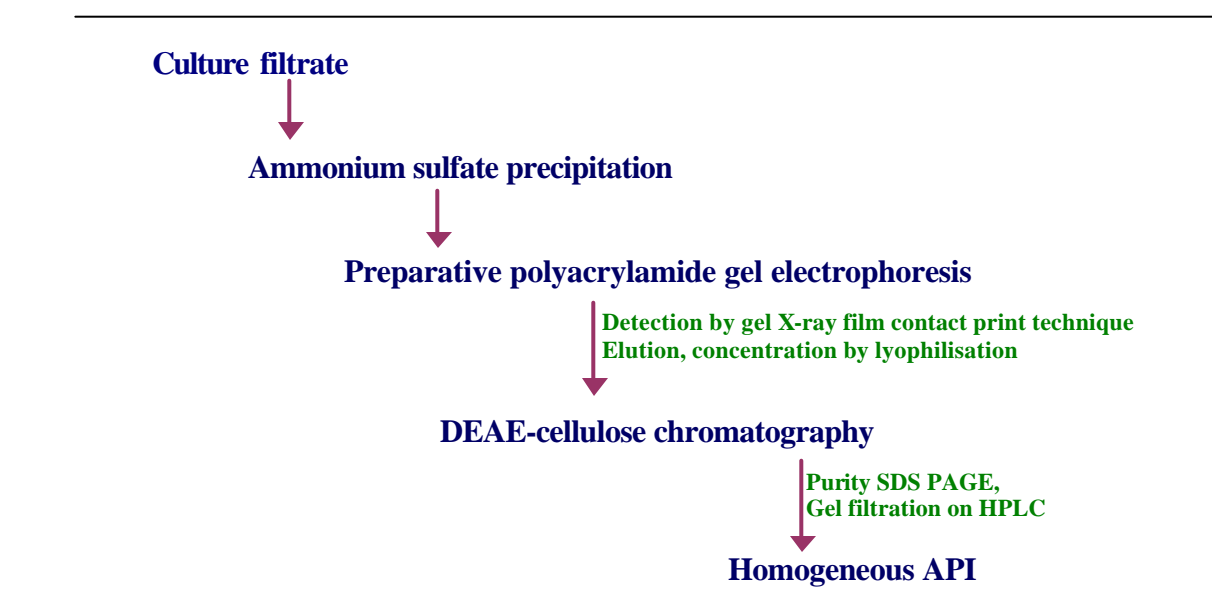
Figure 2. Flow diagram of the protocol used in the purification of API

Table 1. Purification of alkaline protease inhibitor (API)

Step	Fraction	Total Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold Purification
I.	Culture filtrate	1200	148	650	4	-
II.	Ammonium sulfate precipitation	10	24	534	22	5
III.	Polyacrylamide Gel Electrophoresis	4	2.5	406	162	37
IV.	DEAE-Cellulose Chromatography	2	1	164	164	37

Biochemical Properties of API

The molecular mass of the native API as determined by gel filtration on HPLC column was 28 kD whereas that by SDS-PAGE was 13.1 kD indicating the presence of two subunits having identical molecular weights. The isoelectric point (pI) of the purified inhibitor was 3.8. The inhibition profile of API indicated competitive type of inhibition (Fig. 5). A K_i value of 2.5 x 10^{-9} M was obtained against subtilisin suggestive of strong inhibition by API. The physicochemical properties of API are summarized in Table 2. The amino acid composition of the purified API is given in Table 3. As revealed from the table the amino acid composition showed an abundance of Ala, Val, Gly, and Asp accounting for about 50% of the total amino acid content The oxidation of Trp by NBS resulted in a progressive decrease in absorption at 280 nm (Fig. 6). The number of Trp residues oxidized per mole of API was calculated to be 2. Two free sulfhydryl groups and five disulfide linkages were detected per mole of inhibitor. The crude and purified API exhibited similar pH and temperature stability profiles, as shown in Chapter 2, API is stable at pH 2 and in the pH range of 6-12 while there is a loss in activity around pH 3-4. API is stable in the temperature range of 40-95°C, at 95°C it is stable for 30 min after which there is a rapid loss of its inhibitory activity.

Table 2. Physicochemical properties of purified API

Property of API	Value obtained
Molecular weight by	
SDS PAGE	13,100 daltons
Gel filtration on HPLC	28,000 daltons
Amino acid composition	27,082 daltons
pI	3.8
Mode of inhibition of Subtilisin	Competitive
K_i	2.5x10^{-9} M
pH stability	2, 6-12
Temperature stability	40-95°C
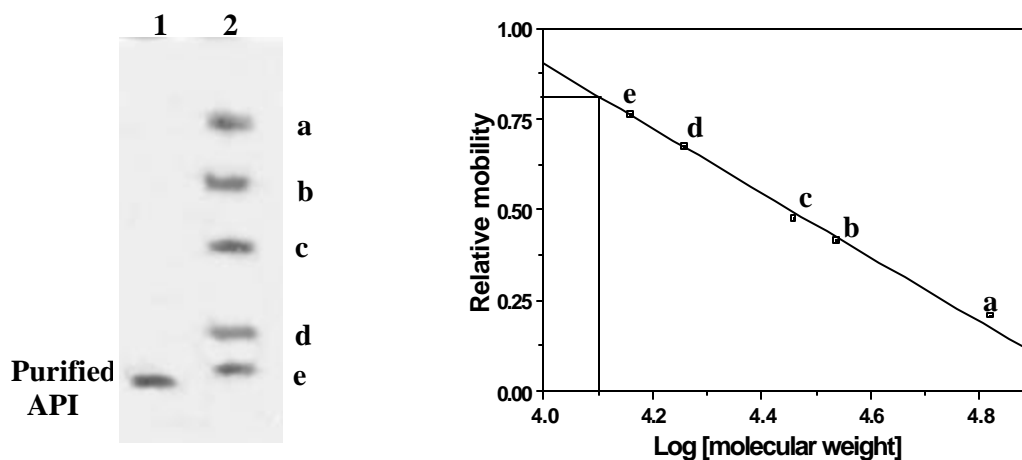
Figure 3. SDS PAGE of API.
The samples were mixed with the tracking dye containing bromophenol blue, β-ME and SDS. Lane 1, purified API (25 μg). Lane 2, molecular weight markers: (**a**), BSA (66,000), (**b**), Pepsin (34,700), (**c**), Carbonic anhydrase (29,000), (**d**), β-lactoglobulin (18,400) and (**e**) Lysozyme (14,400); The bands were visualized by staining with coomassie blue R-250.
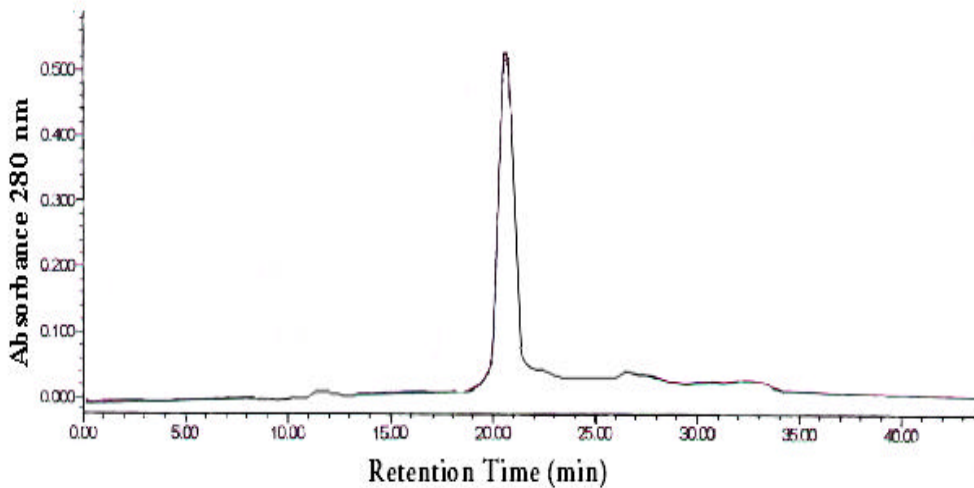
Figure 4. Gel filtration of API using HPLC
Elution profile of API by 50 mM potassium phosphate buffer at a flow rate of 0.5 ml/min.
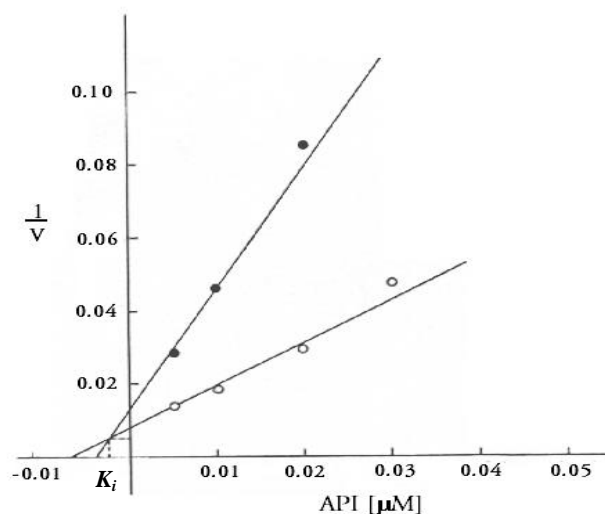
Figure 5. Determination of K_i by the Dixon method

Enzymatic activity of subtilisin was estimated using casein at (o) 5 mg/ml, (●) 10 mg/ml, respectively, at different concentrations of API. The reciprocal of substrate hydrolysis by subtilisin was plotted as a function of inhibitor concentration. The straight lines indicated the best fits for the data obtained. The Inhibition constant K_i. was determined from the point of intersection of the plots.

Table 3: Amino acid composition of API

Amino acid	No. of residues / mol of protein	
	API	SSI
Asx	19	18
Glx	14	12
Thr	14	16
Ser	14	18
Pro	12	16
Gly	27	22
Ala	42	36
Val	27	26
Met	2	6
Iso	1	0
Leu	12	18
Tyr	5	6
Phe	5	6
His	4	4
Lys	4	4
Arg	14	8
Cys [a]	12	8
Trp [b]	2	2

a Estimated by DTNB titration
b Determined by spectrophotometric method of Spande and Witkop
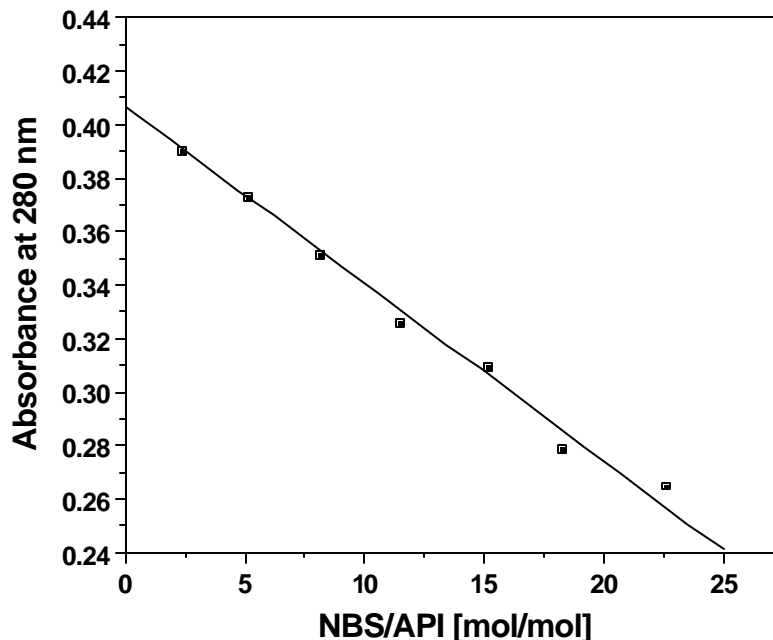
Figure 6. Oxidation of API by NBS.

Aliquots (5 μl) of NBS (1 x 10^{-2} M) were successively added to the purified API (2.25 x 10^{-3} M). After each addition, the decrease in absorption at 280 nm was measured. The number of tryptophan residues were calculated by the method of Spande and Witkop.

Stoichiometry of binding of API with alkaline protease from *Conidiobolus* sp.

API specifically shows inhibition towards alkaline proteases such as subtilisin, proteinase K from *Triticharium album limber* and the alkaline protease from *Conidiobolus* sp. The binding interaction of API with the alkaline protease from *Conidiobolus* sp. was probed using activity measurements and fluorescence titration of the protease with API. Inhibition of the alkaline protease as a function of API concentration followed a sigmoidal relationship. As revealed from the figure (Fig. 7A), the molar combining ratio of API to alkaline protease at the point of complete inhibition was 1:2. The active site of the alkaline protease contains a Trp residue and the intrinsic fluorescence exhibits a λ_{max} at 340 nm (Tanksale, *et al.,* 2000). The quantum yield of the protein fluorescence due to binding of API to the alkaline protease decreased gradually in a saturation manner and indicated a molar combining ratio of 1:2 (Fig. 7B).

(A)

(B)
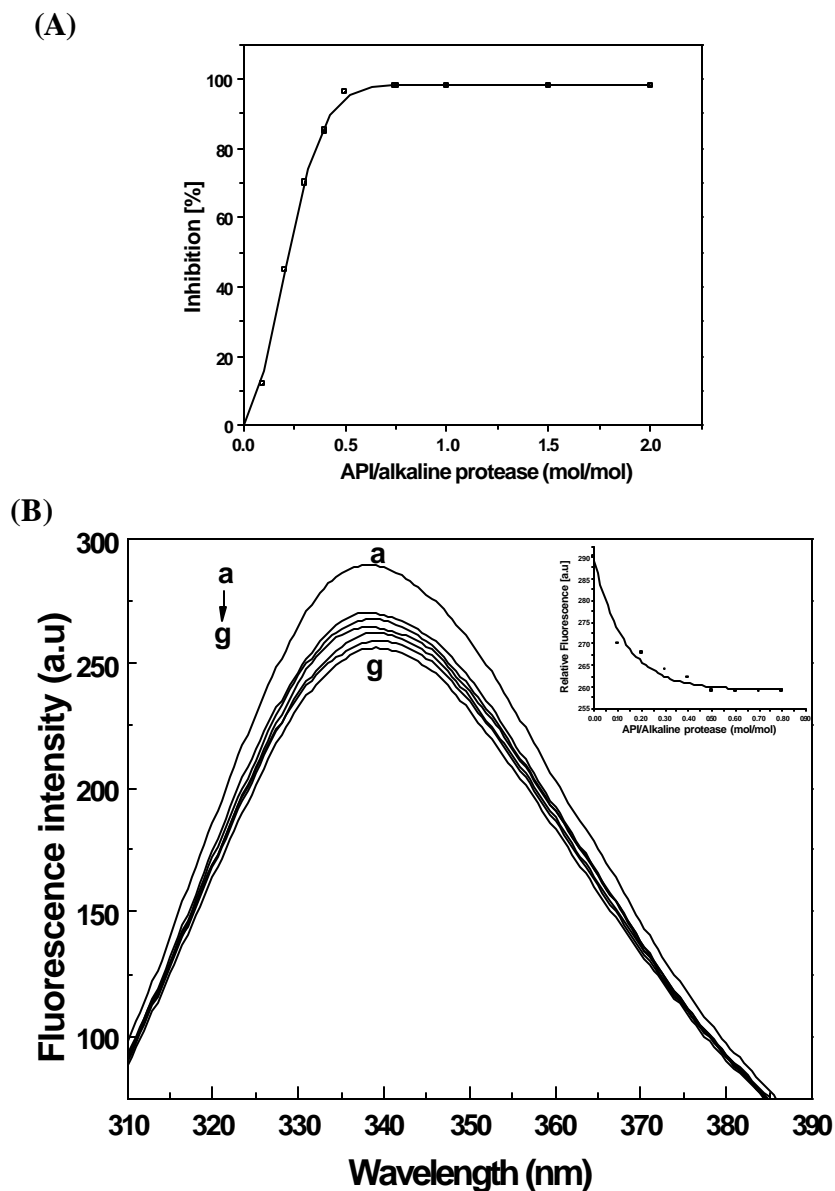
Figure 7. Stoichiometry of binding of API with alkaline protease from *Conidiobolus* sp.
A) The proteolytic activity of the purified alkaline protease was determined in the presence of increasing concentrations of API. The % inhibition of the protease activity was calculated from the residual enzymatic activity. The sigmoidal curve indicates the best fit for the % inhibition data obtained. The molar combining ratio at the point of complete inhibition was obtained from the graph. **B)** The fluorescence quenching of alkaline protease with API is shown. Alkaline protease was excited at 295 nm, and the emission was monitored from 300 to 400 nm. Titration of the enzyme $(3.5 \times 10^{-10}$ M) in phosphate buffer (0.05 M) pH 7.5 was performed by the addition of increasing concentration of the inhibitor to the enzyme solution. Each scan represents a different molar ratio of API to alkaline protease: (**a**) 0:1, (**b**) 0.1:1; (**c**) 0.2:1; **d**) 0.3:1;(**e**) 0.4:1; (**f**) 0.5:1; (**g**) 0.6:1. The inset shows decrease in the fluorescence quantum yield as a function of the concentration of API.

Discussion

Gram-positive filamentous bacteria of the genus *Streptomyces* are ubiquitous soil organisms characterized by a morphologically complex life cycle. In contrast to the Gram-negative bacteria like *E. coli*, Gram-positive bacteria lack a cell wall and are therefore able to secrete proteins directly into the external milieu. Most of the extracellular inhibitory proteins discovered so far have been isolated from *Streptomyces* sp. and classified as members of the SSI family and are designated as SSI- like (SIL proteins) (Taguchi, *et al.,* 1993). The microbial strain producing extracellular API was identified to be a *Streptomyces* sp. and therefore API belongs to the SSI family of proteins. Purification of the SSI-like proteins is generally carried out by conventional chromatographic techniques that are tedious and time consuming. However, the use of preparative polyacrylamide gel electrophoresis followed by gel-X-ray film contact print technique for detection of inhibitor band offers a simple, rapid and sensitive method for purification of the alkaline protease inhibitor. The use of gelatin coating on the X-ray film as a substrate for the protease activity facilitates the detection of inhibition. API was purified 37 fold over the culture filtrate in a single step of purification.

Purified API moved as a single band indicating its homogeneity. The molecular weight of API by SDS-PAGE was 13.1 kD while that by HPLC was 28 kD indicating API to be a homodimeric protein of two identical subunits. The molecular weight calculated according to the amino acid composition of API is consistent with that determined by HPLC. Majority of the SSI-family proteins reported are homodimeric and usually possess molecular weights in the range of 20-25 kD (Kourteva and Boteva, 1989; Miura, *et al*., 1994). *Streptomyces* subtilisin inhibitor (SSI) is a homodimeric protein of 23 kD. The amino acid composition of API showed an abundance of Ala, Val, Gly, and Asp similar to that reported for SSI (Ikenaka, *et al.,* 1974). API was stable in a pH range of 6 to 12 and at pH 2, however, lost its activity around pH 3-4. Thus, the reduced activity of API in the pH range of 3-4 may be attributed to its decreased solubility in the pH range around the pI (3.8). In addition to the inhibition of alkaline proteases such as subtilisin some of the SIL-family members show varying specificity of inhibition and are reported to inhibit chymotrypsin, trypsin and some other serine proteases (Taguchi *et al*., 1993a) however API exhibited an inhibition specifically towards alkaline proteases. The binding interaction of API with the alkaline protease revealed a molar combining ratio of 1:2 of API and the alkaline protease.

The unique specificity of inhibition towards alkaline proteases of the proteinaceous API together with its broad pH and temperature stability make it an ideal candidate for its exploration in various biotechnological applications especially as a biocontrol defense protein for the protection of plants against pest and pathogen infestations. Being of microbial origin it can be conveniently subjected to various recombinant techniques with minimum genetic manipulations.

References

Bradford, M. N. (1976) *Anal. Biochem*. **72**, 248-254.

DelMar, E. G., Largman, C., Brodick, J. W., and Geokas, M. C. (1979) *Anal. Biochem*. **99**, 316-320.

Dixon, M. (1953) *Biochem. J.* **55**, 170-171.

Habeeb, A. F. S. A. (1972) *Methods Enzymol*. **25**, 457-464.

Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., Sato, S., and Murao, S. (1974) *J. Biochem.* **76,** 1191-1209.

Kunitz, M. (1947) *J. Gen. Physiol*. **30**, 291-310.

Laemmli, U. K. (1970) *Nature* **227**, 680-685.

Laskowski, M., and Kato, I. (1980) *Ann. Rev. Biochem*.**49**, 593-626.

Kojima, S., Terabe, M., Taguchi, S., Momose, H., and Miura, K. (1994) *Biochem. Biophys. Acta* **1207**, 120-125.

Kourteva, Y., and Boteva, R. (1989) *FEBS Lett.* **247,** 468-470.

Pichare, M. M., and Kachole, M. S. (1994) *J. Biochem. Biophys. Methods* **28**, 215-224.

Sato, S., and Murao, S. (1973) *Agric. Biol. Chem.* **37,** 1067-1074.

Seidl, D. S., Abreu, H., Jaffe, W. G. (1978) *FEBS Lett.* **92,** 245-250.

Seidl, D. S., Abreu, H., Jaffe, W. G. (1982) *Int. J. Pept. Protein Res.* **19,** 153-157.

Spande, T. F., and Witkop, B. (1967) *Methods Enzymol*. **11**, 498-506.

Svendsen, J. B., Hejgaard, J., and Chavan, J. K. (1984) *Carlsberg Res. Commun.* **49,** 493-502.

Taguchi, S., Kikuchi, H., Suzuki, M., Kojima, S., Terabe, M., Miura, K., Nakase, T., and Momose, H. (1993) *Appl. Environ. Microbiol.* **59,** 4338-4341.

Taguchi, S., Kikuchi, H., Kojima, S., Kumagai, I., Nakase, T., Miura, K and Momose, H. (1993a) *Biosci. Biotech. Biochem.* **57,** 522-524.

Tanksale, A. M., Vernekar, J. V., Ghatge, M. S., and Deshpande, V. V. (2000) *Biochem. Biophys. Res. Commun*. **270,** 910- 917.

Tashiro, M., Asao, T., Nakano, H., Takahashi, K., and Kanamori, M. (1991) *Agric. Biol. Chem.* **55,** 265-267.

Vartak, H. G., Paranjpe, S. V., Bodhe, A. M., and Jagannathan, V. (1980) *Arch. Biochem. Biophys.* **204,** 129-133.

Vesterberg, O. (1972) *Biochem. Biophys. Acta* **257**, 11-19.

Williams, S. T., and Davies, F. L. (1967) *J. Gen. Microbiol*. **48**,171-177.

Yoshikawa, M., Iwasaki, T., Fujii, M., and Oogaki, M. (1976) *J. Biochem.* **79,** 765-773.

Yoshikawa, M., Yokota, K., and Hiraki, K. (1985) *Agric. Biol. Chem.* **49,** 367-371.

Chapter 4

API as a Novel Antifungal Protein: Protease Inhibitory Activity as the Biochemical Basis of Antifungal Activity

Summary

Besides its antiproteolytic activity, API was found to exhibit antifungal activity (*in vitro*) against several phytopathogenic fungi such as *Fusarium*, *Conidiobolus, Alternaria, Rhizoctonia* and also against *Trichoderma,* a saprophytic fungus. Retardation in the rate of hyphal growth extension was observed in the presence of API. *Fusarium oxysporum* f. sp. *ciceri* was found to be the most sensitive to inhibition requiring 0.5 µg/disc as a minimum inhibitory dose (MID). The fungal strains *F. oxysporum* f. sp. *ciceri* and *Conidiobolus* sp. produce extracellular alkaline protease(s) when grown in a liquid medium. The serine alkaline protease(s) are known to be vital for the growth and development of the above fungal strains; therefore, the antiproteolytic activity of API could be conveniently correlated to its antifungal activity. Parallel enrichment of both the antiproteolytic and antifungal functions obtained during purification of API indicated its bifunctional behavior. The inactivation of API at 95°C for 30 min resulted in the concomitant loss of the two activities revealing their presence on a single molecule. Chemical modification of API with NBS resulted in the complete loss of antiproteolytic and antifungal activities, with no gross change in conformation implying the involvement of a Trp residue in the active site of the inhibitor and the presence of a single active site for the two activities. Treatment of API with DTT abolished both the activities although the native structure of API remained virtually unaffected, indicating the catalytic role of the disulfide bonds. Inactivation of API either by active site modification or by conformational changes leads to the concurrent loss of both the antiproteolytic and antifungal activities. Based on the correlation between antiproteolytic and antifungal activities during co-purification, heat inactivation, chemical modification and its binding interaction with the fungal protease, we have demonstrated that the dual function of API is a consequence of its ability to inhibit the essential alkaline protease.

Introduction

Increasing population and diminishing availability of cultivable land have posed serious problems to agriculture. The situation is worsened as the plant is invariably subjected to a variety of biotic and abiotic stresses. Among the biotic stresses, the major constraints are the pest and pathogen attacks. Fungal diseases are one of the principal causes that contribute substantially to the overall loss in crop yield. Fungal pathogens employ diverse strategies to infect their host plants. To colonize plants, fungi have evolved mechanisms to invade plant tissues, to optimize growth in the plant, and to propagate. Bacteria and viruses, as well as some opportunistic fungal parasites, often depend on natural openings or wounds for invasion. In contrast, many phytopathogenic fungi have evolved mechanisms to actively traverse the plant's outer structural barriers, the cuticle, and the epidermal cell wall. To gain entrance, fungi generally secrete a cocktail of hydrolytic enzymes including cutinases, cellulases, pectinases, xylanases, and proteases (Goodenough *et al*., 1991; Knogge, 1996).

