MOLECULAR ASPECTS OF A FUNGAL ALKALINE PROTEASE

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MOLECULAR ASPECTS OF A FUNGAL ALKALINE PROTEASE

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By

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DEDICATED TO...

MY PARENTS

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CERTIFICATE

Certified that the work incorporated in the thesis entitled "**MOLECULAR ASPECTS OF A FUNGAL ALKALINE PROTEASE**" submitted by **Ms. Aparna M. Tanksale** was carried out by the candidate at the National Chemical Laboratory, Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

(**Vasanti. V. Deshpande**) **January 2001**

Research guide
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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**MOLECULAR ASPECTS OF A FUNGAL ALKALINE PROTEASE**" submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Pune has not been submitted by me for a degree to any other university or institution. This work was carried out at the National Chemical Laboratory, Pune, India.

(**Aparna M. Tanksale**)
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And above all, I thank God almighty……...

APARNA TANKSALE

ABBREVIATIONS

β-ME	β-mercaptoethanol
3,4-DCI	3,4-dichloroisocoumarin
ANS	8-Anilino-napthalene-1-sulfonic acid
BSA	Bovine serum albumin
CD	Circular dichroism
DEPC	Diethylpyrocarbonate
DFP	Di-isopropyl fluorophosphate
DTNB	Dithiobisnitrobenzene
DTT	Dithiothreitol
E-64	L-3carbozytrans2,3-epoxypropyl-leucylamido(4-guanidine)butane
GH	Guanidine hydrochloride.
HNBB	Hydroxy nitro benzyl bromide
IEF	Isoelectric focussing
NAI	N-acetylimidazole
NBS	N-bromosuccinimide
NEM	N-ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
PCMB	p-Chloromercuribenzoate
PHMB	p-Hydroxy mercuribenzoate
PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TLCK	Tosyl-L-lysine chloromethyl ketone
PG	Phenylglyoxal
OPTA	O-pthalaldehyde
WRK	Woodward's Reagent K
TNBS	Trinitrobenzenesulfonic acid
TEMED	N,N,N',N' tetramethyl ethylenediamine
DAN	Diazoacetyl-DL-norleucine methyl ester
EPNP	1,2-Epoxy-3-(p-nitrophenoxy) propane
sAAPF-pNA	N-succinyl-L-Ala-Ala-Pro-Phenylala-p-nitroanilide

TPCK	Tosyl-L-phenylalanine chloromethyl ketone
5-IAF	5-Iodoacetamidofluorescein

ABSTRACT

Proteases are one of the highest value commercial enzymes as they have extensive applications in food, pharmaceutical, detergent, and dairy industries and serve as important tools in determination of structure of proteins and polypeptides. The biotechnological promise of proteases makes them an ideal candidate for structure-function relationship studies. Alkaline proteases hold a great potential for application in leather and detergent industries due to the increasing awareness of developing environmentally friendly technologies. Fungal alkaline proteases offer a distinct advantage over the currently used bacterial proteases in terms of the ease of preparation of microbe-free enzyme as against the cost-intensive filtration technology required for the isolation of a bacterial enzyme. A fungus *Conidiobolus macrosporus* (NCIM 1298) isolated in our laboratory produces high amounts (30U/ml) of an alkaline protease (APC). Its activity at highly alkaline pH, broad substrate specificity, and the ease of cell-free enzyme preparation suggest that it has a potential for industrial application. Higher thermostability is often a primary goal for improving the properties of an industrial enzyme. A comprehensive understanding of the active site of the enzyme and of the mechanism of its inactivation is essential for delineating its structure-function relationship. The present investigation, therefore, was undertaken to study some of the molecular and biochemical aspects of the protease.

The highlights of the work done are as follows:

1. Structure-function relationship of the APC: purification of APC and characterization of the amino acid residues at or near the active site.
2. Characterization of unfolding and refolding pathways of the protease.
3. Thermal stability of the alkaline protease:

   (i) Thermal inactivation of the alkaline protease and use of various additives to improve the thermal stability.

   (ii) Immobilization of alkaline protease for reuse and increased thermal stability.

Chapter 1: General Introduction:

This part comprises of literature survey with reference to proteases, their occurrence, mechanism of action, functions, and applications.