Host pathogen interaction

Plants have evolved an array of defense mechanisms to combat pathogen attack, which are either constitutive or inducible. The most rapid and efficient pathogen-induced defense response is the hypersensitive response that is characterized by localized cell and tissue death at the site of infection and induction of intense metabolic alterations in the cell surrounding necrotic lesions (Baker *et al*., 1997). These local responses trigger non-specific resistance throughout the plant, known as systemic acquired resistance, that provides protection against infection by a broad range of pathogens (Sticher, *et al.*, 1997). The various pathogen-induced defense responses that contribute to efficient confinement of the pathogen include: 1) cell wall reinforcement by deposition and crosslinking of polysaccharides, proteins, glycoproteins and insoluble phenolics. (2) Stimulation of secondary metabolic pathways, which yield small compounds with antibiotic activity (the phytoalexins) and also defense regulators such as salicylic acid, ethylene and lipid-derived metabolites; (3) Accumulation of a broad range of defense-related proteins and peptides. (Fritig, *et al*., 1998)

Antimicrobial proteins and peptides

Accumulation of a group of proteins called the pathogenesis-related (PR) proteins represents the major quantitative change in protein composition that is strongly induced after infection. Table 1 presents the PR protein families that have been characterized from different

plant species and classified mainly according to sequence similarities (Van Loon, *et al.*, 1993). In addition to PR proteins, small peptides with antimicrobial properties also accumulate in infected plants (Broekaert, *et al*., 1997).

Most PR proteins have a damaging action on the structures of the parasite. PR-1 and PR-5 family members interact with the plasma membrane (Niderman, *et al*., 1995; Abad, *et al*., 1996). There is, in fact, little information on the mechanism of interaction of the proteins of families 1 and 5 with the plasma membrane even though the crystal structures of a tomato PR-1 protein (Fernandez, *et al*., 1997) and of a maize PR-5 protein (Batalia, *et al*., 1996) have been recently elucidated. These structural studies revealed that PR-5 proteins have an electrostatically polarized surface that may be responsible for antifungal activity by interacting with a membrane ion channel, a water channel or an osmotic receptor (Batalia, *et al*., 1996). The class 5 PR proteins are homologous to the sweet protein thaumatin from *Thaumatococus danielli*. PR-5 proteins have been reported to create transmembrane pores and therefore named as permatins (Vigers, *et al*., 1991). The well characterized antifungal proteins osmotin and zeamatin from maize belong to the PR-5 class.

Table 1 Antimicrobial pathogenesis-related proteins in plants.

PR protein family	Enzymatic activity	Target in pathogen
PR-1	?	Membrane?
PR-2	1, 3-β-glucanase	Cell wall glucan
PR-3	Endochitinase	Cell wall chitin
PR-4	Endochitinase	Cell wall chitin
PR-5	?	Membrane
PR-6	Proteinase inhibitor	Proteinase
PR-7	Proteinase	?
PR-8	Endochitinase	Cell wall chitin
PR-9	Peroxidase	
PR-10	RNAase?	?
PR-11	Endochitinase	Cell wall chitin
Unclassified	α-Amylase Polygalacturonase inhibitor protein (PGIP)	Cell wall α-glucan Polygalacturonase

Another well characterized target of plant antimicrobial proteins is the cell walls of fungi and bacteria. The β-1,3 glucanases (PR-2 family) and chitinases (PR-3, PR-4, PR-8 and

PR-11 families) can attack the fungal cell wall since the substrates of these two enzymes, β-1,3 glucans and chitin are the constituents of the cell wall of most higher fungi. Some enzymes from the pathogen are involved in pathogenesis and are also targets for plant defense proteins. Peroxidase (PR-9 family) has indirect antimicrobial activity by catalyzing oxidative crosslinking of proteins and phenolics in the plant cell wall, and thus protects the host from degradation by the pathogen's hydrolytic enzymes. The role of other enzymes such as proteinases and RNases in plant defense is not yet clear. Plants also synthesize inhibitors of fungal polygalacturonases which are considered as pathogenicity factors (Desiderio, *et al*., 1997). The PR-6 family represents the proteinase inhibitors which are induced during the hypertensive response (Geoffroy, *et al*., 1990)

In addition to the PR proteins listed in Table 1, small antimicrobial peptides active against fungi and bacteria have been isolated from plants (Broekaert, *et al*., 1997). The peptide families, thionins, plant defensins and lipid transfer proteins are induced upon infection and thus share similarity with the PR proteins. These peptides exert their detrimental effect at the level of the plasma membrane of the target microoganism but are lkely to act via different mechanisms.

Strategies developed for the control of fungal diseases

Agronomical industries employ a wide variety of chemical antifungal agents to overcome the problem of fungal infections, which plays a vital role in controlling the agricultural economy. However, chemical pesticides are associated with several drawbacks such as lack of specificity, increased incidence of development of resistance upon prolonged application and deleterious effect on human health together with the environmental hazards inherent with residual toxicity. Therefore, there is an immediate need to develop alternative mechanisms to combat these biotic stresses for enhancing the productivity of plants. One of the mechanisms involves plant breeding, which aims towards the manipulation of host plant resistance against the major plant pathogens. Host resistance may be modified by the use of interspecific and intergeneric hybridization to incorporate resistance traits from resistant germplasm. This approach, however is often time-consuming and involves the identification of resistant germplasm from plant introductions and seed banks, followed by wide hybridization which may be achieved through conventional crosses and screening. Moreover, intergeneric and even interspecific crosses can be very difficult to achieve, and they may

require more complicated manipulations such as embryo rescue and *in vitro* culture, or somatic hybridization, in which the fusion of somatic protoplasts is used to circumvent extreme sexual incompatibilities (Gleddie and Keller, 1989).

Molecular genetic approaches aimed at identifying resistance traits or at directly modifying the host plant's resistance status offer an interesting alternative. The search for molecular markers linked or associated with disease and pathogen resistance traits, for instance, is a key goal of many plant pathologists since such markers greatly aid in the indirect selection of resistant germplasm and tracking of resistant progeny. In parallel, the advent of plant genetic transformation now offers the possibility of manipulating host plant resistance to pathogens by the use of appropriate resistance genes (Shah, *et al*., 1995). In this case plants with 'built-in' resistance may be designed by the addition of defense related proteins that interact with specific receptors or interfere with some metabolic functions in the target organism. Two kinds of resistance may be obtained by this approach:

   1. Complete resistance, where the trait introduced kills the target pest, and

   2. Relative resistance or tolerance, where the novel trait only partly protects the plant.

Complete resistance is seen where the genes encoding toxic (pesticidal) molecules are integrated into the plant genome. This approach forms a resistance mechanism based upon antibiosis, viz, the pathogen recognizes the host plant, attacks or invades, and is prevented from further invasion by some toxic metabolite. Relative resistance or tolerance also suggests that the pathogen recognizes the host, invades and attacks, but is prevented from causing a serious or full-scale host infection due to factors, which interfere with specific metabolic processes.

Although until now most emphasis has been put on the design of 'killer plants,' partial resistance shows increasing potential. Like any other pesticide, transgenic plants expressing toxic proteins will exert a strong selection pressure on the target pest population, which may result in the rapid development of resistant populations in the field and thus decrease the effectiveness of the modified plants when they are used as a sole control method (Brattsen, 1991). The effect of plants expressing antimetabolic proteins, in contrast, would be more diffuse, leading to reduced fitness of the target pest without exerting a high selection pressure on the target population. As a result, these plants would not provide complete control of the target pests, but instead alter their overall fitness in the environment. Such plants could prove

to be useful, especially when used in combination with other control approaches. Pathogens with reduced fitness could be more susceptible, for instance, to biological control agents used in the field, thereby contributing to the improvement of their efficacy (Gleddie and Michaud, 2000).

Protease inhibitors as defense tools against plant pathogens

By virtue of their ability to inhibit a wide variety of proteolytic enzymes, PIs were proposed as a tool to control pest and pathogenic organisms as diverse as herbivorous insects, parasitic nematodes and human microbial pathogens (Schuler, *et al*., 1998; Atkinson, *et al*., 1995; Henskens, *et al*., 1996). Until now little is known about the potential of PIs in the control of plant pathogens, but one can speculate that blocking the activity of plant fungal or bacterial pathogen proteases could eventually decrease their fitness by the alteration of some yet uncharacterized, but useful physiological functions. Extracellular proteases may be important in many pathogens for basic processes like dietary protein hydrolysis, penetration of host tissues or zymogen activation. While their role during the infection of plant tissues still remains to be elucidated, the secreted proteases of plant pathogens represent potential target molecules for the design of pathogen-resistant transgenic plants. Protease inhibitors have received increased attention because of their small size, abundance and stability (Ryan, 1990). In this perspective, the use of protease inhibitor (PI)-encoding genes may appear of particular interest in the development of plants, which are partly resistant to pathogens. Several studies examined the nature and the role of protease inhibitors in plant pathogenic fungi. The pathogen *Phytophthora infestans*, was shown to induce the synthesis of three chymotrypsin inhibitors in infected potato tubers (Valueva, *et al*., 1998). These serine PIs were purified from infected tissues, and subsequently shown to inhibit fungal zoospore germination and hyphae development *in vitro*, suggesting an antifungal role for these PIs in potato tubers. Inhibitors of trypsin and chymotrypsin purified from cabbage leaves, were shown to inhibit spore germination and germ tube elongation of *Botrytis* and *Fusarium* species when added to fungal cultures *in vitro* (Lorito, *et al*., 1994) In another study trypsin/chymotrypsin inhibitors purified from buckwheat (*Fagopyrum esculentum*) seeds were able to inhibit spore germination and mycelium growth of *Alternaria alternata* (Dunaevskii, *et al*., 1994), while a cysteine PI purified from the pearl millet (*Pennisetum glaucum*) was shown to affect fungal spore and mycelium growth of several pathogenic species including

Trichoderma reesei (Joshi, *et al*., 1998). There are many reports of transgenic plants expressing protease inhibitors that act primarily as insect growth retardants (Hilder, *et al*., 1987; Johnson *et al*., 1989; Gatehouse, *et al*., 1997).

The recent introduction of transgenic plants containing pest and pathogen resistance genes has launched a new era in agriculture. The developing technology allows the transfer of a genetic trait into plants from vastly different species. Exploration of novel antifungal proteins and the genes encoding them from not only plants but also other sources such as microorganisms can lead to the combinatorial expression of several defense proteins, which could be a promising strategy to engineer plants with enhanced and broad-spectrum resistance. In the present chapter we present the characterization of the novel antifungal property of API against agronomically important fungal pathogens and the biochemical basis for its bifunctional nature.

Materials and Methods

Materials

Potato dextrose, bactoagar and other microbiological media components were from Himedia, India. N-bromosuccinimide (NBS), dithiothreitol (DTT), N-acetyl imidazole (NAI), phenyl glyoxal (PG), diethyl pyrocarboxylate (DEPC), phenyl methyl sulfonly fluoride(PMSF), Woodward's reagent K (WRK) and trinitrobenzenesulfonic acid (TNBS) were from Sigma Chem. Co. USA. All other chemicals used were of analytical grade.

Fungal isolates

The fungal strains *Fusarium monoliforme* (NCIM 1099, 1100), *Alternaria solani* (NCIM 887, 888), *Conidiobolus* sp. (NCIM 1298), *Asperigillus flavus* (NCIM 535), *Trichoderma reesei* (NCIM 992, 1052, 1186) and *Fusarium oxysporum* (NCIM 1008, 1043, 1072) were from our in house culture collection unit, National Collection of Industrial Microorganisms (NCIM) Pune, India. *Fusarium oxysporum* f. sp. *ciceri* was obtained from ICRISAT, Hyderabad, India. *Rhizoctonia solani* and *Pythium* sp. were obtained from Niku Bioresearch Laboratories, Pune, India. The alkaline protease from the *Conidiobolus* sp. was purified as described (Tanksale *et al.,* 2000).

Assay for protease inhibitory activity

The antiproteolytic activity of API was determined by the caseinolytic assay (Kuntiz, 1947) as described in chapter 2. The protein content was determined by Bradford method using BSA as a standard (Bradford, 1976).

Bioassays for determination of antifungal activity

Inhibition of growth of fungi by API was studied using a hyphal extention inhibition assay as described by (Roberts and Selitrennikoff, 1986). Fungal mycelium from freshly grown culture was spot inoculated at the centre of a petriplate and incubated at 28°C for 48h. Sterile filter paper discs impregnated with suitable samples of API were placed on the periphery of the advancing fungal mycelium. The plates were further incubated at 28°C and observed for crescents of retarded mycelial growth. *Fusarium oxysporum* f. sp. *ciceri* and *Conidiobolus* sp. was grown in 250 ml Erlenmeyer flask containing 50 ml of Sabouraud's dextrose broth supplemented with 2% soyabean meal for 4 to 5 days at 28°C. The fungal cells were separated by filtration and the filtrate was checked for the presence of alkaline protease by caseinolytic assay as described earlier. The fungal strains were also grown on solid Sabouraud's dextrose medium supplemented with 2% soyabean meal in the presence and absence of phenyl methyl sulphonyl flouride (PMSF) (5 mM).

Chemical modification of API

API (100 μg) was incubated with various chemical modifiers (10 mM) in potassium phosphate buffer (0.05 M) pH 7.5 in a reaction volume of 500 μl at room temperature for 20 min. The chemically modified protein was subjected to gel filtration on Sephadex G 10 column (1 x 20 cm) equilibrated with potassium phosphate buffer (0.05 M) pH 7.5, to remove the excess of modifying agent and the antiproteolytic and antifungal activity of the modified API was determined as described above.

Fluorescence and circular dichroism analysis

The fluorescence measurements were performed with a Perkin-Elmer LS-50 spectrofluorimeter at 25°C using a slit width of 7.5 mm in a 1 cm path length quartz cuvette, with an excitation wavelength of 295 nm. Measurements were performed in triplicate. In the enzyme-inhibitor interaction studies, the fluorescence spectrum of only API was subtracted from the corresponding spectrum of enzyme plus inhibitor.

The far UV-CD spectra of the native and modified inhibitor (10 μg) in potassium phosphate buffer (0.05 M) pH 7.5 were recorded between 200-250 nm on a Jasco J-715

spectropolarimeter at 25°C using 10 mm pathway cuvette in 0.1 nm increment. Spectra are represented as an average of 5 scans with the baseline subtracted. CD spectra were analyzed using the k2D program for protein secondary structure prediction (Andrade, *et al*., 1993; Merelo, *et al*., 1994).

HPLC analysis

The quaternary structure of native and modified API was monitored by size exclusion HPLC on Protein-Pak 300SW HPLC column (7.8 mm x 300 mm) using a Waters liquid chromatograph. The column was pre-equilibrated with potassium phosphate buffer (0.05 M) pH 7.5, and the samples were analyzed at a flow rate of 0.5 ml/min and monitored at a wavelength of 280 nm.

Results

Antifungal activity of API

Crude inhibitor preparation was checked for its ability to inhibit fungal mycelial extension. The fungi namely *Fusarium oxysporum, Fusarium monoliforme, Conidiobolus* sp., *Alternaria solani, Trichoderma reesei, Rhizoctonia solani* exhibited moderate to good inhibition whereas *Aspergillus flavus* and *Pythium* sp. did not show crescents of retarded growth even with very high dose of API. *Fusarium oxysporum* f. sp. *ciceri* exhibited highest sensitivity to inhibition requiring 30 µg/disc (1mg/ml) as minimum inhibitory dose (MID) while *Conidiobolus* sp. was inhibited with a MID of 50 µg/disc (2 mg/ml). *Trichoderma reesei* (NCIM 1186) required a MID of 100 µg/disc (4 mg/ml) while other strains required much higher doses of about 10 mg/ml. Purified API showed a 100 fold increase in antifungal activity over the crude inhibitor preparation against *F.oxysporum* requiring a MID of 0.5 µg/disc (Fig. 1).

(A)   (B)
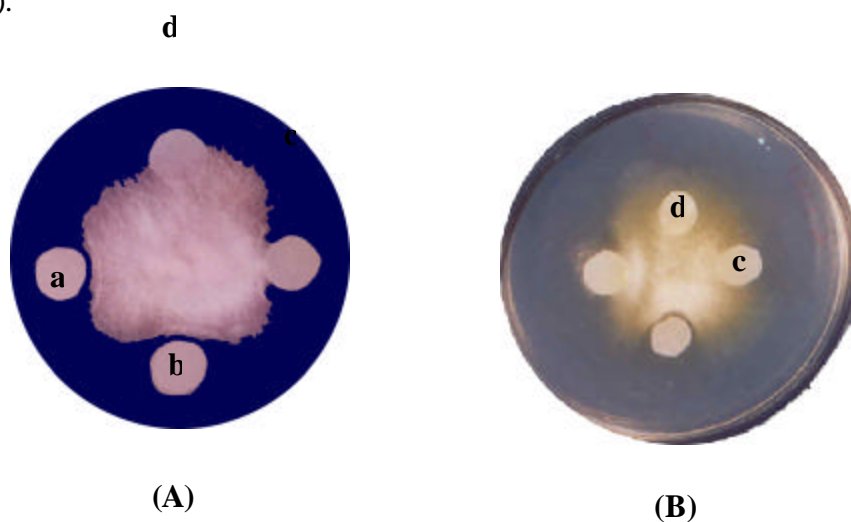
Figure1: Antifungal activity of API against *Fusarium oxysporum* f. sp. *ciceri and Trichoderma reesei*
A. Growth inhibition of *Fusarium oxysporum* f . sp. *ciceri* using different concentrations of purified inhibitor (a) 2 µg, (b) 1 µg, (c) 0.5 µg and (d) 0 µg per disc. **B.** Growth inhibition of *Trichoderma reesei* (a) 10 µg, (b) 5 µg, (c) 2.5 µg and (d) 0 µg per disc.

The fungal strain *F.oxysporum* f. sp. *ciceri* and *Conidiobolus* sp. produce extracellular alkaline protease(s) when grown in a liquid medium. Inhibition of the alkaline protease

activity was observed in the presence of API by the caseinolytic assay. In the presence of PMSF (5 mM), retardation in the growth rate and hyphal extension was observed indicating that the protease is essential for the growth and development of the organism. The alkaline protease from *Conidiobolus* sp. has been purified to homogeneity (Tanksale *et al.*, 2000) and its interaction with API was studied to decipher the correlation of the anifungal and antiproteolytic activites.

Chapter 4

Co-purification of antiproteolytic and antifungal activities

API (10 µg) obtained after successive steps of purification (Chapter 3) viz. culture filtrate (specific activity 4 U/mg), ammonium sulfate precipitation (22 U/mg) and preparative PAGE (162 U/mg) when tested by plate assay against *Conidiobolus* alkaline protease displayed an increase in the zone of inhibition of casein hydrolysis as the specific activity increased indicating the extent of purification (Fig. 2A). Similarly, the antifungal activity of API determined at the corresponding steps of purification resulted in increased inhibition of hyphal extension (Fig. 2B). Thus, during purification, increase in the antiproteolytic activity

paralleled the enrichment of antifungal activity suggesting their coexistence on a single molecule.

(A) **(B)**

Figure 2: Copurification of antiproteolytic and antifungal activity
A. Antiproteolytic activity of API: The effect of purification of API on the inhibition of alkaline protease from *Conidiobolus* sp. is demonstrated by the plate assay using skimmed milk agar. Increase in zone of inhibition of casein hydrolysis is observed with the successive steps of purification of API (10 µg) in phosphate buffer (0.05 M) pH 7.5. (**a**) Sterile distilled water control, (**b**) culture filtrate (specific activity 4U/mg), (**c**) ammonium sulfate precipitation (22 U/mg), (**d**) preparative PAGE (162 U/mg). **B.** Antifungal activity of API: The parallel enrichment of antifungal activity of API against *Conidiobolus* sp. with purification is shown. *Conidiobolus* sp. was spot inoculated on potato dextrose agar and retardation of fungal growth extension was observed with the successive steps of purification of API (50 µg) in phosphate buffer (0.05 M) pH 7.5 as above.

Simultaneous loss of antiproteolytic and antifungal activities upon heat inactivation of API

API is stable over a wide temperature range of 50 to 85°C for 10 h. At 95°C, API lost its antiproteolytic and antifungal activity after 30 min. The structural changes induced due to heat inactivation were followed by fluorescence and circular dichroism measurements. API exhibits a λ_{max} of 339 nm when excited at 295 nm characteristic of the presence of its tryptophanyl residues in a non-polar environment (Fig. 3A). The heat inactivated API showed a prominent red shift of 15 nm and a 25% decrease in fluorescence quantum yield in comparison to the native protein revealing the exposure of the Trp residues as a result of change in the tertiary structure. The active site of the alkaline protease contains a Trp residue and the intrinsic fluorescence exhibits a λ_{max} at 340 nm (Tanksale *et al.*, 2000). The binding of API to the alkaline protease results in the fluorescence quenching of the enzyme. Interaction of thermally inactivated API with the alkaline protease did not result in the quenching of fluorescence of the protease, indicating the inability of API to bind to the enzyme. API displays a strong and characteristic CD spectrum in the far-UV region with two negative minima at 209 nm and 220 nm (Fig. 3B). The inhibitor is an α/β type of protein consisting of intermixed segments of α-helix and β-sheet, with 27, 14 and 59% of α-helix, β-sheet and random coil structure respectively (Table 2). Concomitant with the loss in the tertiary structure of API by heat inactivation, there was a substantial change in the secondary structure as revealed by the CD spectrum (Fig. 3B, Table 2). A considerable decrease was observed in the α-helical content, which appears to be the most labile structural element of API. These results concluded that disruption in the native structure by heat inactivation leads to the loss of its biological property.

TABLE 2: CD analysis of native and modified API

Sample	% α	% β	% Random coil
Native	27	14	59
NBS-modified	30	12	58

|			
Heat-modified	8	37	55
DTT-modified	40	16	44

(A)   **(B)**
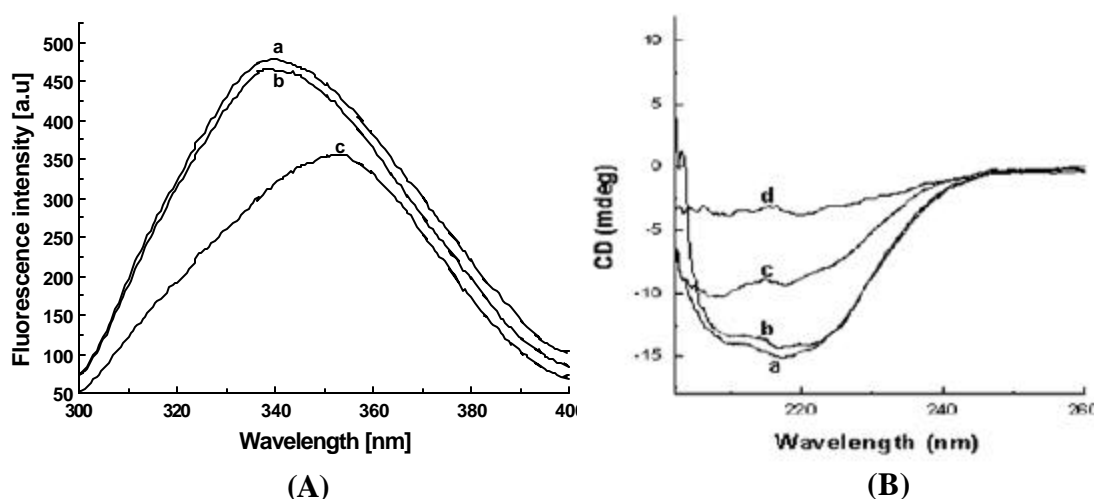
Figure 3: Effect of modification on the structure of API.
     A. Fluorescence spectra of native and modified API: API (15 μg) in phosphate buffer (0.05 M) pH 7.5 was excited at 295 nm and the emission was monitored from 300 to 400 nm. (**a**) Native API., (**b**) API treated with DTT (10 mM) for 15 min at room temperature., and (**c**) API heated at 95°C for 30 min. **B**. Far UV-CD spectra of native and modified API: The far UV-CD spectra of API (10 μg) in phosphate buffer (0.05 M) pH 7.5 are shown. (**a**) native API., (**b**) API treated with NBS (0.1mM) for 30 min at room temperature., (**c**) API treated with DTT (10 mM) for 15 min at room temperature., and (**d**) API heated at 95°C for 30 min.

Tryptophan is essential for antiproteolytic and antifungal activities

     Chemical modification of API with various group specific modifiers such as PMSF, NAI, WRK, DEPC, TNBS, or PG at a concentration of 10mM had no effect on its antiproteolytic and antifungal activity. However, modification of API by NBS (0.1 mM) resulted in the loss of its antiproteolytic and antifungal activities. Treatment with NBS may lead to the modification of Cys, Tyr or Trp. However, decrease in absorption of NBS-modified API at 280 nm eliminates the possibility of thiol group modification by NBS (Chap. 3, Fig. 6). Complete loss of activity of API by NBS but not by NAI negates the modification of Tyr residues. Thus, the Trp residues of API play a crucial role for its bifunctional property. The structural changes in the NBS-modified API were monitored by fluorescence and CD studies. The tryptophanyl fluorescence of NBS-modified API resulted in a progressive quenching of about 80%, as a function of NBS concentration without any shift in the emission maximum (Fig. 4). The CD spectra of the native and NBS-modified API were identical

indicating negligible effect of modification of Trps on the secondary structure (Fig. 3B). NBS-modified API fails to quench the tryptophanyl fluorescence of alkaline protease implying its inability to bind to the active site of the enzyme. This suggests that the loss of antiproteolytic and antifungal activity of NBS-modified API is due to its incapability to form a functional complex with the target enzyme, concluding the essential role of Trp residues in the enzyme-inhibitor binding.
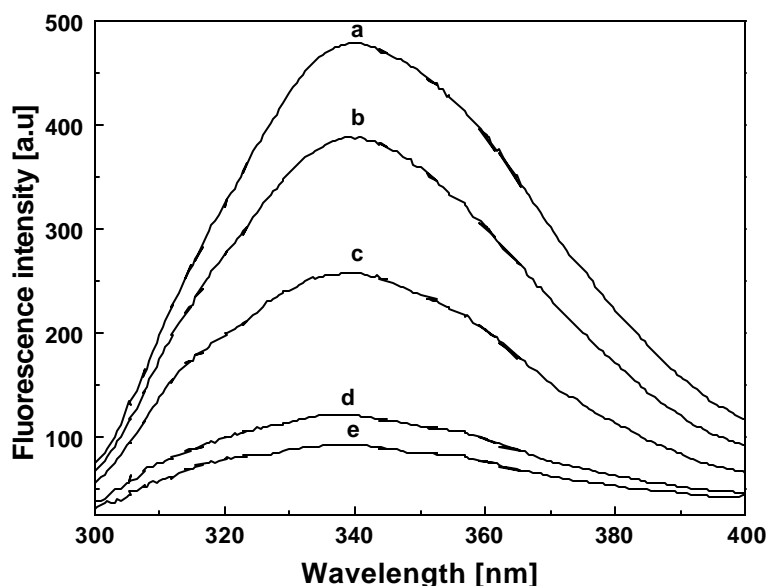
**Figure. 4: Fluorescence titration of API with NBS.**
API (2.25×10^{-3} mM) was excited at 295 nm and the effect of NBS on the fluorescence emission of API from 300 to 400 nm was monitored by the addition of increasing amounts of NBS (10×10^{-3} M). The spectra represent the API to NBS molar ratios of (**a**) 1:0.(**b**) 1:4.6., (**c**) 1: 6.9., (**d**) 1:11.5., and (**e**) 1: 16.2.

Functional role of disulfide linkages for the antiproteolytic and antifungal activity of API

API is a homodimeric protein containing five disulfide linkages. To investigate the functional role of disulfide linkages, API was treated with DTT. The inhibitor lost its antiproteolytic and antifungal activity when treated with DTT (10 mM) at room temperature for 15 min. The secondary structure of DTT-modified API showed a slight increase (13%) in the α-helical content (Fig. 3B) (Table 2). The tertiary and quaternary structure remained unperturbed as revealed by fluorescence (Fig. 3A) and size exclusion HPLC studies

respectively (Fig. 5). The DTT-modified API quenched the tryptophanyl fluorescence of the alkaline protease indicating that the binding coordinates of API are undisturbed by DTT modification. Thus, the modification of API by DTT brings about changes in the functional features rather than in gross conformation.
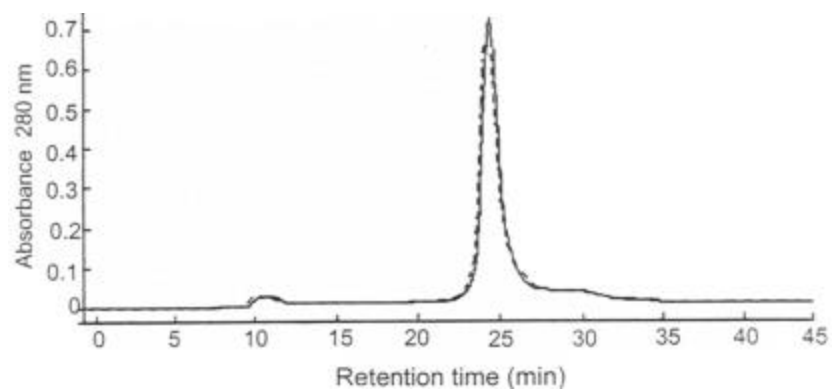
Figure 5: Size exclusion HPLC profile of native and DTT modified API
        The figure shows the elution profile of API on a Protein-Pak 300SW HPLC column pre-
        equilibrated with phosphate buffer (0.05M) pH 7.5. (—), native API and (---), API treated
        with DTT (10mM) for 15min at room temperature

Discussion

Protease inhibitors are widely distributed within the plant kingdom. Some inhibitors are constitutively expressed in seeds and storage organs while others are induced upon wounding in leaves (Green and Ryan, 1972). The protease inhibitors play an important role in the protection of plant tissues from pest and pathogen attack by virtue of an antinutritional interaction. Many insects that feed on plant material possess neutral or alkaline guts and depend predominantly on serine proteases for digestion of food material. Thus, serine protease inhibitors have potential usefulness as defense tools in protecting crop plants against invading pests (Ryan, 1990). Plants producing proteins with the ability to inhibit the growth of fungi *in vitro* have been reported by many workers (Terras, et al., 1992; Linh and Huynh, 1994; Kumari and Chandrasekhar, 1994). Lorito *et al*., in 1994 have reported, for the first time, trypsin and chymotrypsin inhibitors from cabbage foliage exhibiting antifungal activity against *Botrytis cinerea* and *Fusarium solani* f. sp. *pisi* (Lorito *et al.,* 1994). The inhibitors supressed spore germination, germ tube elongation and caused leakage of cytoplasmic contents in an *in vitro* bioassay. Cysteine protease inhibitor from pearl millet with antifungal activity specifically towards *Trichoderma reesei* has been reported (Joshi *et al*., 1998). API displayed potent antifungal activity (*in vitro*) against *F.oxysporum* f. sp. *ciceri. F. oxysporum* is a wilt-causing facultative root pathogen of legumes. It colonizes the conducting strands and blocks them completely resulting in wilting which leads to heavy losses in crop yield. API was also found to inhibit the growth of other fungi such as *Conidiobolus, Alternaria* and *Rhizoctonia.* Thus the bifunctional property of API can be exploited as a biocontrol strategy to enhance resistance against various fungal pathogens.

Understanding the biochemical basis of the antiproteolytic and antifungal activities of API and elucidation of the structure-function relationship of its interaction with the alkaline protease are essential for its use as a candidate bifunctional protein in transgenic research. The co-purification of the antiproteolytic and antifungal activity of API suggested their presence on a single molecule. To delineate the mechanism of protease inhibition and in turn its correlation with the antifungal property, it is important to assign the factors that determine the interaction of API with the target enzyme viz. the alkaline protease. The presence of a Trp residue in the active site of the alkaline protease from *Conidiobolus* has been exploited to monitor the interaction between the enzyme-inhibitor complex. Heating of API, led to a

substantial alteration in its secondary and tertiary structure, with a consequent loss in its capacity to bind to the alkaline protease, culminating in the inactivation of both antiproteolytic and antifungal activities. Chemical modification of API by a Trp specific modifier NBS, resulted in the loss of its functions as a consequence of its inability to form a functional enzyme-inhibitor complex. Thus the Trps of API play an essential role in maintaining the hydrophobicity of the binding pocket and in its efficient binding to the target enzyme.

It has been reported that the Cys^{71} - Cys^{101} disulfide bond of *Streptomyces* subtilisin inhibitor (SSI) present in the vicinity of the reactive site scissile bond Met^{73} - Val^{74}, is more accessible to reduction by DTT than the other disulfide bonds (Uchida *et al*., 1991). The half-reduced SSI retained 65% of inhibitory activity of native SSI and maintained a conformation similar to that of fully oxidized SSI. In order to delineate the functional role of disulfide linkages in API we have reduced API with DTT. DTT-modified API retained its ability to bind with the protease as revealed by reduction in the fluorescence of enzyme-inhibitor complex. The structural hierarchy of DTT-modified API in terms of secondary, tertiary and quaternary structure was virtually unaffected as suggested by CD, fluorescence, and size exclusion chromatography respectively. Kojima *et. al*. have demonstrated the requirement of a disulfide linkage for inhibitory activity of SSI, by eliminating the disulfide bridge near the reactive site and by replacing both Cys residues with Ser residues (Kojima *et al*., 1993). The conformational rigidity owing to the presence of a disulfide bridge near the reactive site has been suggested to be crucial for inhibitory action of the protein. Although, there was no significant structural change in API upon manipulation of the disulfide linkages there was a simultaneous loss of both the antiproteolytic and antifungal activity validating the significance of disulfide linkages for the activity of API. Thus, either a disturbance in the enzyme-binding or catalytic site of API as in NBS- or DTT-modified API respectively or the thermal unfolding of the native 3D structure, which perturbs both the enzyme-binding and catalytic sites, results in the loss of the protease inhibitory activity and in turn its antifungal activity. Our results, therefore, consolidate the functional duality of API to be effectuated through the inhibition of a vital protease.

Studying plant defense responses and devising newer and ecofriendly strategies for plant protection against pests and pathogens is today one of the most dynamic areas of

research in plant science. Identification of novel proteins, which can confer sustained resistance, is an essential prerequisite to the application of gene transfer techniques for development of pest and pathogen resistance in crop plants. There is a considerable interest in using the proteinase inhibitors as potential defense proteins. The proteinase inhibitors used so far in developing insect-resistant transgenic plants were active against the neutral proteinases such as trypsin (Ryan, 1990) whereas mid gut proteinases of major lepidopteran insects such as *Helicoverpa armigera* have been shown to be highly active at pH 10 (Harsulkar *et al*., 1999). The API described in the present studies is active at pH 10 and is stable in a broad pH range of 6 to 12. Its antiproteolytic activity in addition to its antifungal property against important phytopathogenic fungi such as *Fusarium oxysporum* have made API, a novel bifunctional defense protein for its potential use in the transgenic research. The resistance of plants to pest and pathogen attack can be enhanced by expressing foreign protease inhibitors in transgenic plants. Fidelity of such an approach has been demonstrated. Hilder *et al*, have reported a gene encoding a cowpea trypsin inhibitor which when transferred to tobacco, enhanced resistance to herbivorous insect pests (Hilder *et al*, 1987). Broglie *et al*, have shown that transgenic tobacco plants exhibited increased resistance against the fungus *Rhizoctonia solani* by the constitutive expression of genes encoding proteins shown to possess *in vitro* antifungal activity (Broglie *et al*, 1991). The genes encoding Bt insecticidal proteins have been expressed in transgenic tobacco, tomato and cotton plants (Vaeck, *et al*., 1987; Fischhoff, *et al*., 1987; Perlak, *et al*., 1990). A critical factor required for transgenic plants is the proper expresssion of the resistance gene. The *Bacillus* genes are A/T rich while plants tend to have a higher G/C content. Owing to the high A/T content, the Bt genes show reduced expression in plants. Improvement in the expression of insecticidal proteins has been accomplished by increasing the G/C content of their encoding genes and/or by using plant preferred codons (Perlak, *et al*., 1991). The genus *Streptomyces* is known to have a high G/C content. The API having antifungal activity is produced by *Streptomyces sp* NCIM 5127. Therefore, the expression of the gene encoding API in transgenic plants could be achieved effectively to render the plants resistant to phytopathogenic fungi with minimum genetic manipulations.

The API from *Streptomyces* sp. NCIM 5127 is stable over a wide pH and temperature range. Therefore, one can envision the direct application of API as a biocontrol agent for the

protection of plants against phytopathogenic fungi by encapsulation for surface application or can be sprayed directly. The seeds of plants can be protected from fungal pathogen attack during germination in soil by coating them with a formulated preparation of API. Furthermore, the API being of microbial origin offers an attractive and economical process for its rapid and convenient production. Moreover, it would be easier to manipulate the microbial protease inhibitor genes as compared to those from plants.

REFERENCES

Andrade, M. A., Chacon, P., Merelo, J. J., and Moran, F. (1993) *Protein Engg*. **6**, 383-390.

Atkinson, H. J., Urwin, P. E., and Hansen, E. and McPherson, M. J. (1995) *Trends Biotechnol*. **13**, 369--374.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. (1997) *Science* **276**, 726-733.

Bradford, M. N. (1976) *Anal. Biochem*. **72**, 248-254.

Brattsen, L. B. (1991) *Arch. Insect. Biochem. Physiol*. **17**, 253-267.

Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., Desamblanx, G. W., Osborn, R. W. (1997) *Crit Rev Plant Sci* **16**, 297-323.

Chen, Z. Y., Brown, R. L., Lax, A. R., Cleveland, T. E., and Russin, J. S. (1999) *Appl. Environ. Microbiol*. **65**, 1320-1324.

Desiderio, A., Aracri, B., Leckie, F., Mattei, B., Salvi, G., Tigelaar, H., Vanroekel, J. S. C., Baulcombe, D. C., Melchers, L. S., Delorenzo, G., and Cervone, F. (1997) *Mol. Plant–Microbe Interact.* **10**, 852-860.

Dunaevskii, Y., Pavlyukova, E., and Belyakova, G. (1994) *Biochemistry (Moscow)* **59**, 739-743.

Fischhoff, D. A., Bowdish, K. S., Perlak, F. J., Marrone, P. G., McCormick,S. H., Neidermeyer, J. G., Dean, D. A., Kusano-Kretzmer, K., Mayer, E. J.,Rochester, D. E., Rogers, S. G., and Fraley, R. T. (1987) Bio/Tech. 5, 807- 813.

Fritig, B., Heitz, T., and Legrand, M. (1998) *Curr. Opin. Immun.* **10,** 16-22.

Gatehouse, A. M. R., Davison, G. M., Newell, C. A., Merryweather, A., Hamilton, W. D. O., Burgess, E. P. J., Gilbert, R. J. C., and Gatehouse, J. A. (1997) *Mol. Breed.* **3,** 49-63.

Gleddie, S., and Keller, W.A. (1989) *J. Tissue Culture Meth.* **12**, 157-162.

Gleddie, S., and Michaud, D. (2000) Recombinant Protease Inhibitors in Plants, book edited by Dominique Michaud.

Goodenough, P. W., Clark, D. C., Durrant, A. J., Gilbert, H. J., Hazlewood, G. P., and Waksman, G. (1991) *FEBS Lett*. **282**, 355-358.

Green, T. R., and Ryan, C. A. (1972) *Science* **175**, 776-777.

Harsulkar, A. M., Giri, A. P., Gupta, V. S., Sainani, M. N., Deshpande, V. V., Patankar, A. G., and Ranjekar, P. K. (1998) *Electrophoresis* **19**, 1397-1402.

Henskens, Y. M. C., Veerman, E. C. I., and Nieuw, A., Amerongen, A. V. (1996) *Biol. Chem*. **377**, 71-86.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F., and Boulter, D. (1987) *Nature* **300**, 161-163.

Johnson, R., Narvaez, J., An, G., and Ryan, C. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9871-9875.

Joshi, B. N., Sainani, M. N., Bastawade, K. B., Deshpande, V. V., Gupta, V. S., and Ranjekar, P. K. (1999) *Eur. J. Biochem*. **265**, 1-9.

Joshi, B. N., Sainani, M. N., Bastawade, K. B., Gupta, V. S., and Ranjekar, P. K. (1998) *Biochem. Biophys. Res. Commun*. **246**, 382-387.

Knogge, W. (1996) *Plant Cell* **8**, 1711-1722.

Kojima, S., Kumagai, I., and Miura, K. (1993) *J. Mol. Biol*. **230**, 395-399.

Kumari, S. R., and Chandrashekhar, A. (1994) *J. Sci. Food. Agric*. **64**, 357-364.

Kunitz, M. (1947) *J. Gen. Physiol*. **30**, 291-310.

Laskowski, M., and Kato, I. (1980) *Ann. Rev. Biochem*.**49**, 593-626.

Linh Vu, and Huynh, Q. K. (1994) *Biochem, Biophys. Res. Commun*. **202,** 666-672.

Lorito, M., Broadway, R. M., Hayes, C. K., Woo, S. L., Noviello, C., Williams, D. L., and Harman, G. E. (1994) *Mol. Plant. Microbe Interact.* **7**, 525-527.

Merelo, J. J., Andrade, M. A., Prieto, A., and Moran, F. (1994) *Neurocomputing* **6**, 443-454.

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., and Fischhoff, D. A. (1990) Bio/Tech. 8, 939-943.

Perlak, F. J., Fuchs R. L., Dean, D. A., McPheron, S. L., and Fischhoff, D. A. (1991) *Proc. Natl. Acad. Sci. U.S.A*. **88**, 3324-3328.

Perlak, F. J., Fuchs R. L., Dean, D. A., McPheron, S. L., and Fischhoff, D. A. (1991) Proc. Natl. Acad. Sci. 88, 3324-3328.

Roberts, W. K., and Selitrennikoff, C. P. (1986) *Biochem. Biophys. Acta* **880**, 161-170.

Ryan, C. A. (1990) *Ann. Rev. Phytopathol*. **28**, 425-449.

Sato, S., and Murao, S. (1974) *Agri. Biol. Chem*. **38** (3), 587-594.

Schuler, T. H., Poppy, G. M., and Kerry, B. R. (1998) *Trends Biotechnol*. **16**, 168-175.

Shah, D. M., Rommens, C. M. T., and Beachy, R. N. (1995) *Trends Biotechnol*. **13**, 362-368.

Sticher, L., Mauch-Mani, B., and Metraux, J.P. (1997) *Annu Rev Phytopathol* **35,** 235-270.

Tanksale, A. M., Vernekar, J. V., Ghatge, M. S., and Deshpande, V. V. (2000) *Biochem. Biophys. Res. Commun*. **270,** 910- 917.

Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., Van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. (1992) *J. Biol. Chem.* **267**, 15301-15309.

Uchida, K., Miyake, Y., and Kainosho, M. (1991) *J. Biomolecular NMR*. **1**, 49-64.

Vaeck, M.,Reynaerts, A., Hofte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M.,Van Montagu, M., and Leemans, J. (1987) Nature 328, 33-37.

Valueva, T. A., Revina, T. A., Kladnitskaya, G. V., and Mosolov, V. V. (1998) *FEBS Lett*. **426**, 131-134.

Van Loon, L. C., Pierpoint, W. S., Boller, T., Conejero, V. (1994) *Plant Mol. Biol. Rep.* **12**, 245-264.

Vigers, A. J., Roberts, W. K., and Selitrennikoff, C. P. (1991) *Mol. Plant-Microbe Interact.* **4,** 315-323.

Chapter 5

Protein Disulfide Isomerase Accelerated Oxidative Refolding of API:
Effect of Macromolecular Crowding on Refolding Kinetics

Summary

The spontaneous refolding and reactivation of API upon reduction and denaturation occurs to a limited extent mainly due to its propensity to aggregate and its dependence on the regeneration of the native disulfide linkages. With higher concentrations of API, reactivation decreases concomitant with the increase in aggregation as revealed by light scattering and HPLC analysis of the refolding products. Protein disulfide isomerase (PDI), a catalyst of oxidative protein folding accelerates the refolding rates and reactivation yields due to its isomerase activity and chaperone like properties. The oxidative refolding pathway of API has been investigated using PDI. At lower concentrations of API, catalytic amounts of PDI are effective in accelerating the reactivation rates and yields indicating its isomerase function. With higher concentrations of API, reactivation yield decreased and catalytic amounts of PDI failed to promote efficient reactivation. However, at a 10 fold molar excess of PDI, the yield is almost doubled and reactivation rates are also increased indicating that PDI functions as a chaperone preventing aggregate formation and as an isomerase, which promotes the correct formation of disulfide bonds in API. To simulate the intracellular environment, we have also studied the influence of macromolecular crowding agents on the protein refolding kinetics of reduced and denatured API. Concentration dependent decrease in the refolding yields was obtained in the presence of crowding molecules (50-250 g/L). The addition of PDI under crowded conditions counteracted the decrease in reactivation significantly and improved yields and rates of API were obtained. Our results on the chemoaffinity labeling reinforced the role of PDI as a foldase displaying both its chaperone and isomerase activities in the oxidative refolding of API.

Introduction

Understanding the nature of events by which an unfolded protein attains its functional three dimensional structure is the most challenging problems in structural biology today. Folding experiments *in vitro*, using chemically denatured proteins, have demonstrated that the information for the three-dimensional structure of a protein is ultimately determined by its amino acid sequence (Anfinsen, 1973). Indeed, because protein folding translates the linear genetic code into three dimensions, it has been called the second, and more intractable, half of the genetic code. Despite considerable effort expended on understanding the relationship between amino acid sequence and native structure, a comprehensive solution to this problem has remained elusive. The native state is the thermodynamically most stable conformation of a polypeptide. To reach the native state, the unfolded polypeptide does not sample randomly all possible conformations, but rather proceeds via one or more pathway(s) in which rapid formation (on the order of milliseconds) of compact folding intermediates restricts the conformational space available to the polypeptide (Dill and Chan, 1997; Dobson, *et al.*, 1998; Dinner, *et al.*, 2000). It appears that removal of hydrophobic side chains away from the aqueous solution to form the hydrophobic core of the folded protein is a major driving force for formation of folding intermediates. One of the major complications encountered in both *in vivo* and *in vitro* protein folding is that of protein misfolding and aggregation (Goldberg *et al.*, 1991, Dobson and Ellis, 1998). The efficiency of folding depends on the kinetic competition between the pathways leading to correct folding and aggregation (Goldberg *et al.*, 1991). It is now generally well accepted that, in most cases, the folding and assembly of nascent polypeptides to functional proteins in the cell are assisted by a whole array of molecular chaperones (Hartl, 1996, Gething and Sambrook, 1992; Frydman, 2001) and folding catalysts (Gilbert, 1997). *In vivo,* the proper interplay between unfolded proteins, folding catalysts and molecular chaperones normally ensures a proper balance between productive and non-productive pathways. Chaperones increase the folding efficiency by suppressing the tendency of unfolded proteins to aggregate and generally do not increase the overall rate of folding. Folding catalysts, on the other hand act on covalent bonds and accelerate the slow chemical steps such as proline isomerization, disulfide bond formation and rearrangement. The correct formation of disulfide bonds is essential for the proper folding of a large number of proteins. Therefore, the oxidative refolding studies are of considerable importance from a physiological

point of view because of their relevance to the folding of disulfide containing proteins *in vivo*. The folding of small, single domain proteins devoid of disulfide bonds or with intact native disulfide bonds has been studied extensively (Kim and Baldwin, 1990). However there is a relative lack of oxidative refolding data and only a few disulfide containing proteins have been investigated in detail, most remarkably bovine pancreatic trypsin inhibitor (BPTI) (van Mierlo *et al*., 1992; Weissman and Kim, 1991), lysozyme (Radford *et al*., 1992; van den Berg *et al*., 1999), α-lactalbumin (Lindner *et al*., 1997), and ribonuclease A (Rothwarf and Scheraga, 1998).

Both eukaryotes and prokaryotes have evolved systems that catalyze the formation and isomerization of disulfide bonds. The folding catalyst namely protein disulfide isomerase (PDI), a multifunctional 55 kDa protein present in near millimolar concentrations in the lumen of the endoplasmic reticulum, has been studied extensively for its essential role in native disulfide bond formation and rearrangement of secreted proteins (LaMantia and Lennarz, 1993; Freedman *et al*., 1994). PDI has two domains with homology to the small, redox-active protein, thioredoxin. The thiol/disulfide centers of the two thioredoxin-like domains function as two independent active sites (Freedman *et al*., 1994). The PDI catalysed oxidative refolding has been investigated for many monomeric proteins (Tang *et al*., 1994; van den Berg *et al*., 1999; Vinci *et al*., 2000) however, there have been no reports of the role of PDI on the regeneration of disulfide bonds in oligomeric proteins. PDI, in cooperation with its isomerase activity, is also known to display molecular chaperone-like activity by suppressing aggregation and increasing reactivation and therefore referred to as a 'foldase' that promotes protein folding and catalyzes formation of native disulfide bonds (Wang, 1998). The role of PDI as a foldase has been demonstrated during the oxidative refolding of disulfide containing proteins such as lysozyme (Puig and Gilbert, 1994) and acidic phospholipase A_2 ($APLA_2$) (Yao *et al*., 1997) and as a chaperone for proteins without disulfide bonds such as D-glyceraldehyde-3-phoshate dehydrogenase (GAPDH) (Cai *et al*., 1994) and rhodanese (Song and Wang, 1995). The prokaryotic counter part of PDI is the battery of enzymes of the Dsb family that are present in the periplasm of Gram-negative bacteria (Bader *et al*., 1998). DsbA is a protein-folding catalyst that acts as the direct donor of disulfides to newly synthesized periplasmic proteins. DsbC catalyses the rearrangement of disulfide bonds in concert with DsbA for the formation of native disulfides (Chen *et al*., 1999). Recently, a new

member of the Dsb family, DsbG, has been identified and has been shown to possess disulfide isomerase activity (Shao *et al*., 2000). In addition to the isomerase activity the Dsb proteins similar to PDI are also known to display molecular chaperone like properties to promote the proper folding of unscrambled proteins.