Chapter 2: Structure-function relationship of an alkaline protease (APC) from *Conidiobolus macrosporus* (NCIM 1298):

Conidiobolus macrosporus (NCIM 1298) produces about five electrophoretically distinct extracellular alkaline proteases. In the present studies, one of the major proteases (APC) having highest electrophoretic mobility and activity has been purified to homogeneity using gel-X-ray film contact print technique followed by DEAE-cellulose chromatography. The purified APC showed a single band on SDS-PAGE and eluted as a single peak upon gel filtration on HPLC as well as on isoelectric focussing confirming homogeneity of the preparation. The optimum pH of the enzyme is pH 10.0 and it exhibited maximum activity at 40°C. The enzyme was stable over a wide range of pH (5.0-12.0) and at 45°C for 1 h at pH 7.5. APC showed a molecular weight of 28,500 and 27,000 by SDS-PAGE and gel-filtration respectively and a pI of 9.8. The molecular weight was further confirmed by amino acid analysis, which revealed that the APC consists of 263 amino acid residues. The protein was 11% glycosylated. It was inhibited by *Streptomyces* subtilisin inhibitor (SSI) but not by soybean-trypsin inhibitor. APC was able to hydrolyze substrates such as casein, hemoglobin, gelatin, myoglobin, ovalbumin, and BSA indicating its broad substrate specificity. The enzyme was inhibited by metal ions such as Cu^{2+}, Hg^{2+}, and Fe^{3+}.

Inhibition of the APC by Woodward's reagent K (WRK) (10 mM), diethylpyrocarbonate (DEPC) (5 mM) and phenylmethylsulfonyfluoride (PMSF) (10 μM) indicated the presence of Asp, His and Ser respectively in the active site of the alkaline protease. There was no effect of EDTA (10 mM) on the enzyme activity. Inactivation of the enzyme by PMSF was not reversed by treatment with β-mercaptoethanol. The enzyme was inhibited by PHMB (10 μM) and the inhibition was reversed by treatment with thiol containing compounds. These results confirm that APC is a serine alkaline protease and not a cysteine or metalloprotease. In addition, the protease was also inhibited by N-

bromosuccinimide (NBS) (10μM) pointing to the presence of Trp residue in the active site. The presence, microenvironment and proximity of the essential Trp with the essential His and Cys residues in the active site of the alkaline protease were deciphered for the first time using chemical modification, chemo-affinity labeling and fluorescence spectroscopy. Kinetics of inactivation by N-bromosuccinimide (NBS) and p-hydroxymercuri-benzoate (PHMB) revealed that a single Trp and Cys are essential for the activity besides the Asp, His and Ser residues of the catalytic triad. Full protection by casein against inactivation of the enzyme by NBS and quenching of Trp fluorescence upon binding of the enzyme with NBS, substrate (sAAPF-pNA) or *Streptomyces* subtilisin inhibitor (SSI) confirmed the participation of the Trp residues at the substrate/inhibitor binding site of the alkaline protease. The microenvironment around the Trp residues was probed by studying the quenching of fluorescence by various solute quenchers. Comparison of the K_{sv} values for the charged quenchers CsCI (1.66) and KI (7.0) suggested that overall Trp microenvironment in the protease is electropositive. The proximity of Trp with His was revealed by sigmoidal shape of the fluorimetric titration curve with a pK_a of 6.1. Both protonated and modified form (DEPC treated) of histidine could quench the Trp fluorescence but quenching by the protonated form was greater. The vicinity of essential Cys with the Trp was demonstrated by the resonance energy transfer between the intrinsic fluorophore (Trp) and the 5-iodoacetamide-fluorescein labeled Cys (extrinsic fluorophore). Our results on the proximity of Trp with essential His and Cys thus confirm the presence of Trp in the active site of the APC.

Chapter 3: Characterization of intermediates on the unfolding and refolding pathways of the APC:

Section I: Interaction of the molten-globule state of APC with the molecular chaperone, a -crystallin:

α-Crystallin, the major eye lens protein with sequence homology to small heat shock proteins, acts like a molecular chaperone by suppressing the aggregation of damaged crystallins and proteins. To gain an insight into its chaperoning ability, we used a protease as the model system that is known to require a propeptide (intramolecular chaperone) for its proper folding. Under the conditions of low ionic strength (0.05 M),

the APC unfolds at pH 2.0 through a partially unfolded 'I' state at pH 3.5 which undergoes transition to a molten-globule like 'I_A' state in the presence of 0.5 M sodium sulfate. At higher temperature, the 'I_A' state showed a complete loss of structure and was prone to aggregation. α-Crystallin bound to this 'thermally stressed I_A state' and suppressed its irreversible denaturation due to aggregation. α-Crystallin-bound enzyme exhibited native-like secondary and tertiary structure demonstrating for the first time the interaction of α-crystallin with the molten-globule state of the protease. ANS-binding studies revealed the involvement of hydrophobic interactions in the formation of the complex of α-crystallin and APC. Unfolding of the protease in presence of α-crystallin and its subsequent refolding by dilution to pH 7.5 resulted in an intermediate state having partial tertiary and secondary structure. α-Crystallin added to the already unfolded APC was unable to refold the protein pointing to a clear difference in the mechanism of chaperoning action of propeptide and α-crystallin. Our results show that α-crystallin blocks the unfavorable pathways that lead to irreversible denaturation of the APC and keeps it in a near-native, folding-competent intermediate state.

Section II: *In vitro* refolding of the mature form of APC in the absence of propeptide:

The unfolding of the APC by guanidine hydrochloride (GH) and its refolding were studied using spectroscopic techniques such as CD, fluorescence, and size-exclusion chromatography. The unfolding was complete at 2.75 M GH and followed a two-state mechanism. The ΔG_{H2O} value or conformational stability of the native state was found to be 2.87 Kcal/mol. The reversibility of the unfolded state was found to be dependent on the denaturant concentration. The APC unfolded by GH concentration of upto 3.5 M was able to refold merely by dilution; only 5% refolding was observed for the APC denatured with 4 M GH. Increase in ionic strength of the refolding buffer, however, accelerated the refolding rate for these slow refolding species with the maximum of 44% activity recovered in presence of 0.1M $MgSO_4$. Addition of sodium sulfate instead of $MgSO_4$ could not assist refolding revealing the role of Mg^{2+} ions in the process of refolding. This is the first report of *in vitro* refolding of an alkaline protease in the absence of a propeptide.

Chapter 4: Thermal stability of the APC:

Section I: Unfolding precedes autoproteolysis during thermal inactivation of APC:

Thermal inactivation of APC was studied both at its stability pH (7.5) and optimum pH (10) in the presence and absence of additives. The enzyme was stable at 40°C at pH 7.5 for 1 h. There was a progressive loss in activity with increasing temperature and complete loss of activity occurred at 60°C. The kinetics of inactivation of the APC at various temperatures at pH 7.5 does not follow the first-order kinetics. Thermal inactivation was accompanied by the conformational changes in the protein structure as revealed by the diminished α-helical content and red shifted fluorescence spectrum of the enzyme. The effect of various additives to enhance the thermal stability of APC by means of modifying its microenvironment was studied. Glycerol (50 %) and sorbitol (25 %) offered 43 and 49 % protection respectively against inactivation of the enzyme at 55°C, at pH 7.5. Glycine (1 M) or magnesium sulfate (100 mM) offered 25 - 30% protection. Stabilization of the enzyme by sugar alcohols or by glycine was due to the prevention of the unfolding of the protein tertiary structure, as revealed by the decrease in the extent of red shift of fluorescence spectra of the enzyme. At 55°C at pH 10.0, the loss in enzyme activity was too rapid to be measured. The APC undergoes autoproteolysis under these conditions as evident by the decrease in the intensity of the protease band upon SDS-PAGE. No thermal stabilization was offered by the additives at this pH indicating that they prevent the unfolding but not the autoproteolysis of the APC.

Section II: Immobilization of the alkaline protease: reuse and improved thermal stability:

Alkaline protease from a fungus *Conidiobolus macrosporus* (NCIM 1298) was immobilized on an inexpensive support, polyamide, using glutaraldehyde as a bifunctional agent. The immobilized enzyme showed a higher optimum temperature of 50°C as compared to that of the free enzyme (40°C). The polyamide-bound enzyme showed maximum activity in the pH range of 8-9, whereas the optimum pH of the soluble enzyme was pH 10. The efficiency of immobilization was 58% under the optimal conditions of pH and temperature. The immobilized enzyme was stable over a wide range of pH (5.0 - 12.0) and exhibited an increased thermostability (50% activity at 60°C).