The *in vitro* refolding experiments represent a good model for deciphering the mechanisms by which a nascent polypeptide chain achieves its native conformation and is generally performed using dilute protein solutions to prevent aggregation and increase the folding rates (Yon, 2001). Most *in vitro* studies of protein folding have used small, single-domain proteins that undergo cooperative and reversible folding reactions. However, the cellular interior is highly crowded and dynamic environment due to the presence of a large number of soluble and insoluble macromolecules (Ellis, 2001; Minton, 2000). Thus, a significant portion of the cell volume is physically occupied and unavailable to other molecules resulting in steric repulsion and excluded volume effect (Minton, 2001). In order to simulate protein folding *in vivo*, the refolding of proteins *in vitro* in the presence of biologically suitable concentrations of crowding agents will be of physiological relevance and will shed further insight into the intricate details of protein folding in cellular enviroment. The effects of macromolecular crowding on protein folding have been reported for a few proteins (van den Berg *et al*., 2000; Martin and Hartl, 1997; Li *et al*., 2001).

In an attempt to decipher the role of PDI on the refolding of oligomeric proteins, we have investigated the oxidative refolding of the alkaline protease inhibitor. API is a dimeric protein containing five disulfide linkages that are shown to be vital for its catalytic activity (Chapter 4). The spontaneous refolding of reduced and denatured API occured to a limited extent due to its dependence on the correct formation of the essential disulfide linkages and its propensity to aggregate. In the present chapter we have demonstrated that PDI functions as a foldase displaying both its isomerase and chaperone activities in the oxidative refolding of API by accelerating the refolding rates and increasing the reactivation yields by decreasing aggregation. These results were reinforced by the fluorescence chemoaffinity labeling of PDI and its interaction with API. In addition, we have studied the influence of macromolecular crowding agents viz. bovine serum albumin (BSA) and polyethylene glycol 10,000 on the refolding kinetics of API in presence of PDI.

Materials and Methods

Materials

Dithiothreitol (DTT), guanidine hydrochloride (GdnHCl), glutathione reduced (GSH) and oxidized (GSSG), bovine serum albumin (BSA), polyethylene glycol (PEG) 10,000 and protein disulfide isomerase (PDI) from bovine liver were obtained from Sigma Chemical Co., U.S.A. All other reagents used were of analytical grade.

Unfolding and refolding of API

API was completely reduced and denatured by incubating at 37 °C for 4 h in phosphate buffer (0.05 M), pH 7.5, containing DTT (0.02 M) and GdnHCl (6 M). It was thoroughly dialyzed against KCl-HCl buffer (0.1 M), pH 2.0, for 3 h followed by dialysis against 0.01 M of the same buffer for 16 h at 4 °C. The reduced and denatured API was distributed in aliquots and stored at -20°C. The number of thiols in the API was determined using DTNB (Habeeb, 1972). Reactivation was initiated by rapid dilution of API to 100 fold in refolding buffer consisting of 100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.5, in the presence of 1 mM GSH/ 0.5 mM GSSG at 28°C unless otherwise specified [The ratio of GSH to GSSG has been determined to be 2 in the endoplasmic reticulum (Hwang *et al*., 1992) and therefore, the buffer is referred to as a redox buffer]. The stock solutions of GSH/GSSG (50 mM) in water were made freshly prior to use and added to the refolding solution before the addition of API. The effect of PDI on the reactivation of reduced and denatured API was studied by addition of API to refolding solutions containing varying concentrations of PDI. Aliquots of the refolding mixture were removed at different time intervals and assayed for anti-proteolytic activity. The reactivation yields are the averages of at least three different experiments. Reactivation of API in the presence of crowding agents (50-250 g/l) such as bovine serum albumin (BSA) and polyethylene glycol (PEG) in refolding buffer was carried out to mimic the physiological conditions. The kinetics of reactivation of API in the absence and presence of crowding agents and/or PDI were determined at 28°C for 4 h.

Fluorescence measurements

Steady state fluorescence was recorded on a Perkin-Elmer luminescence spectrofluorimeter LS50B at 28°C using a slit width of 5 mm in a 1 cm path length quartz cuvette. The intrinsic fluorescence spectra were recorded at an excitation wavelength of 280

nm and the emission was recorded in the range of 300-500 nm. Measurements were performed in triplicate and suitable baseline corrections were carried out.

Fluorescent chemoaffinity-labeling of protein disulfide isomerase:

PDI (50μM) was modified by treatment with 100 fold molar excess of 5-iodoacetamidofluorescein (5-IAF) in 50mM potassium phosphate buffer, pH 7.5 for 24 h in dark. At pH 7.5 iodoacetamide specifically reacts with free –SH groups. The labeled protein was separated from the free reagent by passage through a column of Sephadex G-10. Fractions showing absorbance at 280 and 490 nm were pooled and concentrated by lyophilisation. The fluorescence spectrum of 5-IAF labeled PDI was monitored at an excitation wavelength of 490 nm and emission wavelength of 527 nm.

PDI (50μM) was treated overnight with 50-fold molar excess of isatoic anhydride (IAN) in 50 mM potassium phosphate buffer, pH 7.5 at room temperature. The excess reagent was removed by passing the mixture through the column of Sephadex G-10. The degree of labeling was determined spectrophotometrically using an extinction coefficient of 4,600 M^{-1} cm^{-1} at 330 nm for the anthraniloyl chromophore (Churchich, 1993).

Measurement of protein aggregation

The aggregation during protein refolding was followed by Rayleigh light scattering experiments with the spectrofluorimeter at 28°C. Both excitation and emission wavelengths were set at 385 nm and the time-dependent change in the scattering intensity was determined.

The refolding products of reduced and denatured API were analyzed by size exclusion HPLC on Protein-Pak 300SW HPLC column (7.8 mm x 300 mm) using a Waters liquid chromatograph. The column was pre-equilibrated with potassium phosphate buffer (0.05 M) pH 7.5 and the samples were analyzed at a wavelength of 280 nm and at a flow rate of 0.5 ml/min.

Results

Reactivation yield as a function of API concentration

The complete reduction and denaturation of API in the presence of 20 mM DTT and 6 M GdnHCl at 37°C was attained after 4 h and resulted in the dissociation of the dimer with the total disruption of the native disulfide bonds as revealed by the DTNB titration, which yielded 12 sulfhydryl groups. The spontaneous reactivation of reduced and denatured API (rd-API) (0.5 µM) initiated by a 100-fold dilution in refolding buffer in the absence of reduced/oxidized glutathione (GSH/GSSG) resulted in a 5-10 % yield of API. A maximum reactivation yield of 40-45% of API was obtained in 3 h in the presence of 1 mM GSH and 0.5 mM GSSG (Fig. 1A). However, higher concentrations of GSH/GSSG failed to enhance the reactivation.
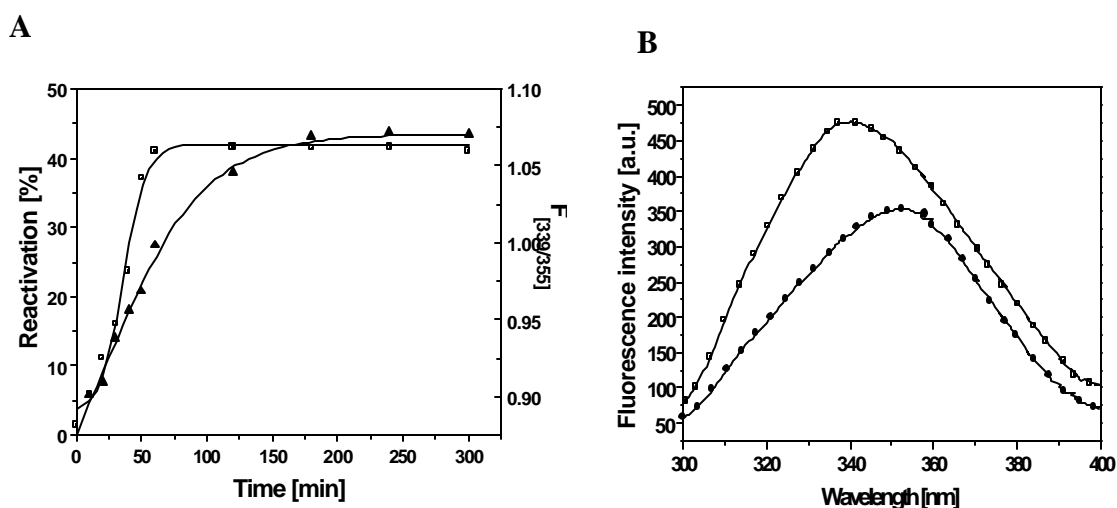

Figure 1: Reactivation and refolding of rd-API.
A. The reactivation of rd-API (0.5 µM) was initiated in redox buffer and the recovery of anti-proteolytic activity (▲) was determined by the caseinolytic assay. The curve represents the best fit of the reactivation data that are expressed as mean ± S. D. (*n* = 3-5). The regain of native tertiary structure of rd-API (1 µM) was monitored by the ratio of the fluorescence emission spectrum at 339 and 355 nm ($F_{339/355}$) (■) at an excitation wavelength of 280 nm. **B.** API (1 µM) was excited at 280 nm and the fluorescence emission spectra were monitored from 300-400 nm, native API (■) and rd-API (●).

The intrinsic fluorescence of API was exploited to investigate the conformational changes induced during the unfolding and refolding pathway. Fluorescence emission

spectrum of native API displays a λ_{max} of 339 nm when excited at 280 nm (Fig. 1B). The rd-API exhibited a prominent red shift of 15 nm and a 25% decrease in fluorescence quantum yield. The ratio of the relative fluorescence intensity at 339 and 355 nm ($F_{339/355}$) of native API is 1.06. A decrease in this ratio is indicative of a red shift due to the unfolding of the tertiary structure and can be used as a probe to monitor changes in the native tertiary structure (Pawar and Deshpande, 2000). Upon spontaneous refolding of rd-API the $F_{339/355}$ required only 50-60 min to attain a constant value of 1.06 corresponding to the native API, however, the inhibitor acquired its maximum activity after 3 h (Fig. 1A). At lower concentrations of rd-API (0.01-0.25 μM), a maximum reactivation yield of 50-55% was obtained. However, the reactivation yield markedly decreased from 55% to 4% as the concentration of API increased from 0.25 to 3 μM (Fig. 2).
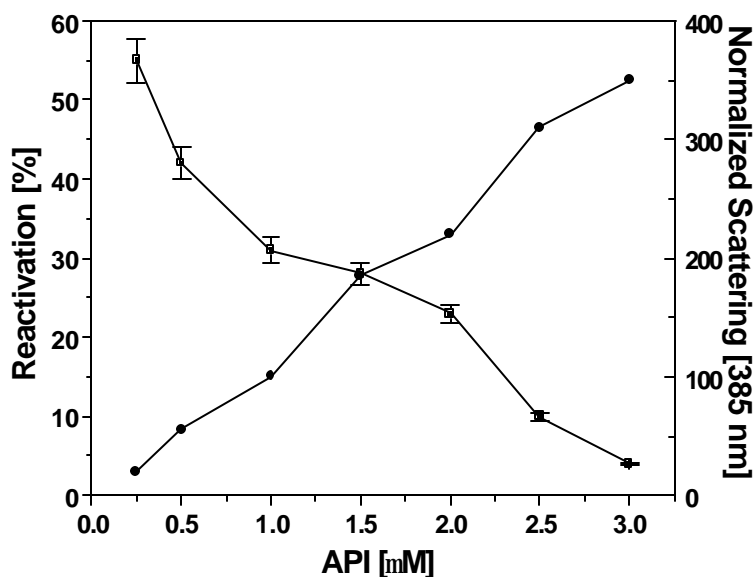

Figure 2: Concentration dependent reactivation of reduced and denatured API.
The concentration dependent reactivation of rd-API was carried out in redox buffer for 3 h. The recovery of inhibitory activity (■) and aggregation by light scattering at 385 nm (●) were determined.

Propensity of rd-API for aggregation

We have utilized the Rayleigh light scattering analysis to monitor the aggregation during the refolding of rd-API. The susceptibility of API to aggregate as probed by the light scattering experiments increased with increasing protein concentration (0.25-3 μM) and

resulted in the decrease in the reactivation yield (Fig. 2). In spite of very weak aggregation of rd-API observed in the concentration range of 0.1-0.5 µM, the reactivation yields were only 45-55% (Fig. 2). Therefore, the refolding products of rd-API (0.25) µM were analyzed by gel filtration using HPLC. Native API appeared as a dimer of 28 kDa (Fig. 3, peak a) with a retention time of 20.5 min, while rd-API appeared as a monomer of 13.5 kDa (Fig. 3, peak b), with a retention time of 24.8 min. The elution profile of rd-API analyzed after 3 h of refolding showed peaks corresponding to the position of native dimer, unfolded monomer and an additional peak (Fig. 3, peak c) in the void volume with the retention time of 11.6 min. This additional peak may be generated due to the formation of heterogeneous oligomeric folding products that are not large enough to be detected by simple light scattering. The ratio of the oligomeric refolding products to the refolded and unfolded API was 10:55:35, which serves to
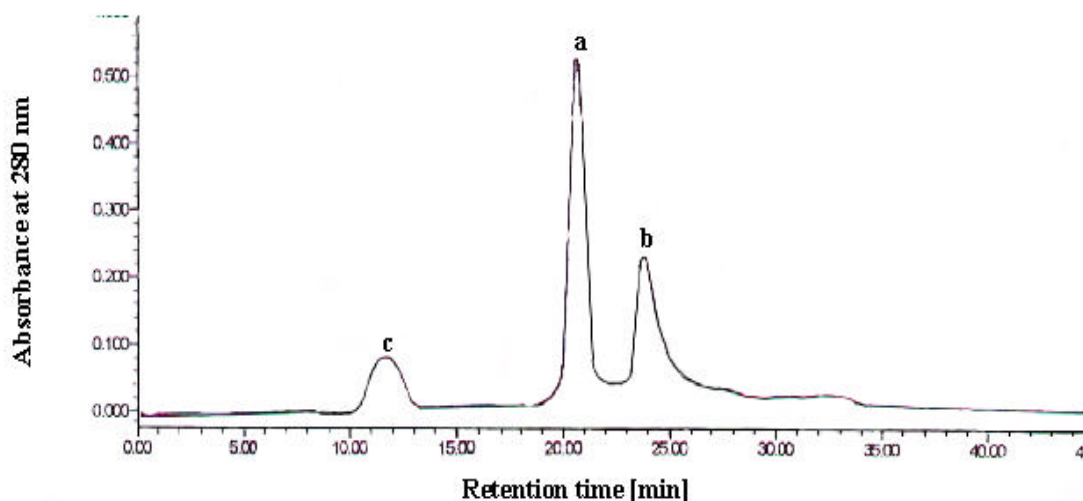

implicate that the decreased reactivation yield in the concentration range of 0.1-0.5 µM of API can be partially attributed to the formation of inactive soluble oligomeric folding products.

Figure 3: Analysis of the refolding products of API.
The elution profile of the refolding products of API (0.25 µM) was analyzed on a Protein-Pak 300SW HPLC column at 28°C after 3 h of initiation of refolding in redox buffer, the peak corresponding to native API, (a); unfolded monomer (rd-API), (b); and oligomeric aggregates (c).

PDI-accelerated refolding of rd-API

The uncatalyzed spontaneous refolding of rd-API led to only a partial recovery of the inhibitory activity. The protein folding catalyst PDI dramatically influenced the reactivation and refolding of rd-API. The refolding of lower concentrations of rd-API (0.01-0.1 µM) showed only 50-55% recovery although they were not prone to aggregate (Fig 2). The presence of only a catalytic amount of PDI (0.025 µM) significantly increased the recovery of
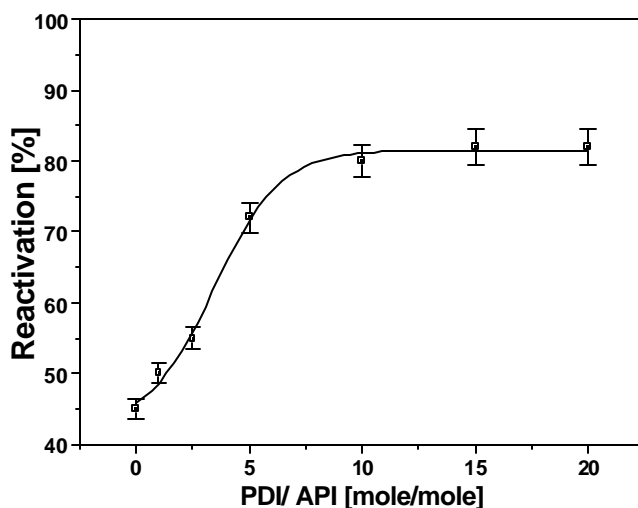
rd-API (0.05 µM) to 90%. Surprisingly, at higher concentrations of rd-API (0.5-3 µM) even a stoichiometric amount of PDI was unable to promote reactivation substantially. However, PDI at a large stoichiometric excess increased the recovery of rd-API (0.5 µM) from 45% to 85% (Fig 4).
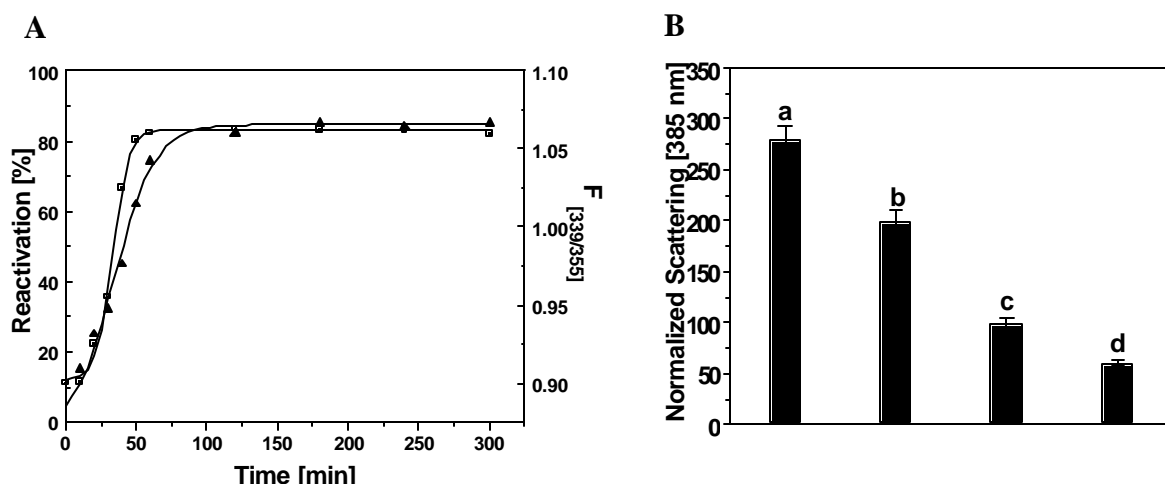
Figure 4: Effect of PDI on the reactivation of rd-API.
Reactivation of rd-API (0.5 µM) in the presence of increasing amounts of PDI was carried out in redox buffer for 3 h and the recovery of inhibitory activity (■) was determined.

A 10-fold molar excess of PDI accelerated the rate of reactivation of rd-API and doubled the reactivation yield as compared to the un-assisted reactivation (Fig. 5A). The regain in the $F_{339/355}$ required almost the same time during catalyzed and uncatalyzed refolding of API although the reactivation was only partially achieved in the uncatalyzed refolding. These observations revealed that the presence of PDI facilitated a faster

isomerisation of the disulfide linkages to regenerate the native disulfides and resulted in increased reaction rates leading to enhanced reactivation yields.

As revealed by the light scattering, the spontaneous uncatalyzed oxidative refolding of rd-API (2 µM) resulted in the formation of aggregates leading to a low recovery of 23%. In concert with its isomerase function, PDI is known to exhibit chaperone-like property by suppressing aggregation. The efficiency of PDI to suppress aggregation of rd-API increased with increasing PDI/rd-API ratios and was in agreement with the increase in reactivation (Fig. 5B). At higher concentrations of rd-API, only a large molar excess of PDI was able to prevent irreversible aggregation and misfolding, which resulted in increased reactivation indicating that it functions as a chaperone. These observations indicated that together with the increase in the reactivation yields, acceleration of the reaction rates by PDI is essentially dependent on its isomerase activity, which assists in the slow chemical steps of disulfide bond formation and rearrangement in API in conjunction with its chaperone activity.


Figure 5: Influence of PDI on the refolding and aggregation of reduced and denatured API.
    A, The time course of reactivation and refolding of rd-API (0.5 µM) in presence of PDI (10 µM) was performed in redox buffer at 28°C. The recovery of inhibitory activity (▲) and the $F_{339/355}$ (■) were monitored. **B**, The aggregation of rd-API (2 µM) in the presence of varying API/PDI ratios was monitored by light scattering at 385 nm. The molar ratios of rd-API to PDI were **a**, 1: 0; **b**, 1:1; **c**, 1: 5 and **d**, 1: 10.

Interaction of fluorescent labeled PDI with rd-API

PDI contains two active site cysteine residues, which are known to function in thiol/disulfide exchange for the regeneration of native disulfide linkages (Freedman *et al.,* 1994). In an attempt to decipher the mechanism of isomerase and chaperone functions of PDI, we have differentially labeled PDI using the chemoaffinity fluorescent labels 5-iodoacetamidofluorescein (5-IAF) and isatoic anhydride (IAN). 5-IAF is known to specifically react with the free SH groups of proteins (Tanksale *et al*., 2000). The reactivation of rd-API in the presence of 5-IAF labeled PDI (IAF-PDI) was only 45 %. Thus, IAF-PDI failed to exhibit its isomerase activity due to the modification of the essential cysteine residues as revealed by the inability of IAF-PDI to enhance the reactivation of rd-API as compared to the native PDI. To investigate the conformational interactions between the PDI and substrate protein, we have analyzed the fluorescence emission spectrum of IAF-PDI-rd-API complex. The IAF-PDI displays a λ_{max} at 527 nm, which does not interfere with that of rd-API since rd-API does not emit at this wavelength. Therefore, the changes in the emission spectra of IAF-PDI will exclusively reflect the conformational changes due to the binding of the substrate protein. As revealed from the emission spectrum of the complex (Fig. 6A), there is rapid quenching in the fluorescence yield at the λ_{max} indicating the binding of labeled PDI with the rd-API. The binding of rd-API to the PDI indicated its intact chaperone function, however, the absence of further reactivation of API, suggested the loss of its isomerase activity.
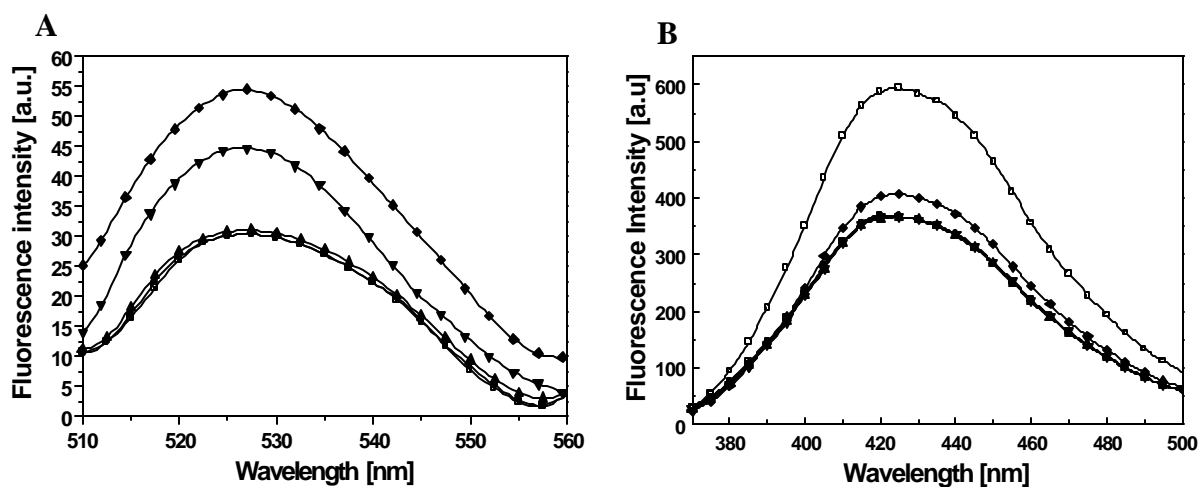
Figure6: Interaction of chemoaffinity-labeled PDI with rd-API.
   A, PDI labeled with 5-IAF (IAF-PDI) (◆) was excited at a wavelength of 490 nm and the time dependent change in fluorescence emission at 527 nm was monitored during its interaction with rd-API. Fluorescence spectrum of IAF-PDI-rd-API complex after, 5 min (▼); 10 min (▲); 15 min (●) and 20 min (■). **B,** PDI was labeled with isatoic anhydride (IAN-PDI) (□) and excited at a wavelength of 330 nm and the time dependent change in fluorescence emission at 420 nm was monitored in the presence of rd-API. IAN-PDI-rd-API complex after, 5 min (◆); 10 min (▼); 15 min (▲); 20 min (●) and 30 min (■).

These results were further corroborated by labeling of PDI with another chemoaffinity fluorescence label isatoic anhydride (IAN). IAN reacts with the nucleophilic groups of the proteins to yield o-aminobenzoyl protein conjugates (Churchich, 1993). The derivatized proteins exhibit an absorption band centered at 330 nm together with an emission band covering the spectral range of 360-500 nm. The emission maximum of the IAN-labeled PDI (IAN-PDI) exhibited a λ_{max} at 420 nm (Fig. 6B). The intact chaperone activity of IAN-PDI was demonstrated by its capacity to bind with the rd-API, and subsequent fluorescence quenching of the IAN-PDI-rd-API complex. Accelerated reactivation of rd-API (70 %) in the presence of IAN-PDI indicated its functional isomerase activity.

Kinetics of reactivation of rd-API under crowded conditions in the presence of PDI

In vitro reactivation of rd-API was carried out in the presence of crowding agents to mimic the intracellular environment. Under crowded conditions, the reactivation yields of API decreased substantially. Addition of BSA in the concentration range of 50-150 g/L to the refolding mixture of rd-API, did not affect the reactivation yields (Fig. 7), however, higher concentrations of BSA resulted in the decrease in the formation of correctly folded API as revealed by the decrease in the reactivation yields. In contrast, in the presence of PEG, a concentration-dependent decrease in the reactivation yields was obtained from 45% to 15%.
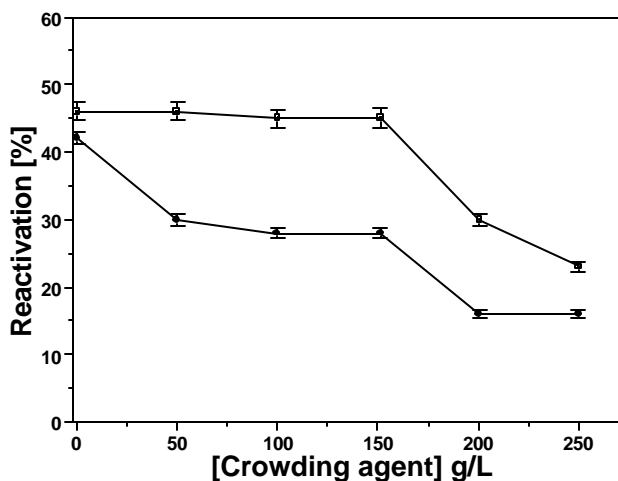
Figure 7: Effect of macromolecular crowding agents on the reactivation of API.
The reactivation of rd-API was carried out in redox buffer at 28°C in the presence of increasing concentrations of BSA (■) and PEG (●) as crowding agents.

This indicated that BSA and PEG differentially influence the reactivation yields of rd-API. In order to understand the influence of these crowding agents on the refolding rates, the kinetic analysis of the refolding of rd-API (0.5 μM) was carried out in the presence of BSA and PEG. The time course of rd-API reactivation in redox buffer followed first-order reaction kinetics with a rate constant of 0.60 s^{-1} with a maximum yield of 44 % (Table I). It was evident that the presence of crowding agents did not alter the order of the reaction but decreased the reactivation rates and yields. In presence of 150 g/L BSA the reactivation yield remained unchanged, but the rate constant decreased to 0.38 s^{-1} (Fig. 8A). Addition of higher concentrations of BSA (250 g/L), revealed a lag phase with no reactivation for the initial 60 min, however, after 180 min only, a maximum recovery of 20% was obtained with a rate constant of 0.34 s^{-1}. On the contrary, in the presence of PEG, the reaction rates increased significantly though a substantial decrease in the reactivation yields was obtained with increasing concentrations of PEG (Fig. 8B). The reactivation yields decreased to 33 % in the presence of 150 g/L PEG and only a 15 % recovery was obtained with 250 g/L PEG.

Table I
Influence of PDI on the reactivation kinetics of API in the presence of crowding agents

Crowding Agent	Concentration [g/L]	Reactivation Yield [%]		Rate constant [s^{-1}]	
		- PDI	+ PDI	- PDI	+ PDI
	0	44.5 ± 1.5	85.5 ± 2.5	0.60 ± 0.05	1.20 ±0.05
BSA	150	42.5 ± 1	66 ± 2	0.40 ± 0.03	0.90 ±0.05
BSA	250	20.0 ± 1.0	44.5 ± 1.5	0.35 ± 0.02	0.80 ± .04
PEG	150	33.0 ± 1	47 ± 2.5	0.65 ± 0.05	1.4 ± 0.07
PEG	250	15 ± 0.5	26.5 ± 1.5	0.80 ± 0.05	1.6 ± 0.07

Experiments were carried out as described in the legend to Fig. 8. Data are expressed as mean ± S. D. (*n*= 3-5)

The role of PDI in assisting the refolding rates of rd-API under crowding conditions was analyzed. Addition of PDI (10 µM) markedly accelerated the reactivation rates and considerably increased the yields in the presence of crowding agents. Moreover, the addition of PDI distinctively enhanced the reactivation in the initial slow phase and reduced the reactivation time from 240 min to 70-80 min. In the presence of BSA, both the reactivation yields and rates of API almost doubled by the addition of PDI (Table I) (Fig. 8C). Although PDI had a dramatic effect on the rate constant, it resulted only in a partial recovery of activity (Fig. 8D) in the presence of PEG.
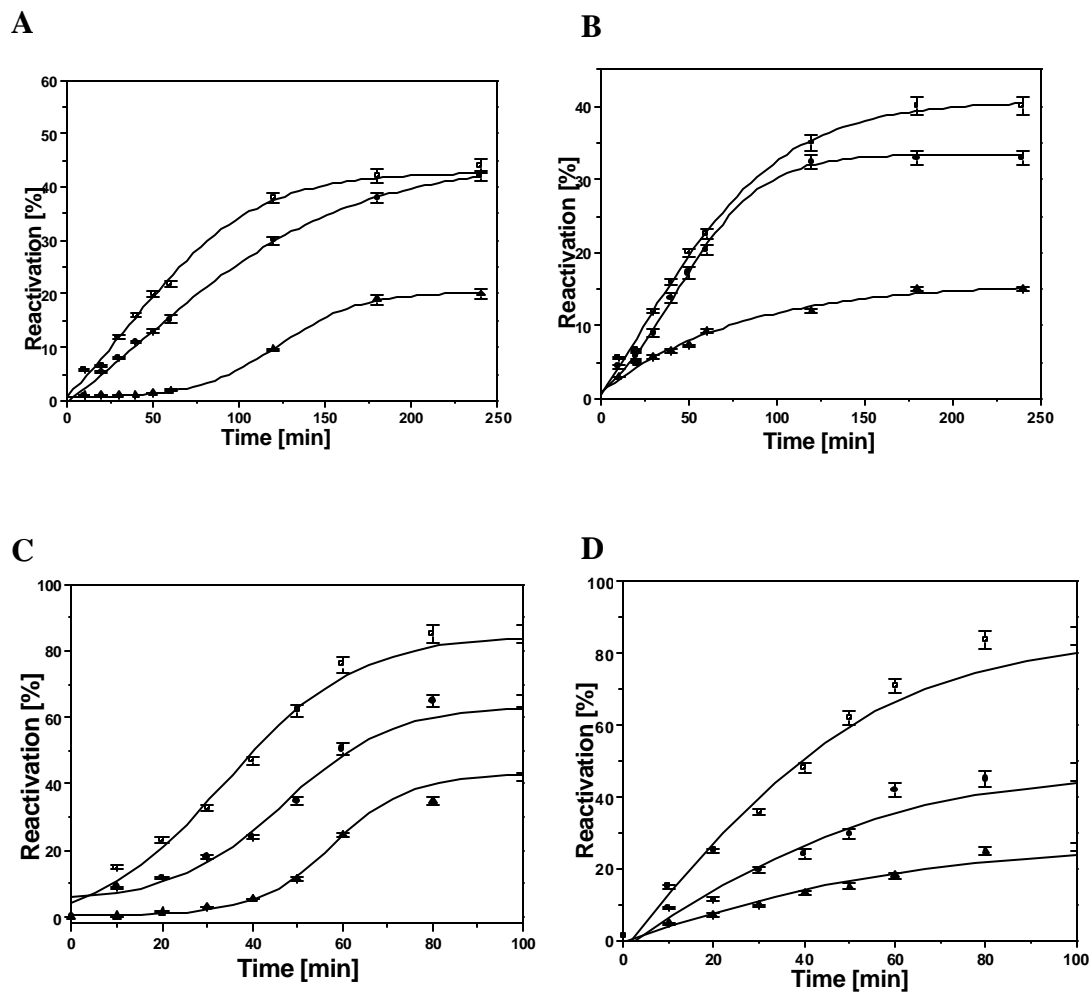
Figure 8: Kinetics of reactivation of API under crowded conditions in presence and absence of PDI.

The time course of reactivation of rd-API (0.5 µM) in redox buffer at 28°C was carried out under crowded conditions in the presence and absence of PDI. **A**, in presence of BSA at 0 (■), 150 (●) and 250 (▲) g/L. **B**, in presence of PEG at 0 (■)., 150(●) and 250 (▲) g/L. **C**, in presence of PDI (10 µM) and BSA at 0 (■)., 150 (●) and 250 (▲) g/L and **D**., in presence of PDI (10 µM) and PEG at 0 (■)., 150 (●) and 250 (▲) g/L. The reactivation data are expressed as mean ± S. D. (*n* = 3-5).

Discussion

Disulfide bonds confer conformational and thermodynamic stability to proteins and are essential for their biological activity (Woycechowsky and Raines, 2000). Formation/isomerisation of disulfide bonds is the rate-limiting step and is therefore critical for the proper folding of proteins. The two processes of disulfide formation and peptide folding are intimately interdependent and work in cooperation for the generation of the native conformation of disulfide-containing proteins (Wang, 1998). Delineating the mechanistic details underlying the efficient oxidative refolding of proteins appears to be an important consideration for defining how proteins fold *in vivo*. Although the oxidative refolding of a handful of small single domain proteins has been reported, there is a paucity of reports describing the refolding of oligomeric proteins containing disulfides linkages. API being a bifunctional inhibitor exhibiting antiproteolytic and antifungal activities it is essential to elucidate the folding pathways that lead to the formation of API in order to gain better insights into its functional mechanisms, therefore, we have studied the oxidative refolding of the dimeric API. Being a dimeric protein the complete reactivation of the reduced and denatured API (rd-API) depends on the association of the monomers in concert with the correct formation of the native disulfide linkages. The spontaneous refolding of API upon dilution into refolding buffer resulted only in partial reactivation. At high API concentrations, refolding yields dropped rapidly concomitant to its propensity to aggregate. Reduced and denatured disulfide containing proteins have a tendency to aggregate during refolding due to non-productive hydrophobic interactions or incorrect non-native disulfide bond formation (Yao, 1997). As the concentration of API decreased weak aggregation is observed due to the formation of soluble aggregates of oligomeric incorrectly folded inactive molecules that cannot be detected by light scattering. The incomplete reactivation of API thus can be attributed mainly to a competition between correct folding and aggregation. Surprisingly, very low concentrations of API that are not prone to aggregation still result in incomplete reactivation although the native tertiary structure is regained within 50 to 60 minutes of refolding. The correct formation of disulfide linkages is crucial for reactivation due to their essential role in catalysis. Therefore, the partial recovery may be due to improperly formed scrambled non-native disulfide linkages and thus may require the assistance of folding catalysts and chaperones.

To verify this notion, attempts were made to refold rd-API in the presence of protein disulfide isomerase (PDI), the oxidative protein folding catalyst (Gilbert, 1997). Refolding of API in the presence of PDI radically improved both yields and rates of refolding as compared to the uncatalyzed refolding indicating an acceleration of thiol/disulfide interchange enhancing the formation of native conformation. The reactivation of dilute API that is not susceptible to aggregation was fully achieved with catalytic amounts of PDI indicating that its isomerase activity was responsible for its efficient refolding. However, with increasing API concentration, stoichiometric amounts of PDI were unable to enhance the refolding efficiency substantially revealing that only isomerase activity of PDI was inadequate. Interestingly, the refolding yields increased exponentially as a function of PDI concentration concomitant with the suppression of aggregation suggesting that PDI also functions as a chaperone. Simultaneously there was an increase in the reactivation rates implying that both chaperone and isomerase activities of PDI operate synergistically to effect efficient refolding of API. The molecular chaperones are the functional class of proteins known to stabilize unfolded and partially folded proteins by interaction through non-specific peptide binding sites to suppress aggregation of denatured proteins with exposed hydrophobic residues (Gething and Sambrook, 1992). PDI along with its two thioredoxin-like catalytic active sites also has a non-specific peptide/protein-binding site (Klappa *et al*., 1998) and at high concentrations, is shown to function like a molecular chaperone (Puig *et al*., 1994). It is therefore referred to as a foldase consisting of both isomerase and chaperone activities (Wang, 1998). The isomerase-independent chaperone activity of PDI has been shown by increasing reactivation yield and decreasing aggregation during refolding of denatured D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cai *et al*., 1994) and rhodanese (Song and Wang, 1995), both of which are devoid of disulfide linkages. The chaperone activity of PDI greatly increases its efficiency as a foldase in promoting protein folding and in catalyzing the formation of native disulfide bonds. It has been reported that PDI assists the oxidative refolding and reactivation of denatured and reduced acidic phospholipase A_2, a monomeric snake venom protein containing seven disulfide bonds (Yao *et al*., 1997). They showed that 90% of the native PDI present in the refolding buffer could be substituted by modified PDI with only chaperone activity and devoid of isomerase activity, indicating that the *in vitro* action of PDI as a foldase consists of both isomerase and chaperone activities. Our results indicated that the presence of

PDI at super stoichiometric concentrations significantly suppressed aggregation exhibiting a chaperone-like activity that diverts API towards productive folding. The efficiency in the suppression of aggregation increases with increasing amounts of PDI and is in agreement with the increase of reactivation of API. Our results showed that for the refolding of API, catalytic amounts of PDI are sufficient to act as an isomerase but required in a large molar excess to act as a chaperone for preventing aggregation and to direct correct folding. These results serves to implicate that as a foldase, PDI displays both isomerase and chaperone activities towards the dimeric API.

Conformational integrity of the active site of an enzyme is essential for its catalysis and investigations on the molecular orientation of the functional groups of active site as well as their microenvironment are the areas of growing scientific interest. Chemo-affinity labeling is a powerful technique to assign the binding sites of enzyme-substrate complexes (Sen *et al.*, 2001), which we have utilized to understand the functional mechanism involved in the interaction of PDI with rd-API. The cysteine specific label 5-IAF modifies the essential redox-active Cys groups at the active sites of PDI and specifically abolished its isomerase activity. The chaperone function of IAF-PDI was unaffected by labeling as revealed by the quenching of fluorescence indicating its binding to rd-API. However, IAF-PDI failed to enhance the reactivation of rd-API signifying that the isomerase activity of PDI is indispensable for reactivation of API. The labeling of PDI with isatoic anhydride (IAN) further substantiated its role as a foldase, wherein the functional integrity of PDI in terms of its chaperone and isomerase functions remained unperturbed due to labeling as demonstrated by the fluorescence quenching of IAN-PDI due to binding of rd-API together with the enhanced reactivation rates and yields of rd-API.

Based on our foregoing results, we propose a representative model of the refolding pathways of rd-API. The schemes illustrate the spontaneous oxidative refolding of rd-API in presence of oxidant initiated by the association of two unfolded or partially folded monomers (U) to form a dimeric transient folding intermediate (D_2). D_2 folds to the native state N_2 via conformational changes involving folding step(s) and disulfide bond formation though with only partial reactivation. Scheme I depicts, the refolding of rd-API at lower concentrations, wherein the intermediate D_2 partitions between two alternative fates, viz. (i) refolding to N_2 and (ii) the formation of non-native disulfides D_{NDS}, yielding incomplete reactivation.

Addition of a catalytic amount of PDI results in the formation of PDI-API complex and PDI promotes the complete reactivation of API through its isomerase activity.

Scheme I. Refolding of rd-API at lower concentrations:
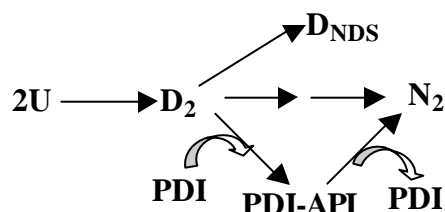
Scheme II. Refolding of rd-API at higher concentrations:
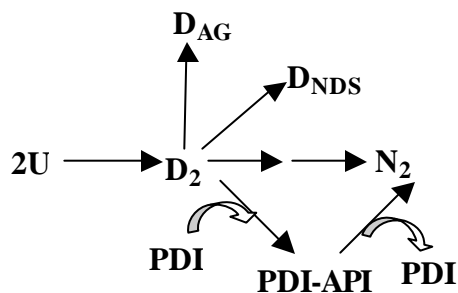
Scheme II shows the refolding of rd-API at higher concentrations, where the intermediate D_2 encounters three competitive pathways, viz. refolding to the native N_2 and the two non-productive pathways leading to the formation of intermediates containing (i) non-native disulfides D_{NDS} and (ii) misfolded aggregates D_{AG}. The addition of PDI to D_2 in stoichiometric excess results in the formation of a PDI-API complex and directs the subsequent refolding of complex PDI-API to native API (N_2). Thus, PDI functions as a foldase displaying both chaperone and isomerase activities efficiently.

In contrast to the *in vitro* refolding conditions wherein the studies are performed using dilute protein solutions, the intracellular environment is highly crowded with macromolecules. It has been shown both theoretically and experimentally that a high

concentration of background macromolecules in the physiological milieu results in volume exclusion and profoundly influences the kinetics and equilibrium of isomerization and association reactions involved in protein folding and assembly (Ellis, 2000). There are two opposite effects of excluded volume on reaction rates (Minton, 2001). If the overall rate of the reaction is limited by the rate at which two reactant molecules encounter each other through diffusional motion, then crowding results in the retardation of diffusional motion and therefore lower reaction rates are obtained. However, if the overall rate of the reaction is limited by the rate at which a transition state complex decays to products, then crowding would facilitate the formation of the transition state by increasing the association. The self-association of monomeric subunits at equilibrium will result in productive reactivation while the non-specific hetero-association will result in aggregation and misfolding. In the present study, we have described the effect of two different crowding agents namely PEG and BSA at concentrations (50-250 g/L) that mimic the intracellular environment, on the reactivation kinetics of API. Increasing concentrations of PEG dramatically enhanced the reactivation rates of rd-API but resulted in low reactivation yields. The crowding theory proposes that macromolecular crowding increases the magnitude of aggregation of proteins specially those that are prone to aggregate during folding. Recently, it has been reported that the refolding of denatured and reduced lysozyme in the presence of crowding agents increased the rates of refolding and was therefore in accord with the excluded volume theory primarily due to increased association rate constants (van den Berg *et al*., 1999). However, the correct refolding of reduced lysozyme was prevented and therefore low refolding yields were obtained. The loss of renaturability was attributed to the enhancement of quasi-irreversible aggregation of unfolded or partially unfolded lysozyme in crowded media. Our results under crowding environment substantiate the excluded volume effects of PEG during the refolding of API that lead to increased association constants resulting in aggregation and decreased reactivation. The influence of a protein crowding agent (BSA) on the refolding of API, however exhibited a different picture, indicating that the effect of crowding on the refolding of proteins are large and diverse, and differ from protein to protein. The first order reactivation kinetics and yield of API remained unaffected, however decreased reaction rates were observed in the presence of BSA. Similar results were obtained during the refolding of glucose-6-phosphate dehydrogenase (G6PDH) and PDI, wherein slower refolding rates were

obtained with high concentrations of crowding agents although the reactivation yields remain unchanged (Li *et al*., 2001). During the GroEL-assisted refolding of rhodanese in the presence and absence of *Xenopus* oocyte extract, comparable refolding yields were obtained but the refolding rate was slower (Burston *et al*., 1996). The effect of crowding on reaction rate is thus complex and depends crucially on the nature of the reaction and on the concentration of crowding agent. As the reactivation kinetics of API, in presence of BSA follows the first order reaction, it indicates the reaction to be transition state limited with the conversion of D_2 to N_2 as the rate-limiting step. Increasing concentrations of BSA decreased both the yields and rates of reactivation. It has been proposed that even for any bimolecular transition state limited reaction, the maximal reaction rate that can be achieved finally depends on the encounter rate of the components and therefore will eventually fall when the concentration of crowding agent becomes too large (Ellis, 2001). The increasing concentrations of BSA also result in the high volume occupancy together with the more specific protein-protein interaction leading to aggregation that might have decreased the productive refolding of API. The addition of PDI, considerably improved the reactivation yields of API in the presence of both the crowding agents. The effectiveness of PDI in improving the recovery of lysozyme at high concentrations of crowding molecules was attributed primarily to its chaperone function (van den Berg *et al*., 1999). In addition, PDI strikingly increased the reaction rates of API and the initial lag phase was essentially abolished during the reactivation in the presence of high concentrations of crowding agents. GroEL/MgATP was shown to accelerate the refolding of G6PDH under crowded conditions and eliminated the initial slow phase (Li *et al*., 2001). Although the two crowding agents influence the reactivation kinetics of API variably, PDI is able to counteract the effects of crowding and enhance both the yield and rates of refolding. PDI is a resident protein of the secretory pathway present in near millimolar concentrations in the lumen of the endoplasmic reticulum and is known to interact with nascent and newly translocated secretory proteins. The lumen of the ER consists of a large number of macromolecules, therefore the refolding of nascent polypeptides chains to functional proteins in the crowding milieu of the ER can be effectively accomplished by PDI employing both its chaperone and isomerase functions. Our results reinforce the role of PDI as a protein folding catalyst for not only small single domain proteins but also its effective role to accelerate the reactivation of dimeric/oligomeric proteins as in the case of the dimeric API.

References

Anfinsen, C. B. (1973) *Science* **181**, 223–30.

Bader, M., Muse, W., Zander, T. and Bardwell, J. (1998) *J. Biol. Chem.* **273,** 10302-10307.

Burston, S. G., Weissman, J. S., Farr, G. W., Fenton, W. A. and Horwich, A. L. (1996) *Nature*, **383**, 96-99.

Cai, H., Wang, C.C. and Tsou, C.L. (1994) *J. Biol. Chem.* **269,** 24550-24552.

Chen, J., Song, J., Zhang, S., Wang, Y., Cui, D. and Wang, C. C. (1999) *J. Biol. Chem.* **274**, 19601-19605.

Churchich, J. E. (1993) *Anal. Biochem.* **213**, 229-233.

Dill, K. A., and Chan, H. S. (1997) *Nat. Struct. Biol.* **4**, 10–19.

Dinner, A. R., Sali, A., Smith, L. J., Dobson, C. M., and Karplus, M. (2000) *Trends Biochem. Sci.* **25**, 331–39.

Dobson, C. M., Sali, A., and Karplus, M. (1998) *Angew. Chem. Int. Ed. Engl.* **37**, 868–93.

Dobson, C. M. and Ellis, R. J. (1998) *EMBO J.* **17,** 5251-5254.

Ellis, R. J. (2001) *Curr. Opin. Struct. Biol.* **11,** 114-119.

Freedman, R. B., Hirst, T. R. and Tuite, M. F. (1994) *Trends. Biochem. Sci.* **19,** 331-336.

Frydman, J. (2001) *Annu. Rev. Biochem*. **70**, 603–47.

Gething, M. J. and Sambrook, J. (1992) *Nature* **355,** 33-45.

Gilbert, H.F. (1997) *J. Biol. Chem.* **272,** 29399-29402.

Goldberg, M. E., Rudolph, R. and Jaenicke, R. A. (1991) *Biochemistry* **30,** 2790-2797.

Habeeb, A. F. S. A. (1972) *Methods Enzymol*. **25**, 457-464.

Hartl, F. U. (1996) *Nature* **381,** 571-580.

Hwang, C., Sinskey, A. J. and Lodish, H. F. (1992) *Science* **257,** 1496-1501.

Kim, P.S. and Baldwin, R. L. (1990) *Annu. Rev. Biochem.* **59,** 631-660.

Klappa, P., Ruddock, L. W., Darby, N. J. and Freedman, R. B. (1998) *EMBO J.* **17,** 927-935.

Kunitz, M. (1947) *J. Gen. Physiol*. **30**, 291-310.

LaMantia, M. and Lennarz, W. J. (1993) *Cell* **74,** 899-908.

Li, J., Zhang, S. and Wang, C. C. (2001) *J. Biol. Chem.* **276,** 34396-34401.

Lindner, R. A., Kapur, A. and Carver, J. A. (1997) *J. Biol. Chem.* **272,** 27722-27729.

Martin, J. and Hartl, F. U. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1107-1112.

Minton, A. P. (2000) *Curr. Opin. Struct. Biol.* **10,** 34-39.

Minton, A. P. (2001) *J. Biol. Chem.* **276,** 10577-10580.

Pawar, S. A. and Deshpande, V. V. (2000) *Eur. J. Biochem.* **267,** 6331-6338.

Puig, A. and Gilbert, H. F. (1994) *J. Biol. Chem.* **269,** 7764-7771.

Radford, S. E., Dobson, C. M. and Evans, P. A. (1992) *Nature* **358,** 302-307.

Rothwarf, D. M., Li, Y. J. and Scheraga, H. A. (1998) *Biochemistry* **37,** 3767-3776.

Sen, A., Chen, Y. –D., Yan, B. and Chalovich, J. M. (2001) *Biochemistry* **40,** 5757-5764.

Shao, F., Bader, M. W., Jakob, U. and Bardwell, J. C. A. (2000) *J. Biol. Chem.* **275,** 13349-13352.

Song, J. L. and Wang, C.C. (1995) *Eur. J. Biochem.* **231,** 312-316.

Song, J. L., Quan, H. and Wang, C.C. (1997) *Biochem. J.* **328,** 841-846.

Tang, B., Zhang, S. Z. and Yang, K. Y. (1994) *J.* **301**, 17-20.