There was a fourteen-fold decrease in the K_m of immobilized enzyme compared to the free enzyme indicating increased affinity for the substrate. The immobilized enzyme was fully active even after twenty-two cycles of repeated use displaying excellent durability. It catalyzed the hydrolysis of ovalbumin and hemoglobin in addition to casein revealing its broad substrate specificity. The polyamide-bound enzyme retained 80% activity at 50°C in presence of 8 M urea exhibiting its stability to the denaturant at elevated temperatures. It was compatible with the detergents such as Rin Shakti, Ariel, and Surf-Excel as deduced by the retention of significant amount of its activity (>57%) in their presence. The improved properties of the immobilized enzyme such as (i) increased stability at higher temperatures, (ii) retention of activity in presence of denaturants and detergents, (iii) broad substrate specificity and (iv) an excellent durability for repeated use make the enzyme a suitable candidate for its application in industries.

PUBLICATIONS

1. Molecular and Biotechnological Aspects of Microbial Proteases.
   M. B. Rao, **A. M. Tanksale**, M. S. Ghatge and V. V. Deshpande
   Microbiology and Molecular Biology Reviews, **62**: 597-635, 1998.
2. Evidence for tryptophan in proximity to histidine and cysteine as essential to the active site of an alkaline protease.
   A. M. Tanksale, J. V. Vernekar, M. S. Ghatge, and V. V. Deshpande
   Biochemical Biophysical Research Communications. **270**: 910-917, 2000.
3. Immobilization of alkaline protease from *Conidiobolus macrosporus* for reuse and improved thermal stability.
   A. M. Tanksale, P. Manish Chandra, M. B. Rao, and V. V. Deshpande.
   Biotechnology Letters **23:** 51-54, 2001.
4. Autoproteolysis precedes thermal inactivation in an alkaline protease from *Conidiobolus macrosporus*.
   M. S. Ghatge, **A. M. Tanksale,** and V. V. Deshpande.
   Communicated.
5. Structure-function relationship of alkaline protease inhibitor: protease inhibitory activity as biochemical basis of the antifungal activity.
   J. V. Vernekar, **A. M. Tanksale,** M. S. Ghatge, and V. V. Deshpande
   Communicated.
6. Interaction of the molten-globule state of an alkaline protease with the molecular chaperone α–crystallin.
   A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande
   Manuscript under preparation.

CONFERENCES / WORKSHOPS

- Evidence for the presence of tryptophan at the active site of an alkaline protease from *Conidiobolus macrosporus* (NCIM 1298)

  A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande

  Poster presentation in the 67th annual meeting of SBC held in Delhi during December 24 to 26, 1998.

- Structure-function relationship of the protease from *Conidiobolus* sp.

  A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande

  Poster presentation in the international symposium: Frontiers in Structural Biology held in Bangalore during August 25-28, 1999.

- Protein structural bioinformatics and genomics.

  Workshop held in National Centre for Biological Sciences, Bangalore on August 28, 1999.

- Biomolecular Structure and Modeling.

  Workshop held in the Bioinformatics Centre, Pune, from December 14-17, 1999.

- Modern perspectives in yeast science and technology.

  Symposium held in National Chemical Laboratory on September 18, 2000.

- Unfolding precedes autoproteolysis during the thermal inactivation of an alkaline protease from *Conidiobolus macrosporus* (NCIM 1298).

  M. S. Ghatge, **A. M. Tanksale**, and V. V. Deshpande

  Poster presentation in the annual meeting of AMI held in Jaipur during October 24 to 28, 2000.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Although they catalyze but a single reaction, the hydrolysis of peptide bond, the various ways they achieve this, their ubiquitous distribution among all life forms, their multiplicity of locations inside, outside and at the surface of cells and, above all, their enormous diversity of function makes them one of the most fascinating groups of enzymes. They are presumed to have arisen in the earliest phases of biological evolution, some billion years ago since the present digestive proteases can be shown to have a common ancestry with those of the microbial origin. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications [1,2]. Proteases represent one of the largest groups of industrial enzymes (amylases and glucose-isomerases being the other two) and account for about 60% of the total worldwide sale of enzymes [3]. 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. The major producers of proteases distributed worldwide are Novo Industries (Denmark), Gist-Brocades (Netherlands), Genencor International and Miles Laboratories (United States).

SOURCES OF PROTEASES:

Since proteases are a physiological necessity for living organisms, they are ubiquitous, and are found in a wide diversity of sources such as plants, animals and microorganisms. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin. Papain is extracted from the latex of *Carica papaya* fruits and is extensively used in industry for the preparation of highly soluble and flavored protein hydrolysates [4]. Keratinases are the proteases, which degrade hair. Digestion of hair and wool is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems [5]. The most familiar proteases of animal

origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins [6,7] which are usually released from their zymogen either by autocatalysis or by the hydrolytic action of other enzymes. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. Chymotrypsin is found in animal pancreatic extract and is used for diagnostic and analytical purposes. Pepsin is an acidic protease found in the stomachs of all vertebrates while rennet is a pepsin-like protease produced in the stomachs of all nursing mammals and is used extensively in the dairy industry to produce a stable curd with good flavor. The specificity of some of these proteases has been listed in Table1.1.

Table 1. 1 Specificity of proteases

Enzyme	Peptide bond cleaved[a]
Trypsin	--Lys (or Arg) ↓ ---
Chymotrypsin, subtilisin	--Trp (or Tyr, Phe, Leu) ↓ ---
Staphylococcus V8 protease	--Asp (or Glu) ↓ ---
Papain	--Phe (or Val, Leu) - Xaa ↓ ---
Thermolysin	---↓ Leu (or Phe) ---
Pepsin	-- Phe (or Tyr, Leu) --↓ Trp (or Phe, Tyr)

[a]The arrow indicates the site of action of the protease. Xaa, any amino acid residue.

Microorganisms represent an excellent source of enzymes due to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales [3] and are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Most commercial proteases mainly neutral and alkaline are produced by the genus *Bacillus*. Fungi elaborate a wider variety of enzymes than bacteria, e.g., *Aspergillus oryzae* produces acid, neutral and alkaline proteases. Fungal acid proteases are useful in cheesemaking industry, neutral proteases in reducing the bitterness of food protein hydrolysates and alkaline proteases in food protein modification. Most of the viral encoded peptidases are endopeptidases of serine, cysteine or aspartic type.

CLASSIFICATION OF PROTEASES:

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) [8]. However proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (I) type of reaction catalyzed, (II) chemical nature of catalytic site, and (III) evolutionary relationship with reference to structure [9]. Depending on their site of action, proteases are grossly subdivided into two major groups, i.e. exopeptidases and endopeptidases (Table 1.2). Based on the functional group at the active site, proteases are further classified into serine, aspartic, cysteine and metallo proteases or of unknown type, e.g., ATP-dependent proteases which require ATP for their activity [10]. Based on their amino acid sequences, proteases are classified into different families [11] and further classified into clans to accommodate sets of peptidases that have diverged from a common ancestor [12].

Exopeptidases act only near the ends of polypeptide chains, further classified as amino- or carboxypeptidases based on their site of action at the N or C terminus respectively. Aminopeptidases liberate a single amino acid residue, a dipeptide (dipeptidyl peptidase) or a tripeptide (tripeptidyl peptidase). The substrate specificities of the enzymes from bacteria and fungi are distinctly different in that the organisms can be differentiated based on the profiles of the products of hydrolysis [13]. Aminopeptidases can also be classified as aminopeptidase N or aminopeptidase A, depending on their preference for neutral (uncharged) or acidic side chains respectively. Most of the aminopeptidases are metallo-enzymes. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups. Other exopeptidases include dipeptidases, which cleave a dipeptide and omega peptidases which release modified residues from N- or C- termini.

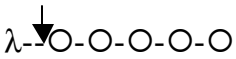
Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N or C termini. They are divided into five subgroups based on their catalytic mechanism, (i) serine proteases, (ii) cysteine

proteases, (iii) aspartic proteases, (iv) metalloproteases and (v) unknown type denoted by the letters S, C, A, M, and U, respectively.