Tanksale, A. M., Vernekar, J., Ghatge, M. S., and Deshpande, V. V. (2000) *Biochem. Biophys. Res. Commun.* **270,** 910-917.

van den Berg, B., Chung, E. W., Robinson, C.V. and Dobson, C. M. (1999) *J.Mol. Biol.* **290,** 781-796.

van den Berg, B., Chung, E. W., Robinson, CV., Mateo, P. L. and Dobson, C. M. (1999) *EMBO J.* **18,** 4794-4803.

van den Berg, B., Wain, R., Dobson, C. M. and Ellis, R. J. (1999) *EMBO J.* **18,** 6927-6933.

van den Berg, B., Wain, R., Dobson, C. M. and Ellis, R. J. (2000) *EMBO J.* **19,** 3870-3875.

van Mierlo, C. P. M., Darby, N. J. and Creighton, T. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6775-6779.

Vinci, M., Ruoppolo, M., Pucci, P., Freedman, R. B. and Marino, G. (2000) *Protein Science* **9,** 525-535.

Wang, C. C. (1998) *Biochemistry (Moscow)* **63,** 407-412.

Weissman, J. S. and Kim, P. S. (1991) *Science* **253,** 1386-1393.

Woycechowsky, K. J. and Raines, R.T. (2000) *Curr. Opin. Chem. Biol.* **4**, 533-539.

Yao, Y., Zhou, Y. and Wang, C. C. (1997) *EMBO J.* **16,** 651-658.

Yon, J. M. (2001) *Braz. J. Med. Biol. Res.* **34**, 419-435.

Chapter 6

Interaction of API with Fungal Alkaline Proteases: Kinetic Parameters Involved in the Inactivation of the Proteases

Summary

API exhibited a slow-tight binding inhibition mechanism towards the fungal alkaline proteases, Proteinase K from *Tritirachium album* limber and the alkaline protease from *Fusarium oxysporum* (FAP) with IC_{50} values of 5.5 ± 0.5 x 10^{-5} M and 6.5 ± 0.5 x 10^{-5} M respectively. The steady-state kinetics revealed time-dependent competitive inhibition of Proteinase K and FAP by API, consistent with a two-step inhibition mechanism: E + I $\leftrightarrows$ EI $\leftrightarrows$ EI*. The first step involved the rapid equilibrium towards the formation of a reversible enzyme-inhibitor complex (EI) with the K_i values of 5.2 ± 0.6 x 10^{-6} M for Proteinase K and 4.5 ± 0.5 x 10^{-6} M for FAP. Subsequently the EI complex isomerizes to a second stable enzyme-inhibitor complex (EI*). The kinetic parameters involved in the two-step inhibition mechanism of Proteinase K-API were evaluated in detail. The rate constant k_5 (9.2 ± 1 x $10^{-3} s^{-1}$) associated with the isomerization of EI to EI*, revealed a faster induction of the localized conformational changes in the EI complex resulting in the clamping down of the enzyme onto the inhibitor. However, the second enzyme inhibitor complex, EI* dissociated at a very slow rate. The dissociation rate constant, k_6 (4.5 ± 0.5 x $10^{-5} s^{-1}$) depicted that API dissociated from the EI* in a much slower rate revealing its tight binding nature. The overall inhibition constant K_i* involved in the slow-tight binding inhibition of Proteinase K by API was 2.5 ± 0.3 x 10^{-7} M. The conformational changes induced in Proteinase K by API were monitored by fluorescence spectroscopy and the rate constants derived were in agreement with the kinetic data. Thus, the conformational changes are the consequences of the isomerization step of EI to EI*. A time dependent quenching of the fluorescence was obtained without any shift in the emission maximum, which represents no changes in the enzyme structure upon binding of API. Based on our results a scheme depicting the slow-tight binding inhibition of Proteinase K by API has been proposed.

Introduction

In recent years, considerable efforts have been expended in the design and synthesis of protease inhibitors, not only to understand about the active site structures and mechanisms of these interesting enzymes but also in generating new therapeutic agents. Specific inhibitors of proteases have proved valuable in a number of applications ranging from mechanistic studies to possible therapeutic uses. The study of the kinetic properties of this class of enzymes frequently has been motivated by their involvement in physiological and biological processes. Determination of the kinetic parameters of the inhibition of proteases will provide insight into the mechanism of the interaction between the enzyme-inhibitor complex. The kinetics of inhibition of several small molecular weight and synthetic inhibitors towards their cognate proteases have been studied extensively (Parisi and Abeles, 1992; Wilmouth, *et al*., 1999), but few studies have been undertaken in sufficient detail to test the parameters involved in the kinetics of proteinaceous protease inhibitors. Of particular importance in this connection are the canonical protease inhibitors employing the standard mechanism of inhibition (Laskowski and Kato, 1980; Otlewski, *et al*., 1999). These inhibitors bind to the active site of the enzymes in the manner of a good substrate very tightly, and are cleaved very slowly. The k_{cat} /K_m value for the hydrolysis of the reactive site peptide bond is very high typical for normal substrates, however, the individual values of k_{cat} and K_m for the inhibitors are several orders of magnitude lower than those for normal substrates. They display very high affinity towards the substrate with low K_i values. The future development of this class of inhibitors for their potential application in therapeutics and biocontrol will undoubtedly depend on application of kinetic techniques that yield quantitative information about the behavior of the inhibitors. Delineating the inhibition mechanism and the reactive site residues of the inhibitors and understanding the binding efficiency will provide further insight for its potential application. When the structure of inhibitor can be correlated with the true dissociation constants for their enzyme-inhibitor complexes, a systematic approach can be made towards the design of more effective inhibitors for a target enzyme using protein engineering.

Considering the physiological importance of the serine alkaline proteases and their role in various physiological and biotechnological processes, there is a lacuna in the studies on the kinetics of the mechanism of inhibition by their naturally occurring protein inhibitors. In this chapter we have studied the parameters involved in the kinetics of inhibition of two

alkaline proteases, Proteinase K and *Fusarium* alkaline protease (FAP) as model enzymes. Proteinase K is a highly active extracellular alkaline serine endopeptidase from *Tritirachium album* limber. It has been named Proteinase K because of its ability to digest native keratin (Ebeling, et al., 1974). It remains active in the presence of low concentrations of SDS and urea. By virtue of this unusual stability, Proteinase K finds immense applications in basic research and biotechnology. *Fusarium oxysporum* is a wilt causing phytopathogenic fungus leading to heavy losses in crop yield. It secretes an extracellular alkaline protease (FAP), which was shown to be indispensable for its growth and development and therefore could be a potential target for the control of this phytopathogen. Determination of the mechanism of inhibition and evaluating the kinetics of inhibition of these two proteases will provide better insights in understanding the mechanism of inhibition and their interactions for their potential applications.

The present chapter deals with the evaluation of the kinetic parameters of API and its inhibition mechanism towards the alkaline proteases Proteinase K and FAP. The steady state kinetics and the conformational modes observed during the binding of the inhibitor to the enzyme as monitored by fluorescence spectroscopy revealed a two-step inhibition mechanism.

Materials and Methods

Materials

Proteinase K from *Tritirachium album* Limber was purchased from Sigma Chemical Co. U.S.A. All other chemicals were of analytical grade.

Production and purification of *Fusarium* alkaline protease (FAP)

The phtyopathogenic fungal strain *Fusarium oxysporum* f. sp. *ciceri* producing the extracellular alkaline protease was obtained from ICRISAT, India. The production of FAP was carried out in Sabouraud's dextrose medium containing soyabean meal (2%) as an inducer for 5-7 days at 28 °C. FAP was purified by polyacrylamide gel electrophoresis method as described (Tanksale *et al*., 2000).

Assay for proteolytic activity and Inhibition Kinetics of Proteinase K and FAP

Proteolytic activity of the fungal proteases was measured by assaying the enzyme activity using casein as a substrate. PK (3 µM) was dissolved in Tris-HCl buffer, 0.05 M containing 1mM CaCl$_2$, pH 8.5 and FAP (2.5 µM) was dissolved in carbonate-bicarbonate buffer, 0.05 M, pH 10.0. The reaction was initiated by the addition of 1 ml of casein (10 mg/ml) to 1ml of enzyme at 37°C for 30 min. The reaction was quenched by the addition of 2 ml of acidified-TCA (5%) and the tubes were kept for 30 min at room temperature before filtering the precipitate through Whatmann no.1 filter paper. Absorbance of the TCA soluble products was measured at 280 nm.

For initial kinetic analysis, the kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant (K_i) and the K_m values were determined as described by Dixon (Dixon, 1953) and by the Lineweaver-Burk's equation by fitting the data into Microcal Origin by non-linear regression analysis. For the Lineweaver-Burk's analysis Proteinase K and FAP were incubated in the presence and absence of API and assayed at increasing concentration of the substrate 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 and the K_i was determined by fitting the resulting data. In Dixon's method, proteolytic activity of Proteinase K and FAP were measured at two different concentrations of substrate as a function of API concentration. 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. Further details of the experiments are given in the respective figure legends.

The progress curve analysis, assays were carried out in a reaction mixture of 1ml containing Proteinase K and FAP in their respective assay buffer and varying concentrations of API and casein (10 mg/ml). 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 estimated by the increase in absorbance at 280 nm. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicates and the average value was considered throughout. Further details of the experiments are given in the respective figure legends.

Evaluation of Kinetic Parameters

Initial rate studies for reversible, competitive inhibition of PK and FAP were analyzed according to Equation 1,

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

where K_m is the Michaelis constant, V_{max} is the maximal catalytic rate at saturating substrate concentration [S], $K_i = (k_4/k_3)$ is the dissociation constant for the first reversible enzyme-inhibitor complex, and I is the inhibitor concentration (Cleland, 1979). The progress curves for the enzymatic activity resulted due to the interactions between API and Proteinase K were analyzed using Equation 2 (Beith, 1995; Morrison, and Stone, 1985).

$$[P] = v_s t + \frac{v_0 - v_s}{k} (1 - e^{-kt}) \qquad (2)$$

where $[P]$ stands for the product concentration at the time t; v_0 and v_s are the initial and final steady-state rates, respectively; and k is the apparent first-order rate constant for the establishment of the final steady-state equilibrium. The reduction in the inhibitor concentration that occurs on formation of the enzyme inhibitor (EI) complex was corrected, which is a prerequisite for tight-binding inhibitors. This is to emphasize that in case of tight binding inhibition, the concentration of EI is not negligible in comparison to the inhibitor concentration, and therefore the free inhibitor concentration is not equal to the added

concentration of the inhibitor. These corrections of the variation of the steady-state velocity were made according to Equation 3 and 4 as described by Morrison and Walsh (1988).

$$v_s = \frac{k_7 SQ}{2(K_m + S)} \qquad (3)$$

$$Q = [(K_i' + I_t - E_t) + 4K_i'E_t]^{1/2} - (K_i' + I_t - E_t) \qquad (4)$$

where $K_i' = K_i^* (1 + S/K_m)$, k_7 rate constant for the product formation, I_t and E_t stands for total inhibitor and enzyme concentration, respectively. The relationship between the rate constant of enzymatic reaction k, and the kinetic constants for the association and dissociation of the enzyme and inhibitor was determined according to Equation 5,

$$k = k_6 + k_5 \left[\frac{I / K_i}{1 + (S / K_m) + (I / K_i)} \right] \qquad (5)$$

The progress curves were analyzed by eqs 2 and 5 using non-linear least-square parameter minimization to determine the best-fit values with the corrections for the tight binding inhibition. The overall inhibition constant is determined as per Equation 6,

$$K_i^* = \frac{[E][I]}{[EI] + [EI^*]} = K_i \left[\frac{k_6}{k_5 + k_6} \right] \qquad (6)$$

For the time-dependent inhibition, in the time range in the progress curves wherein formation of EI^* is small, it is possible to directly measure the effect of the inhibitor on v_0, i.e., to measure K_i directly by applying competitive kinetic analysis. Values for K_i were obtained from Dixon analysis at a constant substrate concentration as described in Equation 7,

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}S} \ (1 + I / K_i) \qquad (7)$$

The rate constant k_6, associated with the dissociation of the second enzyme-inhibitor complex was measured directly from the time-dependent inhibition. Concentrated Proteinase K and API were incubated in a reaction mixture to reach equilibrium, followed by large dilutions in assay mixtures containing near-saturating substrate concentrations. Proteinase K (2 mM) was pre-incubated with equimolar concentrations of API for 120 min in Tris-HCl

buffer, 0.05 M, pH 8.5. The pre-incubated sample (5 µl) was removed and diluted 5000-fold in the same buffer and assayed at 37°C using casein at (150 mg/ml) at different time intervals.

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 300-500 nm at 25°C. The slit widths on both the excitation and emission were set at 5 nm and the spectra were obtained at 100 nm/min.

For the enzyme inhibitor binding studies, titration of Proteinase K with API was performed by the addition of different concentrations of the inhibitor to a fixed concentration of enzyme solution. On the titration curve for each inhibitor concentration, a new enzyme solution was used and all the data on the titration curve were corrected for dilutions and the graphs were smoothened. The magnitude of the rapid fluorescence decrease $(F_0 - F)$ occurring at each API concentration was computer fitted to the Equation 8, to determine the calculated value of K_i and ΔF_{max} (Houtzager, *et al*., 1996).

$$(F_0 - F) = \Delta F_{max}/\{1+ (K_i/[I])\} \tag{8}$$

The first order rate constants for the slow loss of fluorescence k_{obs}, at each inhibitor concentration [I] were computer fitted to the Equation 9 (Houtzager, *et al*., 1996), 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.

$$k_{obs} = k_5[I]/\{K_i + [I]\} \tag{9}$$

The time course of the protein fluorescence following the addition of inhibitor were measured for 10 min with excitation and emission wavelengths fixed at 295 and 340 nm, respectively, with data acquisition at 0.1 s intervals. Corrections for the inner filter effect were performed as described by Equation 10 (Lakowicz, 1983).

$$F_c = F \text{ antilog } [(A_{ex} + A_{em})/2] \tag{10}$$

Where F_c and F stand for the corrected and measured fluorescence intensities, respectively, and A_{ex} and A_{em} are the absorbances of the solution at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering.

Results

Kinetic Analysis of the Inhibition of Proteinase K and FAP

The kinetic parameters involved in the mechanism of inhibition by API were evaluated using Proteinase K and the alkaline protease from *Fusarium oxysporum* (FAP) as model enzymes. Initial kinetic assessments revealed that API is a competitive inhibitor of Proteinase K and FAP with IC_{50} (concentration of the inhibitor required for 50% inhibition of the enzyme) values of $5.5 \pm 0.5 \times 10^{-5}$ M and $6.5 \pm 0.5 \times 10^{-5}$ M respectively (Fig. 1A, B).


(A)

(B)
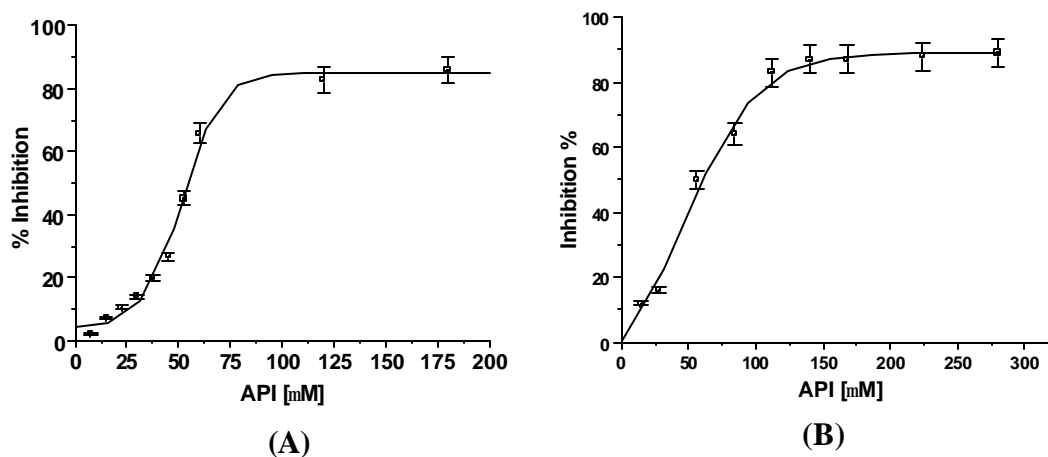
Figure 1. Inhibition of Proteinase K and FAP by API
The proteolytic activity of **(A)** Proteinase K and **(B)** FAP were determined in the presence of increasing concentrations of API. The percent inhibition of the protease activity was calculated from the residual enzymatic activity. The curves indicate the best fit for the percent inhibition data (average of triplicates) obtained.
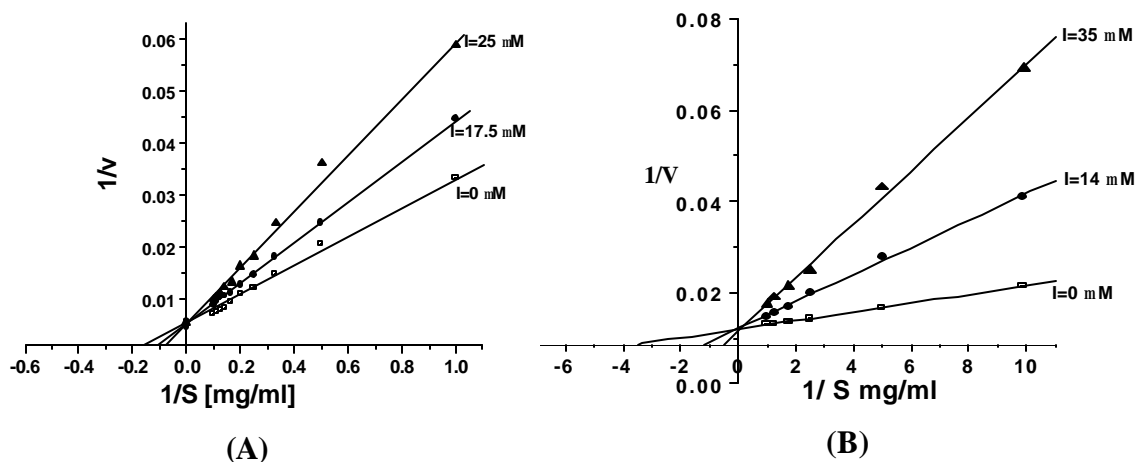
The steady-state rate of proteolytic activity of Proteinase K and FAP reached rapidly in the absence of API, whereas, in its presence a time-dependent decrease in the rate as a function of the inhibitor concentration was observed. The progress curves derived in the presence of API revealed a time range where the initial rate of reaction was similar with that of the absence of the inhibitor, and does not deviate from linearity (Fig. 2A, B), therefore conversion of EI to EI* was minimal. This time range for a low concentration of API was 8 min for Proteinase K and 5 min for FAP, wherein all the classical competitive inhibition experiments performed were used to determine k_4/k_3 which is the K_i value of the (eq 5). The inhibition constant K_i, associated with the formation of the reversible enzyme inhibitor complex (EI) determined from the fits of data to the reciprocal equation were $5.2 \pm 0.6 \times 10^{-6}$ M for Proteinase K and

4.5 ± 0.5 x 10^{-6} M for FAP (Fig.3A, B), which was corroborated by the Dixon method (Fig. 4 A, B).

**(A)**   **(B)**
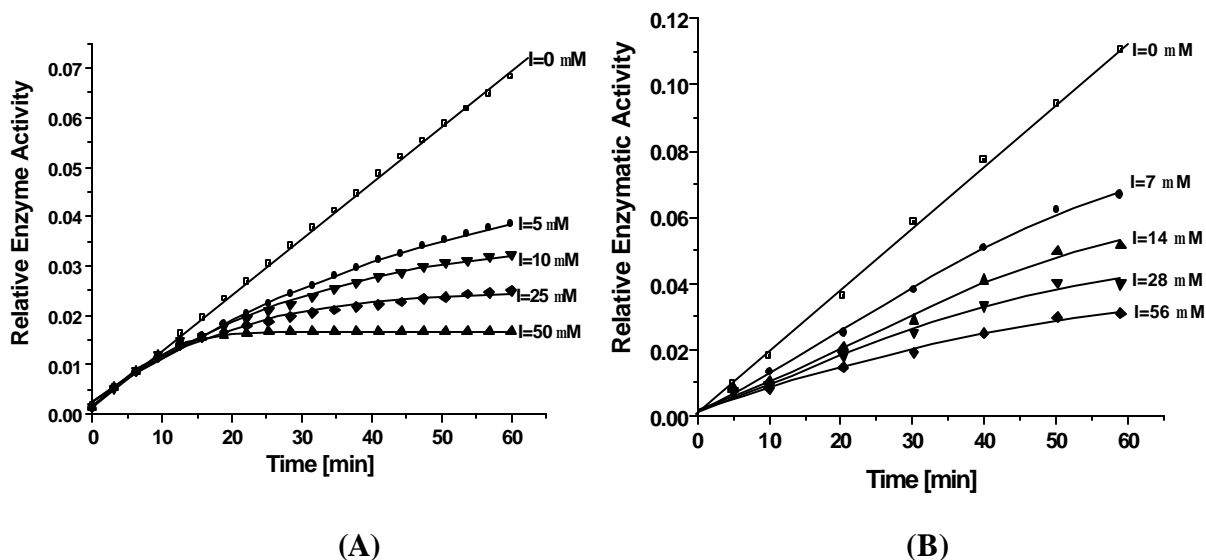
Figure 2. Time course of inhibition of Proteinase K and FAP by API

> Reaction solution contained Proteinase K (A) and FAP (B) in respective buffers at increasing concentrations of API and casein (10 mg/ml). Reactions were initiated by the addition of the enzymes at 37°C. The points represent the hydrolysis of substrate as a function of time at 37°C. The lines indicate the best fits of data obtained from eq 2 and 3. Concentrations of API were 0 μM (■), 5 μM (●), 10 μM (▼), 25 μM (◆), and 50 μM (▲) in A, and 0 μM (■), 7 μM (●), 14 μM (▲), 28 μM (▼), and 56 μM (◆), in B.

(A)   **(B)**

**Figure 3. Initial rate of enzymatic reaction of Proteinase K in the presence of API**

> Initial rate of proteolysis by Proteinase K (A) and FAP (B) are shown. The enzymes were incubated (■) without or with the inhibitor at 17.5μM (●), 25 μM (▲) in A, and at 14 μM (●), 35 μM (▲) in B, and assayed at increasing concentrations of substrate. The straight lines obtained indicated the best fit for the data obtained as analyzed by the Lineweaver-Burk's reciprocal equation and the K_i values were determined from the graphs.
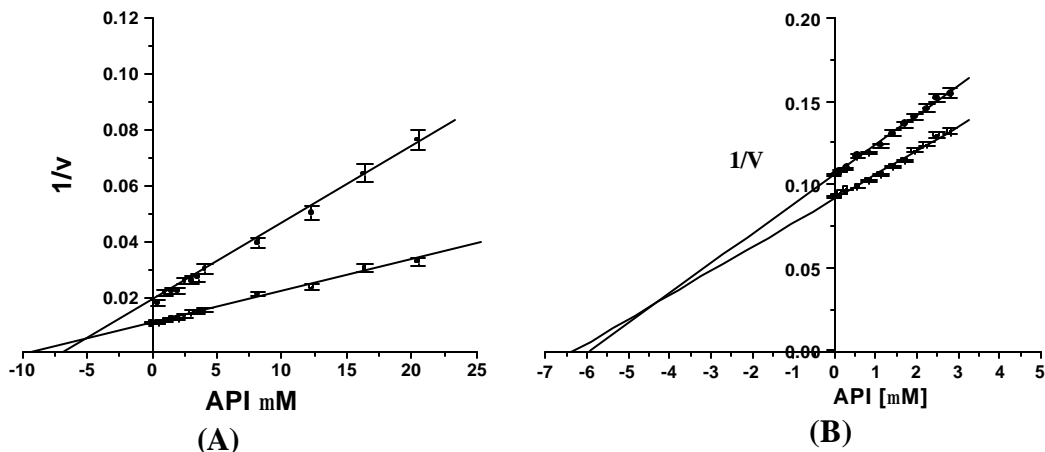
Figure 4. Dixon Plots for the determination of K_i values.
Enzymatic activity of **(A)** Proteinase K and **(B)** FAP were estimated using casein at (■) 10 mg/ml, and (●) 5 mg/ml, respectively, at different concentrations of API. The reciprocal of substrate hydrolysis by Proteinase K and FAP (1/v) were plotted as a function of inhibitor concentration. The straight lines indicated the best fits for the data obtained.

The kinetic parameters involved in the slow-tight binding inhibition of API were evaluated with Proteinase K. The apparent rate constant k, derived from the progress curves of Proteinase K when plotted *versus* the inhibitor concentration followed a biphasic hyperbolic function (Fig. 5), revealing a fast equilibrium precedes the formation of the final slow dissociating enzyme-inhibitor complex (EI*), indicating two-step, slow-tight inhibition mechanism (Scheme I). Indeed, the data could be fitted to equation 5 by non-linear regression analysis, which yielded the best estimate of the overall inhibition constant K_i^* of $2.5 \pm 0.3 \times 10^{-7}$ M.
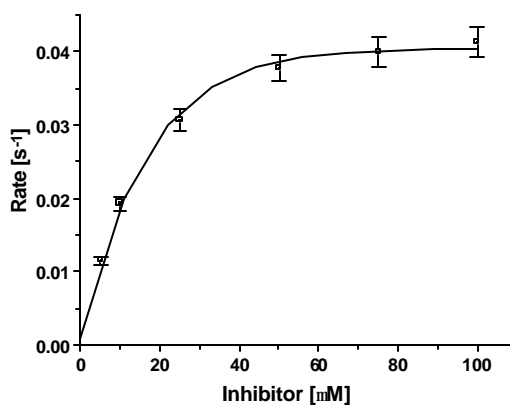

Figure 5. Dependence of Proteinase K inhibition on API concentration

The rate constants k, were calculated from the progress curves recorded following the addition of Proteinase K to the reaction mixture containing casein and API. The solid line indicates the best fit of the data obtained.

In case of slow-tight binding inhibitors, since the EI* complex is stable and dissociates slowly, the rate constant k_6, for the conversion of EI* to EI, was determined, in an alternative method, by pre-incubating high concentrations of enzyme and inhibitor for sufficient time to allow the system to reach equilibrium. Further, large 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 dissociation constant can be determined by the regeneration of enzymatic activity. Under these conditions, v_0 and the effective inhibitor concentration are considered approximately equal to zero and the rate of activity regeneration will provide the k_6 value. The equimolar pre-incubated/equilibrated mixture of Proteinase K and API, was diluted 5,000-fold into the assay mixture containing the substrate at 50 K_m. By least-squares minimization of eq 2 to the data for recovery of enzymatic activity, the determined k_6 value was $4.5 \pm 0.5 \times 10^{-5}$ s$^-$ (Fig. 6), which clearly indicated a very slow dissociation of EI*. The final steady-state rate v_s, was determined from the control that was pre-incubated without the inhibitor.
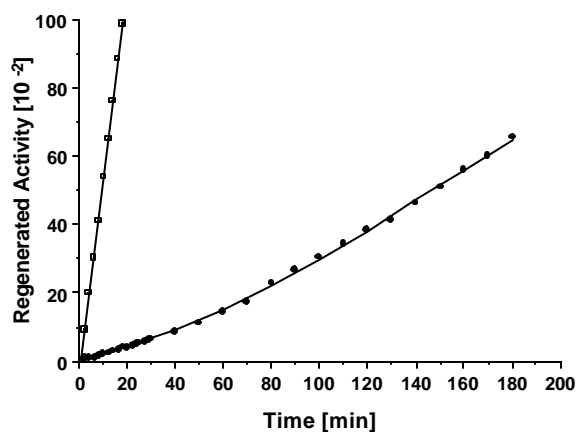
Figure 6. Dissociation rate constant (k_6) for Proteinase K-API complex
Proteinase K (2 mM) was pre-incubated without (■) or with (●) equimolar concentrations of API for 120 min at 37°C. At the specified times indicated by the points, 5 µl of the pre-incubated sample was removed, diluted 5000-fold in the same buffer, and was assayed for the proteolytic activity using casein at 50xK_m. The rate constant associated with the regeneration of activity (k_6) was determined by measuring the absorbance at 280 as described in the text.

Table-I

Inhibition constants of API against Proteinase K

Values of rate constants for Proteinase K inhibition by API were calculated from Scheme I at 37°C in Tris-HCl buffer, 0.05 M, pH 8.5 using casein as the substrate. IC_{50} is from the inhibition profile, K_i was determined from the steady-state time range for the competitive inhibition. k_6 is calculated from the regeneration assay, K_i^* and k_5 were determined from the equations as described in the text.

Inhibition constants	Values
IC_{50}	$5.5 \pm 0.5 \times 10^{-5}$ M
K_i	$5.2 \pm 0.6 \times 10^{-5}$ M
K_i^*	$2.5 \pm 0.3 \times 10^{-7}$ M
k_5	$9.2 \pm 1 \times 10^{-3}$ s^{-1}
k_6	$4.5 \pm 0.5 \times 10^{-5}$ s^{-1}
k_5/k_6	$2.0 \pm 0.2 \times 10^{2}$
$t_{1/2}$	4.27 ± 0.5 h

This clearly indicated a change in the EI* which prohibits the inhibitor to dissociate from the enzyme. The value of the rate constant k_5, associated with the isomerization of EI to EI*, was $9.2 \pm 1 \times 10^{-3}$ s^{-1} as obtained from fits of eq 3 to the onset of inhibition data using the experimentally determined values of K_i and k_6 (Table-I). The overall inhibition constant K_i^* is a function of $k_6/(k_5 + k_6)$ and is equal to the product of K_i 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 4.27 ± 0.5 h, suggesting higher binding affinity of API towards Proteinase K.

Fluorescence changes of Proteinase K due to binding of API and the dependence of emission fluorescence on time dependent binding of inhibitor.

Slow-tight binding inhibitors bind at the active site of the target enzyme and induce localized conformational changes in the enzyme that result in the clamping down of the enzyme into the inhibitor forming a stable enzyme-inhibitor complex. The kinetic analysis of Proteinase K inhibition by API revealed a two-step inhibition mechanism, where the EI complex isomerizes to a tightly bound, slow dissociating EI* complex. To investigate and correlate this isomerization to the conformational modes in the Proteianse K due to binding of API, we have analyzed the fluorescence emission spectra of the protease in the presence and absence of the inhibitor. Proteinase K exhibited an emission maxima (λ_{max}) at 337 nm, as a result of the radiative decay of the π - π^* transition from the Trp residues (Fig. 7). The binding of API resulted in a concentration dependent quenching of the fluorescence with saturation reaching at/above ? of API. The absence of blue or red shift in λ_{max} negated any drastic gross conformational changes in the three-dimension structure of the enzyme due to inhibitor
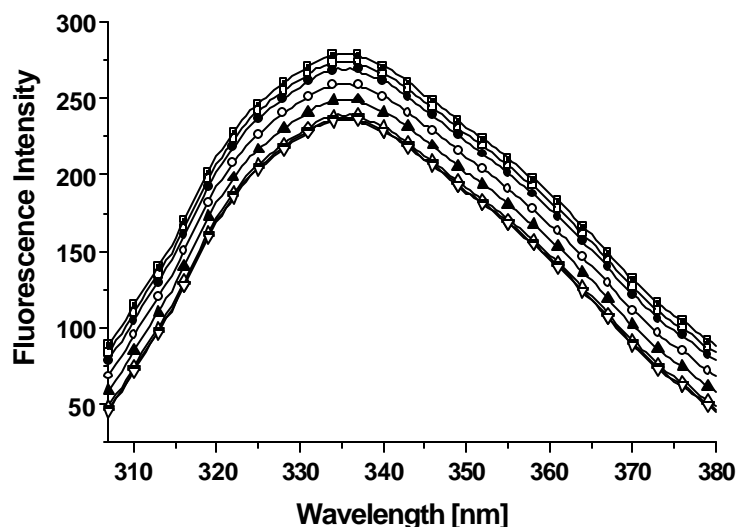

binding.

Figure 7. Steady state fluorescence emission spectra of Proteinase K as a function of API
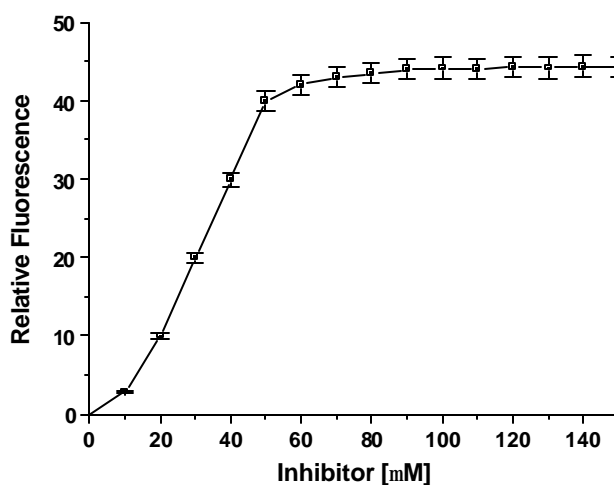
Protein fluorescence was excited at 295 nm and emission was monitored from 300-400 nm at 25°C. Titration was performed by the addition of different concentrations of the inhibitor to a fixed concentration of enzyme. Proteinase K was dissolved in buffer, and the concentrations of API used were 0 μM (■), 15 μM (□), 30 μM (●), 45 μM (O), 60 μM (▲), 75 μM (△), 87.5 μM (◆), 100 μM (▼), 150 μM (∇), 200 μM

The subtle conformational changes induced during the isomerization of EI to EI* was monitored by analyzing the tryptophanyl fluorescence of the complexes as a function of time. Binding of API resulted in an exponential decay of the fluorescence intensity as indicated by a sharp decrease in the quantum yield of fluorescence followed by a slower decline to a stable value (Fig. 8). Further, titration of API against Proteinase K revealed that the magnitude of the initial rapid fluorescence loss ($F_0 - F$) increased hyperbolically (Fig. 9), which corroborated the two-step slow tight binding inhibition of Proteinase K by API. From the data in figure 8, the magnitude of the rapid fluorescence decrease at a specific API concentration was found to be close to the total fluorescence quenching observed figure 7, indicating that the EI and EI* complexes have the same intrinsic fluorescence.
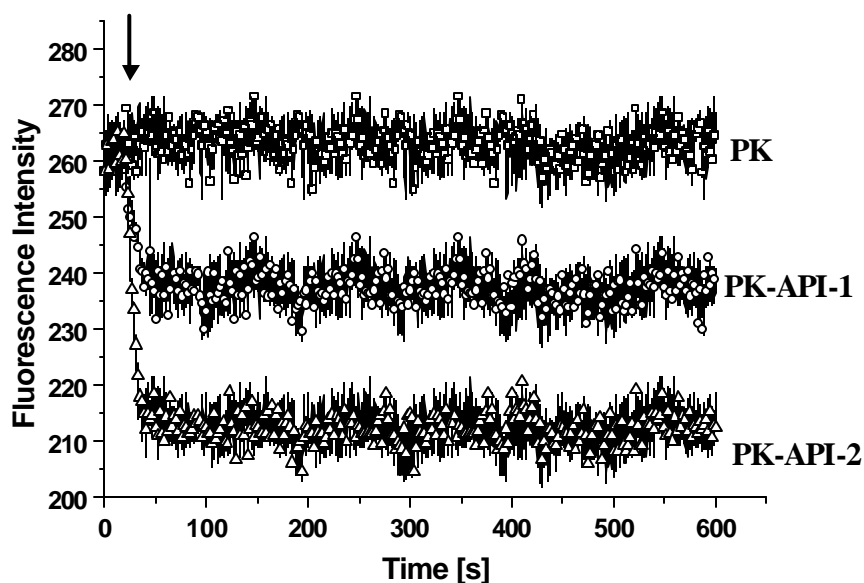
Figure 8. Time dependent Effect of API on the fluorescence quenching of Proteinase K
API was added to Proteinase K at the specified time (indicated by the arrow) and the fluorescence emission was monitored for 600 s, at a data acquisition time of 0.1 s. The excitation and emission wavelength were fixed at 295 and 337 nm, respectively. The data were the average of five scans with the correction for buffer and dilutions. The concentrations of API used were 0 μM (PK), 60 μM (PK-API-1), and 120 μM (PK-API-2).

The K_i value determined by fitting the data for the magnitude of the rapid fluorescence decrease ($F_0 - F$) was 5.8 ± 0.6 x 10^{-5} M and the k_5 value determined from the data derived from the slow decrease in fluorescence was 9.5 ± 1 x 10^{-3} s^{-1}. These rate constants are in good agreement with that obtained from the kinetic analysis, 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*.

Figure 8. Effect of API concentration on the tryptophan fluorescence of Proteinase K
Proteinase K was treated with increasing concentrations of API. The fluorescence was measured at 25°C (excitation 295 nm and emission 337 nm). Each measurement was repeated five times and the average values of the fluorescence intensity at 337 nm were recorded. Control experiments with the buffer and inhibitor were performed under identical conditions. The fluorescence changes ($F - F_0$) were plotted against the inhibitor concentrations. The resulting hyperbola indicates the best fit of the data obtained.

Discussion

We present the first report of a bifunctional proteinaceous inhibitor API, exhibiting slow-tight binding inhibition against the alkaline proteases; Proteinase K and FAP. The inhibitor showed exceptionally high potency against Proteinase K and its interaction with the enzyme indicated its "tight-binding" nature. The two-step inhibition mechanism was corroborated by the equilibrium binding studies of the enzyme and the inhibitor and by the correlation of the kinetic data with the conformational changes induced in the enzyme-inhibitor complexes.

A number of enzymatic reactions do not respond to the presence of competitive inhibitors instantly, but rather display a slow-onset of the inhibition. In some cases the inhibitor interacts slowly with the enzyme, in others the formation of the enzyme-inhibitor complex takes place in a very short time. Such inhibition is called slow-binding inhibition and the inhibitor is refereed to as slow-binding inhibitor (Wolfenden, 1976; Williams and Morrison, 1979; Szedlacsek and Duggleby, 1995; Sculley et al., 1996). From the kinetic point of view, the possible mechanisms for the slow-binding inhibition phenomena are described in Scheme I. Scheme (Ia) assumes that the formation of an EI complex is a single slow step and the magnitude of k_3I is quite small relative to the rate constants for the conversion of substrate to product. However, scheme (Ib) demonstrates the two-step slow-binding inhibition, where the first step involves the rapid formation of a reversible EI complex, which undergoes slow isomerization to a stable, tightly bound enzyme-inhibitor complex, EI*, in the second step. 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 to 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, which has been thoroughly reviewed (Morrison, 1982; Pegg and Itzstein, 1994; Kati, *et al*., 1998; Yiallouros, *et al*., 1998; Ploux, *et al*., 1999). Understanding the basis of the isomerization of EI complex to EI* complex could lead to designing of inhibitors that allow titration of the lifetime of the EI* complex. The future development of slow-tight binding inhibitors will undoubtedly depend on application of kinetic techniques that yield quantitative information about the properties of the inhibitors.

Enzyme-catalyzed reactions, where the concentrations of the enzyme and inhibitor are comparable, and the equilibria are set up rapidly are referred to as tight binding inhibition. Kinetically the slow-binding inhibition can be illustrated by three mechanisms (Scheme I). When an inhibitor has a low K_i value and the concentration of I varies in the region of K_i, both k_3I and k_4 values would be low (Dash, *et al*., 2001). Thus, a simple second-order interaction between enzyme and inhibitor, and low rates of association and dissociation would lead to slow-binding inhibition. Alternatively, a two-step model depicts the rapid formation of an initial collisional complex EI, which slowly isomerizes to form a tightly bound slow dissociating complex EI*. Slow binding inhibition can also arise due to an initial slow inter conversion of the enzyme E, into another form E*, which binds to the inhibitor by a fast step. Understanding the basis of the isomerization of EI to EI* could lead to design of inhibitors that allow titration of the lifetime of the EI*. In case of slow-tight binding inhibition, the inhibitor will inhibit the enzyme competitively at the onset of the reaction, however at increasing concentration of inhibitor, the rate of substrate hydrolysis will decrease hyperbolically as a function of time. In tight binding inhibition corrections have to be made for the reduction in the inhibitor concentration that occurs on formation of the EI complex, since the concentration of EI is not negligible in comparison to the inhibitor concentration and the free inhibitor concentration is not equal to the added concentration of the inhibitor. The kinetic analysis of Proteinase K inhibition provides a unique opportunity for the quantitative determination of these rates and affinities, which can be extended to other slow-tight binding inhibition reactions. The formation of EI complex between Proteinase K and API was too rapid to be measured at steady-state kinetics and was likely to be near diffusion control. However, the isomerization of EI to the second tightly bound enzyme inhibitor complex EI*, was too slow and relatively independent of the stability of the EI. The k_6 values revealed very slow dissociation of the inhibitor from the EI* indicating a highly stable, non-dissociative nature of the second complex. Therefore for slow-tight binding inhibition the major variable is k_6, the first-order rate constant associated with the conversion of EI* to EI, and the apparent inhibitor constant K_i^* depends on the ability of the inhibitor to stabilize the EI*. The half-life as derived from the k_6 value indicated a longer half-life of the EI*, which is an essential parameter for an inhibitor to have biotechnological applications.

The characteristic feature of slow binding inhibition is the induction of conformational changes in the enzyme-inhibitor complex, resulting in the clamping down of the enzyme to the inhibitor, thus leading to the formation of a stable enzyme-inhibitor complex. The two-step inhibition mechanism of Proteinase K by API was reflected in the quenching pattern of the fluorescence of the enzyme-inhibitor complexes. The rate constants derived from the fluorescence analysis of the complexes corroborated the values derived from the kinetic analysis. Therefore, we propose that the initial rapid fluorescence loss reflected the formation of the reversible complex EI, whereas the subsequent slower decrease was correlated to the accumulation of the tightly bound complex EI*. Any major alteration in the three-dimensional structure of Proteinase K due to the binding of API can be ruled out, since there was no shift in the tryptophanyl fluorescence of the complexes. The proteolytic activity decreased linearly with increasing concentrations of API yielding a stoichiometry close to 1:2 (also revealed by fluorescence) expected for the dimeric API, that binds two molecules of enzyme. The agreement of the rate constants concomitant with the fluorescence changes observed during the time-dependent inhibition, lead us to correlate the localized conformational changes in the enzyme-inhibitor complex to the isomerization of the EI to EI*.

The kinetic analyses demonstrated that the inhibition of Proteinase K by API, followed slow-tight binding inhibition mechanism and the induced conformational changes are conveniently monitored by fluorescence spectroscopy. Based on our observations, we conclude that concomitant with the kinetic analysis, fluorescence spectroscopy plays a very important role for the determination of kinetic constants of enzyme inhibition and for the characterization of the mechanism of inhibition of Proteinase K by the slow-tight binding inhibitor API.

Scheme I.

128
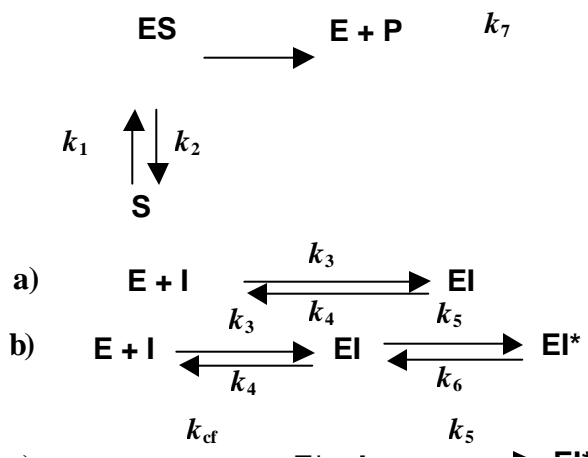
Where, E stands for free enzyme, I is free inhibitor, EI is a rapidly forming pre-equilibrium complex, and EI* is the final enzyme inhibitor complex. Alternately, E may undergo inter conversion into another form E* which binds to the inhibitor by a fast step, where k_{cf} and k_{-cf} stand for the rate constants for forward and backward reaction, respectively, for the conversion of the enzyme.

Scheme I describes two alternative models for the time-dependent inhibition. The mechanism in scheme Ia, where the binding of the inhibitor to the enzyme is slow and tight, but occurs in a single step, is eliminated based on the data of Table-I, because the inhibitor has measurable effect on the initial rates before the onset of slow-tight binding inhibition. Scheme Ic represents the inhibition model where the inhibitor binds only to the free enzyme that has slowly adopted the transition-state configuration can also be eliminated by the observed rates of onset of inhibition. Our foregoing results for the inactivation of Proteinase K are, therefore, consistent with the slow-tight binding mechanism as described in Scheme Ib.

References

Beith, J. G. (1995) *Methods Enzymol.* **248**, 59-84.

Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103-138.

Dash, C., Phadtare, S., Deshpande, V., and Rao, M. (2001) *Biochemistry* **40**, 11525-11532.

Dixon, M. (1953) *Biochem. J.* **55**, 170-171.

Houtzager, V., Oullet, M., Falgueyret, J.-P., Passmore, L. A., Bayly, C., and Percival, M. D. (1996) *Biochemistry* **35**, 10974-10984.

Kati, W. M., Saldivar, A. S., Mohamadi, F., Sham, H. L., Laver, W. G., and Kohlbrenner, W. E. (1998) *Biochem. Biophys. Res. Com.* **244**, 408-413.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

Laskowski, M., Jr. and Kato, I. (1980) *Annu. Rev. Biochem.* **49,** 593-626.

Morrison, J. F. (1982) *Trends Biochem. Sci.* **7**, 102-105.

Morrison, J. F., and Stone, S. R. (1985) *Comments Mol. Cell. Biophys.* **2**, 347-368.

Morrison, J. F., and Walsh, C. T. (1988) *Adv. Enzymol.* **62**, 201-302.

Otlewski, J., Krowarsch, D., and Apostoluk, W. (1999) *Acta Biochimica Polonica* **3,** 531-565.

Parisi, M. F., and Abeles, R. H. (1992) *Biochemistry* **31,** 9429-9435.

Pegg, M. S., and Itzstein, M. von (1994) *Biochem. Mol. Biol. Int.* **32**, 851-858.

Ploux, O., Breyne, O., Carillon, S., and Marquet, A. (1999) *Eur. J. Biochem.* **259**, 63-70.

Sculley, M. J., Morrison, J. F., and Cleland, W. W. (1996) *Biochim. Biophys. Acta.* **1298**, 78-86.

Szedlacsek, S., and Duggleby, R. G. (1995) *Methods Enzymol.* **249**, 144-180.

Tanksale, A. M., Vernekar, J. V., Ghatge, M. S., and Deshpande, V. V. (2000) *Biochem. Biophys. Res. Commun.* **270,** 910- 917.

Williams, J. W., and Morrison, J. F. (1979) *Methods Enzymol.* **63**, 437-467.

Wilmouth, R. C., Kassamally, S., Westwood, N. J., Sheppard, R. J., Claridge, T. D. W., Aplin, R. T., Wright, P. A., Pritchard, G. J., and Schofield, C. J. (1999) *Biochemistry* **38,** 7989-7998.

Wolfenden, R. (1976) *Annu. Rev. Biophys. Bioeng.* **5**, 271-306.

Yiallouros, I., Vassiliou, S., Yiotakis, A., Zwilling, R., Stocker, W., and Dive, V. (1998) *Biochem. J.* **331**, 375-379.

Chapter 7

Inhibitor Induced Thermal Stability of Proteinase K

Summary

Proteases find tremendous applications in various biotechnological industries. Higher thermostability is one of the essential features for the commercial exploitation of enzymes and often a prerequisite for the application of enzymes in several industrial processes. The thermal inactivation of industrially important enzymes can be circumvented by their stabilization through the formation of reversible enzyme-inhibitor complexes. API is stable over a wide temperature range (40-95°C) and loses its activity at 95°C after 30 min. We have selected Proteinase K, the alkaline protease from *Tritirachium album* Limber as the model enzyme to study the thermal stability of enzyme-inhibitor complexes. The thermal stability profile of Proteinase K revealed that it is stable at 65°C for 10 min followed by a rapid loss of activity as a function of time. Interestingly, the stability of Proteinase K is enhanced by the binding of API to the enzyme. The incubation of uncomplexed Proteinase K at 70°C abolished its activity, however the inhibitor bound Proteinase K-API complex retained 50-55% activity. Thermal inactivation is often correlated to the structural and conformational changes in a protein. The structural changes induced in Proteinase K at 70°C were probed by circular dichroism and fluorescence spectroscopy. The binding of API to Proteinase K prevents any changes in the secondary and tertiary structure of the enzyme, thus preventing the thermal denaturation. Our results implicated that the thermal inactivation of alkaline proteases can be prevented by API, and thus can have enormous applications in industries utilizing alkaline proteases at higher temperatures.

Introduction

Biotechnology represents a powerful and versatile technology for delivering environment friendly industrial products and processes (Bull, *et al.*, 1998). The concept and development of enzymatic catalysis in biotechnology marks a paradigm shift for the production and processing of various chemical substances. Enzymes such as proteases, amylases, lipases, cellulases, and xylanases have received significant attention because of their importance in cleaning products, starch processing, and pulp and paper manufacturing (Rao, *et al*., 1998; Kulkarni, *et al*., 1999). In addition to the manufacturing sectors, enzymes are used extensively in processing operations where they have a large impact in reducing environmental pollution. Since natural enzymes are adapted to their particular function in a living cell, in most instances, they are poorly suited for industrial applications that often encounter extremes of pH, temperature and/or salinity. Therefore, increased thermostability is an important factor for the suitability of an enzyme in commercial applications. Enhanced thermal stability offers several advantages such as higher conversion rates, increased substrate solubility, decreased viscosity of the medium, and a reduced susceptibility to microbial contamination leading to increased storage and operational stability.

Enzymes, however, are susceptible to the harsh denaturing conditions of high temperatures that characterize chemical processing and thus, impair their catalytic rates. The thermoinactivation of proteins involves a number of processes such as aggregation, peptide bond hydrolysis, deamidation of aspargine residues, formation of scrambled structures with or without disulfide exchange reactions and dissociation of prosthetic group.

Strategies for improving protein thermostabilization

The conditions in which industrially important proteins are used often differ from their natural environment. Stability is, therefore, a necessary consideration for most proteins and this drives the on-going search for improved biocatalysts. Broadening the industrial applicability of enzymes is currently a very active area of research (Lehmann and Wyss, 2001). Various approaches have been explored for the stabilization of enzymes (Gupta, 1991, Janecek, 1993)

Use of additives

Several external compounds are used to enhance the thermostability of proteins (Gupta, 1991; Gonzalez, *et al*., 1992; Bandivadekar and Deshpande, 1994). Naturally

occurring osmolytes such as polyols, aminoacids, and methylamines are known to provide thermal stability to enzymes without substantive alteration in their catalytic action (Yancey, *et al*., 1982; Santoro, *et al*., 1992; Taneja and Ahmad, 1994). Water has been found to have a great influence on enzyme thermostability (Klibanov, 1989). The addition of sugars, polyhydric alcohols and other polymers such as polyethylene glycol and dextran are shown to effect thermostabilization by strengthening the hydrophobic interactions by modifying the structure of water (Back, *et al*., 1979; Ward and Moo-Youg, 1988). Salts and metal ions represent another important class of additives (Pace and Grimsley, 1988; Coolbear, *et al*., 1992; De Bolle, *et al*., 1997). The binding of substrates, substrate analogues and coenzymes are also shown to enhance the thermal stability by increasing the rigidity of conformation (Segawa and Sugihara, 1984; Gonzalez, *et al*., 1992)

Chemical modification

The stabilization of enzymes by covalent modifications can be achieved by various methods (Mozhaev, et al 1990; Janecek, 1993). The hydrophobic interactions within the protein can be strengthened by modification with nonpolar reagents. Another method involves the introduction of new polar or charged groups that promote the formation of additional hydrogen or ionic bonds and helps in maintaining the structural integrity. The other approach to achieve stabilization by modification is by hydrophilization of the protein surface that prevents the unfavorable hydrophobic contact with water. A 1000-fold increased thermostability of α-chymotrypsin (in comparison with the native enzyme) prepared by its hydrophilization with glyooxylic acid and cyclic anhydrides of aromatic acids has been reported (Melik-Nubarov, *et al*, 1987; Mozhaev, *et al*., 1988)

Chemical cross linking

The stability of proteins and enzymes can be substantially improved by employing the chemical cross linking technique (Wong and Wong, 1992). In this procedure, the molecule is braced with chemical crosslinks either intramolecularly or intermolecularly to another species to reinforce its active conformation. Various chemicals have been used for this purpose, however the use of bifunctional compounds is most promising (Ji, 1983; Gaffney, 1985). These compounds are essentially group specific chemical modifiers and may be classified into zero-length, homobifunctional and heterobifunctional crosslinkers. They react with nucleophilic side chains of amino acids. It has been shown that the use of a series of

bifunctional reagents of different chain lengths have been used to crosslink amyloglucosidase (Tatsumoto, *et al*., 1989). Increased thermostability of α-chymotrypsin was obtained by the cross linking of carbodiimide-activated enzyme with 1,4-tetramethylenediamine (Torchilin, *et al*., 1978).

Enzyme immobilization

Immobilization of an enzyme is defined as the conversion of an enzyme from a water-soluble, mobile state to a water-insoluble, immobile state (Klibanov, 1983). There are several methods of enzyme immobilization such as, covalent attachment to solid supports, adsorption on solid supports, entrapment in polymeric gels and encapsulation (Klibanov, 1979; Monsan and Combes, 1988). Stabilization of enzymes due to immobilization has been ascribed to many factors (Klibanov, 1979). The mutual spatial fixation of enzyme prevents aggregation and autolysis especially of proteases. The immobilization of enzymes increases their conformational rigidity and protects against unfolding. Also, protection from inactivators (pH, oxygen, hydrogen peroxide,'poisons') is conferred as a result of immobilization.

Protein engineering

Protein engineering through site-directed mutagenesis has become a promising alternative strategy for protein stabilization (Nosoh and Sekiguchi, 1988, 1990). Site-directed mutagenesis has enabled engineered proteins to be produced that differ from their wild counterparts only in one or more predefined amino acids. This approach has made it possible to stabilize mesophilic enzymes by manipulating their amino acid sequences. The enhanced stabilization of enzyme structures by protein engineering can be obtained by: 1) Introduction of internal or surface disulfides. Disulfide bonds restrict the degree of freedom for the unfolded state and thereby stabilize the conformation of the folded state. 2) Strengthening the internal hydrophobicity stabilizes the protein thermodynamically and kinetically, since protein folding is driven by the hydrophobic properties of nonpolar amino acids. 3) Increase in internal hydrogen bonding and 4) Increase of surface salt bridges (Janecek, 1993).

At present there are numerous strategies leading to the stabilization of enzymes for their potential exploitation in various biotechnological applications. All these individual methods have their own advantages and demerits. In this chapter we have explored yet another aspect of API in a novel method for the thermostabilization of enzymes by the formation of reversible enzyme-inhibitor complexes. We have used the fungal alkaline protease, Proteinase K from *Tritirachium album* Limber as a model enzyme. The structure-function relationship of the enzyme at higher temperatures upon binding of API has been correlated.

Materials and Methods

Materials

Proteinase K from *Tritirachium album* Limber was purchased from Sigma Chemical Co. U.S.A. All other chemicals were of analytical grade.

Assay for proteolytic activity of Proteinase K

Proteolytic activity of Proteinase K (PK) was measured by assaying the enzyme activity using casein as a substrate. PK (3.57 µM) was dissolved in 1 ml of Tris-HCl buffer, 0.05 M containing 1mM CaCl₂, pH 8.5 and the reaction was initiated by the addition of 1 ml of casein (10 mg/ml) at 37°C for 30 min. The reaction was quenched by the addition of 2 ml of acidified-TCA (5%) and the tubes were kept for 30 min at room temperature before filtering the precipitate through Whatmann no.1 filter paper. Absorbance of the TCA soluble products was measured at 280 nm.

The Proteinase K (357 µM) was incubated with API (185 µM) at 4°C for 1 h to allow formation of an enzyme-inhibitor complex followed by incubation 70°C for different time periods. The complex was diluted 100 fold at 4°C for 30 min and the time dependent regain of residual enzyme activity was determined. Proteinase K treated similarly in the absence of inhibitor served as a control.

Temperature stability of Proteinase K

PK (35 µM) was incubated at different temperatures for 1 h. At the end of incubation, the enzyme was incubated on ice for 15 min and the residual activity was determined.

Effect of thermal denaturation on the structure of Proteinase K

Fluorescence analysis

The fluorescence measurements were performed with a Perkin-Elmer LS-50 spectrofluorimeter fitted with a Julabo F 20 water bath. The samples were analyzed using a slit width of 7.5 mm in a 1 cm path length quartz cuvette, with an excitation wavelength of 295 nm and the emission was recorded from 300-500 nm at a scan speed of 200nm/min. Measurements were performed in triplicate. The time dependent change in the tertiary structure of PK upon thermal denaturation was monitored. PK (3.57 mM) was incubated with API (2 mM) at 4°C for 30 min to allow complex formation and their fluorescence was monitored for 1 h at 37 and 70°C. In the enzyme-inhibitor interaction studies, the fluorescence

spectrum of only API was subtracted from the corresponding spectrum of enzyme plus inhibitor.

Circular dichroism analysis

The effect of thermal denaturation on the secondary structure of PK in the presence and absence of API was determined by far UV-CD spectroscopy. CD spectra were recorded in a Jasco J-715 spectropolarimeter at ambient temperature using a cell of 1-mm path length. Replicate scans were obtained at 0.1 nm resolution, 0.1 nm bandwidth and a scan speed of 50 nm/min. Spectra were average of 6 scans with the baseline subtracted spanning from 250 nm-200 nm in 0.1 nm increment. The CD spectrum of PK (35 µM) was recorded in 50 mM sodium phosphate buffer (pH 7.5) in the absence/presence of API (16 µM) after incubation at 70 °C for 1 h and similarly also at room temperature as control. In the enzyme-inhibitor interaction studies, the spectrum of only API was subtracted from the corresponding spectrum of enzyme plus inhibitor.

Results

Proteinase K, the alkaline protease from *Triticharium album* Limber, is inhibited competitively by API with IC_{50} and K_i values of () and (), respectively (Chapter 6, Section I). We have used this enzyme as a model system to study the enzyme inhibitor interaction at higher temperatures and to explore the potential use of API towards the thermostabilisation of industrially important enzymes.

Temperature stability of Proteinase K

Proteinase K is stable in a temperature range of 40-50°C (Fig 1). However it is less stable at 60 and 65°C. At 65°C there is a rapid loss of activity after 10 min. All denaturation studies were, therefore, carried out at temperatures above 65**°C.**
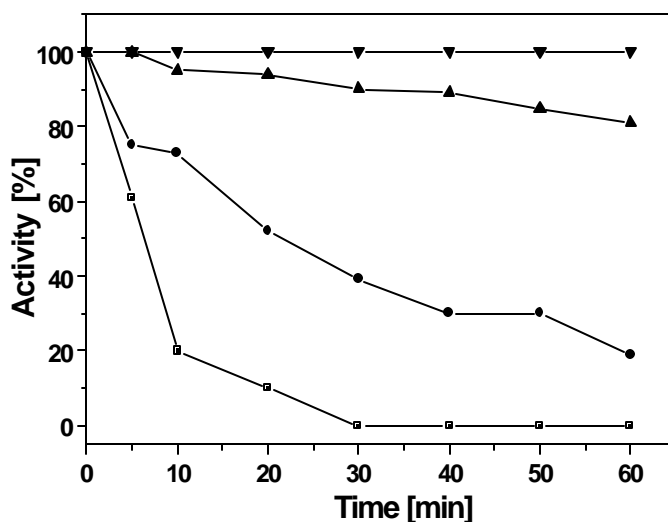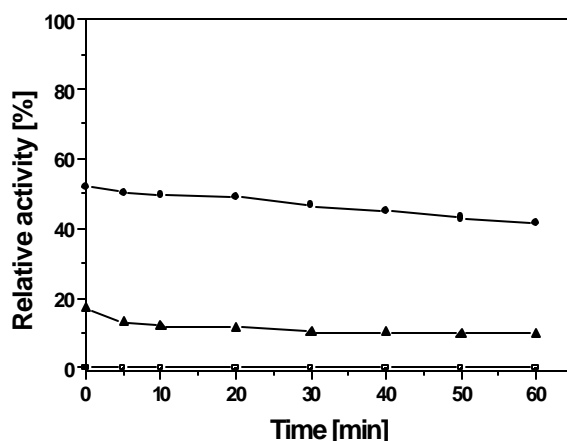

Figure 1: Temperature stability of Proteinase K
Proteinase K was incubated at 40 (▼), 50 (▲), 60 (●) and 65°C (■), for the time specified and the proteolytic activity was determined at 37°C.

Time–dependent regain in activity of Proteinase K in presence of API

In Chapter 6 (Section I), we have shown the formation of a Proteinase K-API (EI) complex which isomerizes to a tight binding (EI*) complex. API was shown to bind to the active site of Proteinase K and dissociated at a very slow rate from the EI* complex thus revealing its tight binding nature. Heating of Proteinase K at 70°C completely abolished its proteolytic activity. However, the binding of API to Proteinase K at 70°C for various time

periods protects the enzyme from inactivation since the binding between enzyme and inhibitor is tight. Further dissociation of the EI* complex resulted in the recovery of about 50%

enzymatic activity at higher temperature since the inhibitor has stabilizing effect on the enzyme (Fig. 2).

Figure 2: Time–dependent Regain of Activity
Proteinase K was incubated with API at 4°C for 30 min to allow formation of complex followed by incubation at 70°C for 60 min (●) and 90 min (▲). The complex was diluted 100 fold and the time dependent regain of activity was estimated. Proteinase K treated similarly in the absence of API (■) served as a control.

Fluorometric analysis of Proteinase K at higher temperature

To decipher the conformational changes induced upon binding of API to Proteinase K, the fluorescence spectra of the enzyme-inhibitor complex was monitored as a function of time. The amino acid sequence of Proteinase K indicated the presence of two Trp residues in its primary structure (Jany et al., 1986). Therefore, the conformational changes induced in the enzyme upon binding of API were followed by exploiting the intrinsic fluorescence of the Trp residues. The tryptophanyl fluorescence spectra of native Proteinase K exhibited an emission maxima (λ_{max}) at ~338 nm (Fig. 3). The heating of Proteinase K at 70°C resulted in a progressive decrease in the tryptophanyl fluorescence with a red shift of 20 nm indicating the unfolding of the enzyme and exposure of the Trp residues from a non polar to polar environment (Fig. 3, 4A). Upon addition of API at 37°C, a rapid decrease in the quantum yield of fluorescence was observed with no shift in the intrinsic fluorescence of Proteinase K indicating the absence of conformational changes in the tertiary structure due to binding. There was a slow decline in the fluorescence intensity, which attained a constant value with time (Fig.4A). The complex of Proteinase K with API at 70 °C exhibited almost a similar

fluorescence pattern as that at 37 °C demonstrating that the binding of API to the enzyme prevents the thermal unfolding of the native structure. The ratio of the relative fluorescence intensity at 338 and 358 nm ($F_{338/358}$) of native Proteinase K is 1.2. A decrease in this ratio is indicative of a red shift due to the unfolding of the tertiary structure and can be used as a probe to monitor changes in the native tertiary structure (Pawar and Deshpande, 2000). As
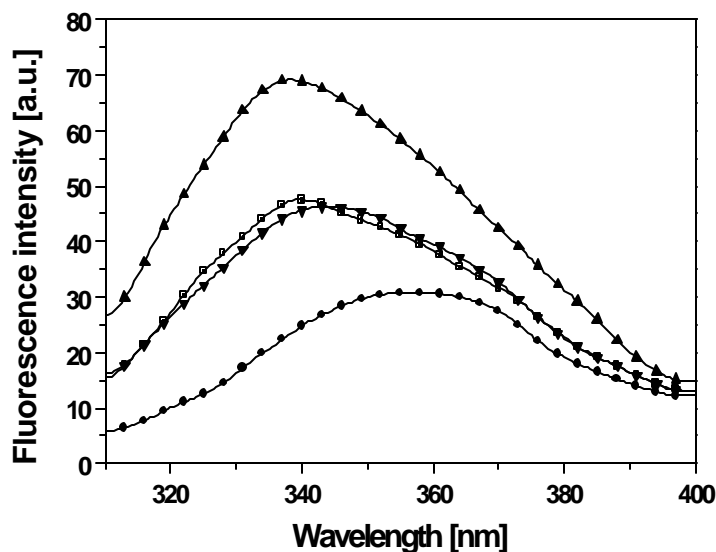
shown in Fig. 4B, there is a rapid decrease in the $F_{338/358}$ ratio of native Proteinase K on heating at 70 °C, however the decrease in the $F_{338/358}$ ratio is significantly prevented by the formation of the Proteinase K-API complex.

Figure 3: Effect of the binding of API on the tertiary structure of Proteinase K.
The fluorescence emission spectra of Proteinase K, native (▲); the complex of Proteinase K-API at 37°C (■) and 70°C (▼); heat treated Proteinase K at 70°C for 1 h (●) are shown.


(A)   **(B)**
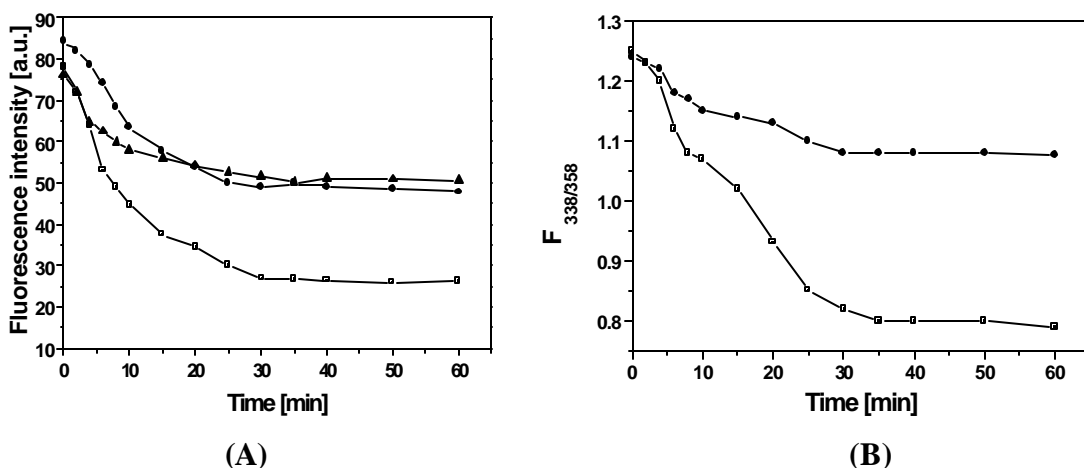
Figure 4: Effect of the binding of API on the tertiary structure of Proteinase K.

A) Proteinase K was excited at 295 nm and the time dependent change in the fluorescence intensity at 338 nm was monitored. Proteinase K treated at 70°C (■); the complex of Proteinase K-API at 37°C (▲) and 70°C (●) are shown. B) The unfolding of native tertiary structure of Proteinase K at 70 °C in absence (■) and presence of API (●) was monitored by the ratio of the fluorescence emission spectrum at 338 and 358 nm ($F_{338/358}$) at an excitation wavelength of 295 nm.

Secondary structural analysis of Proteinase K at higher temperature

In order to evaluate the effect of the inhibitor binding on the secondary structure of Proteinase K at 70°C, we have analyzed the CD spectra of Proteinase K-API complex. The circular dichroism spectra of Proteinase K display a characteristic CD spectrum in the far UV region (Fig. 5). The heating of Proteinase K at 70°C results in the thermal unfolding of the secondary structure with a concomitant loss in activity. Interestingly, the native enzyme and the enzyme-inhibitor complexes exhibited almost a similar profile of negative ellipticity in the far-UV region indicating that the binding of API to Proteinase K helps in maintaining the structural and functional integrity of Proteinase K at high temperatures.
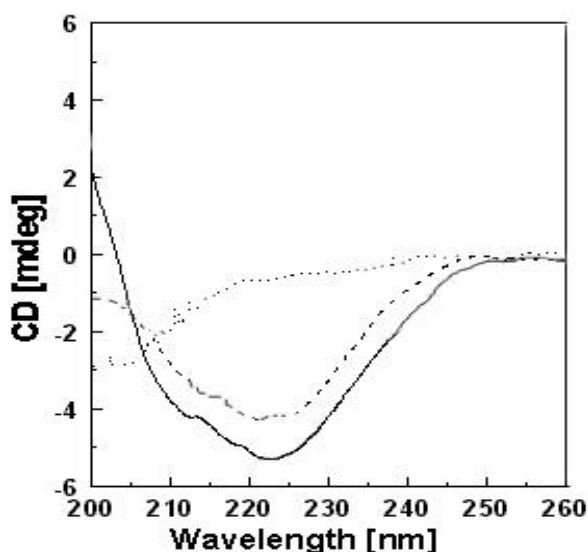
Figure 5: Effect of the binding of API on the secondary structure of Proteinase K.
Far-UV circular dichroism spectra of Proteinase K, native (——); heat treated Proteinase K at 70°C for 1 h in absence (…..) and presence (- - -) of API are shown. Each spectrum represents the average of six scans with the baseline subtracted.

Discussion

Alkaline proteases have immense potential application in leather and detergent industries due to the increasing awareness of developing environment-friendly technologies (Rao, *et al*., 1998). Higher thermal stability is an important factor for the suitability of alkaline proteases for their commercial exploitation. Detailed elucidation of the mechanisms responsible for stabilization and destabilization of enzymes especially at elevated temperatures is of special importance from both a scientific and a commercial points of view. The native conformation of an enzyme is stabilized by intramolecular interactions such as the hydrophobic interactions. Stability in a folded protein is a balance between the stabilizing interactions and the tendency towards destabilization caused by the loss of conformational entropy as the protein adopts the unfolded form. As the temperature rises, the increase in the destabilizing interactions results in protein unfolding and denaturation. Proteinase K undergoes irreversible thermal denaturation at higher temperatures resulting in the loss of its biological function. The stability of Proteinase K can be altered by the binding of API and therefore has opened up a novel strategy for the stabilization of enzymes at higher temperatures. API is a thermostable inhibitor, the presence of five disulfide linkages in API confers the conformational rigidity on the protein and increases the stabilizing interactions. It is an active site directed reversible tight-binding inhibitor of Proteinase K (Chapter 6, Section I), and at higher temperatures the inhibitor stabilizes the enzyme from inactivation. The functional coordinates in Proteinase K are therefore protected from inactivation due to complexation with API.

The binding of API to Proteinase K protects it from thermal unfolding and its subsequent inactivation. This was further corroborated by the structural studies. Thermal inactivation of Proteinase K was accompanied by the loss of secondary and tertiary structure. Unfolding of the enzyme was indicated by the diminished negative ellipicity and the red shift in the emission maximum. Thus, improved stability of the enzyme can be achieved primarily by overcoming the unfolding of the protein at elevated temperatures. Majority of the strategies employed for the stabilization of enzymes are directed towards eliminating (or minimizing) the process of unfolding by strengthening the hydrophobic interactions (Gupta, 1991). As revealed from our results, the binding of API to Proteinase K at higher temperatures stabilizes the enzyme by minimizing the loss of its secondary and tertiary structure. The

binding of API probably strengthens the stabilizing interactions, which confer structural rigidity making the enzyme resistant to unfolding. The stabilization of the alkaline protease, subtilisin by the *Streptomyces* subtilisin inhibitor (SSI) has been reported (Arakawa, *et al.*, 1991). It was demonstrated that subtilisin and SSI mutually enhance each others stability; subtilisin stabilizes SSI against SDS-induced unfolding while SSI stabilizes subtilisin against thermal denaturation. The complex of ProteinaseK-API at elevated temperatures displayed slight changes in the secondary and tertiary structure that could promote the dissociation of the inhibitor from the enzyme upon dilution.

In conclusion, our results demonstrate that the thermal stabilization of alkaline proteases can be enhanced by API, and can have enormous applications in industries utilizing alkaline proteases at higher temperatures. The extension of these studies for the thermostabilization of other commercially important alkaline proteases could lead to the development of a novel cost-effective method utilizing reversible enzyme inhibitors as stabilizing agents.

References

Arakawa, T., Lazenby, K., Kolvenbach, C. G., Horan, T. P., McGinley, M., Rohde, M. F., and Narhi, O. P. (1991) *Agric. Biol. Chem.* **55,** 930-910.

Back, J. F., Oakenfull, D., and Smith, M. B. (1979) *Biochem.* **18,** 5191-5196.

Bandivadekar, K. R., and Deshpande, V. V. (1994) *Biotech. Lett.* **16,** 179-182.

Bull, A. T., Marrs, B., Kurane, R. (Eds.) (1998) Biotechnology for Clean Industrial Products and Processes. Towards Industrial sustainability. Paris: OECD Publications.

Coolbear, T., Whittaker, J. M., and Daniel, R. M. (1992) *Biochem. J.* **287,** 367-374.

De Bolle, X., Vinals, C., Fastrez, J., and Feytmans, E. (1997) *Biochem. J.* **323,** 409-413.

Gaffney, B. J. (1985) *Biochim. Biophys. Acta* **822,** 289-317.

Gonzalez, G., Gonzalez, C., and Merino, P. (1992) *Biotech. Lett.* **14,** 919-924.

Gupta, M. N. (1991) *Biotech. Appl. Biochem.* **14,** 1-11.

Janecek, S. (1993) *Process Biochem.* **28,** 435-445.

Jany, K. D., Lederer, G., and Mayer, B. (1986) *FEBS Lett.* **199,** 139-144.

Ji, T. H. (1983) *Methods Enzymol.* **91,** 508-609.

Klibanov, A. M. (1979) *Anal. Biochem.* **93,** 1-25.

Klibanov, A. M. (1983) *Science* **219,** 722-727.

Klibanov, A. M. (1989) *Trends Biochem. Sci.* **14,** 141-144.

Kulkarni, N., Shendye, A., and Rao, M. (1999) *FEMS Microbiol. Rev.* **23,** 411-456.

Lehmann, M., and Wyss, M. (2001) *Curr, Opin. Biotechnol.* **12**, 371–375,

Melik-Nubarov, N. S., Mozhaev, V. V., Siksnis, V. A., and Martinek, K. (1987) *Biotech. Lett.* **9,** 725-730.

Monsan, P. Combes, D. (1988) *Methods Enzymol.* **137,** 584-598.

Mozhaev, V. V., Melik-Nubarov, N. S., Siksnis, V., and Martinek, K. (1990) *Biocatalysis* **3,** 189-196.

Mozhaev, V. V., Siksnis, V. A., Melik-Nubarov, N. S., Galkantaite, N. Z., Denis, G. J., Butkus, E. P., Zaslavsky, B. Yu., Mestechkina, N. M., and Martinek, K. (1988) *Eur. J. Biochem.* **173,** 147-154.

Nosoh, Y., and Sekiguchi, T. (1988) *Biocatalysis* **1,** 257-273.

Nosoh, Y., and Sekiguchi, T. (1990) *Trends Biotech.* **8,** 16-20.

Pace, C. N., and Grimsley, G. R. (1988) *Biochem.* **27,** 3242-3246.

Pawar, S. A. and Deshpande, V. V. (2000) *Eur. J. Biochem.* **267,** 6331-6338.

Rao, M., Tanksale, A., Ghatge, M., and Deshpande, V. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 597-635.

Santoro, M. M., Liu, Y., Khan, S. M. A., Hou, L., and Bolen, D. W. (1992) *Biochem.* **31,** 5278-5283.

Segawa, S. I., and Sugihara, M. (1984) *Biopolymers* **23,** 2489-2498.

Taneja, S., and Ahmad, F. (1994) *Biochem. J.* **303,** 147-153.

Tatsumoto, K., Oh, K. K., Baker, J. O., and Himmel, M. E. (1989) *Appl. Biochem. Biotechnol.* **20/21,** 293-308.

Torchilin, V. P., Maksimenko, A. V., Klibanov, A. M., Berezin, I. V., and Martinek, K. (1978) *Biochim. Biophys. Acta* **522,** 277-283.

Ward, O. P., and Moo-Youg, M. (1988) *Biotech. Adv.* **6,** 39-69.

Wong, S. S., and Wong, L-J. C. (1992) *Enzyme Microb. Technol.* **14,** 866-874.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) *Science* 217, 1214-1222.