Table 1.2. Classification of proteases

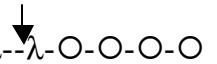
PROTEASES

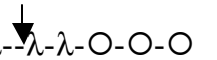
EXOPEPTIDASES

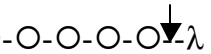


(Based on Actions)

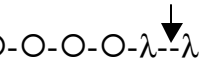
Aminopeptidases
(3.4.11) 

Dipeptidyl peptidase
(3.4.14) 

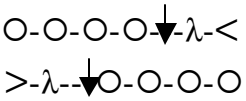
Tripeptidyl peptidase
(3.4.14) 

Carboxypeptidases
(3.4.16-18) 

carboxypeptidase (serine) (3.4.16)
carboxypeptidase (metallo) (3.4.17)
carboxypeptidase (cysteine) (3.4.18)

Peptidyl dipeptidases
(3.4.15) 

Dipeptidases
(3.4.19) 

Omegapeptidases
(3.4.19) 

ENDOPEPTIDASES

(Based on Active sites)

Serine endopeptidases (3.4.21)
Cysteine endopeptidases (3.4.22)
Aspartic endopeptidases (3.4.23)
Metallo endopeptidases (3.4.24)
Endopeptidases of unknown catalytic mechanism (3.4.99)

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

Serine proteases: serine proteases are characterized by the presence of a serine group in their active site. Based on their structural similarities, they have been grouped into 20 families, which have been further subdivided into about six clans with common ancestors [9]. The classification of peptidases based on the evolutionary relationships has been given in Barett et al., [14], and MEROPS – the peptidase database; url: http://www.bi.bbsrc.ac.uk/Merops/merops.htm. Trypsin, chymotrypsin are the well studied proteases of this subgroup. Another interesting feature of the serine proteases is the conservation of glycine residues near the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly [15]. They are recognized by their irreversible inhibition by 3,4-DCI, E.64, DFP, PMSF and TLCK. Some are inhibited by thiol reagents such as PCMB due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. Their isoelectric points are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

Serine alkaline proteases: These are produced by several bacteria, molds, yeasts and fungi. They are inhibited by DFP but not by TLCK or TPCK. They hydrolyze a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of the 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 *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp [16], subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *S. cerevisae* [17] and filamentous fungi such as *Conidiobolus* spp. [18] and *Aspergillus* and *Neurospora* spp. [19].

Subtilisins: Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, Subtilisin Carlsberg produced by *Bacillus licheniformis* and Subtilisin Novo or BPN' produced by *B. amyloliquefaciens* have been identified. Subtilisin Carlsberg is widely used in detergents. The active-site conformation of subtilisins is similar to that of trypsin and chymotrypsin despite the dissimilarity in their overall molecular arrangements. The serine alkaline protease from

the fungus *Conidiobolus coronatus* was shown to possess a distinctly different structure from Subtilisin Carlsberg in spite of their functional similarities [20].

Aspartic proteases: Commonly known as acid proteases, these proteases depend on aspartic acid residues for their catalytic activity. They have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) [21] and have been placed in the clan AA. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes [22]. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly in which Xaa can be Ser or Thr. The aspartic acid proteases are inhibited by pepstatin and diazocompounds such as DAN and EPNP in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin. They can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus, Penicillium, Rhizopus* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* sp.

Cysteine/thiol proteases: The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His residues differs among the 20 families [9]. Generally cysteine proteases are active 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 for glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents.

Metalloproteases: These are the most diverse of the catalytic types of proteases [23] characterized by the requirement of a divalent metal ion for their activity. Out of 30 families of metalloproteases, 17 contain only endopeptidases, 12 contain only exopeptidases and 1 (M3) contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino

acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H. 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 alkaline proteases possess a very broad specificity. Myxobacter 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. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP. Thermolysin, collagenase and elastase are the well studied metalloproteases. Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing, and may be useful in the treatment of diseases such as cancer and arthritis [24].

MECHANISM OF ACTION OF PROTEASES:

The mechanism of action of proteases has been a subject of great interest to researchers as it forms a basis for exploring various ways of modifying its activity to make it suitable for its biotechnological application. Studies of the mechanism of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration. 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 S1' through Sn' toward the C terminus. The residues which they accommodate from the substrate are numbered P1 through Pn and P1' through Pn', respectively (Fig. 1.1).

Protease:	N	Sn ----- $S_3 - S_2 - S_1$ κ $S_1' - S_2' - S_3'$ ----- Sn' C
Substrate:	N	Pn ----- $P_3 - P_2 - P_1$ --¦-- $P_1' - P_2' - P_3'$ ----- Pn' C

Fig. 1.1 Active sites of proteases.

The catalytic site of proteases is indicated by κ and the scissile bond is indicated by --¦--.

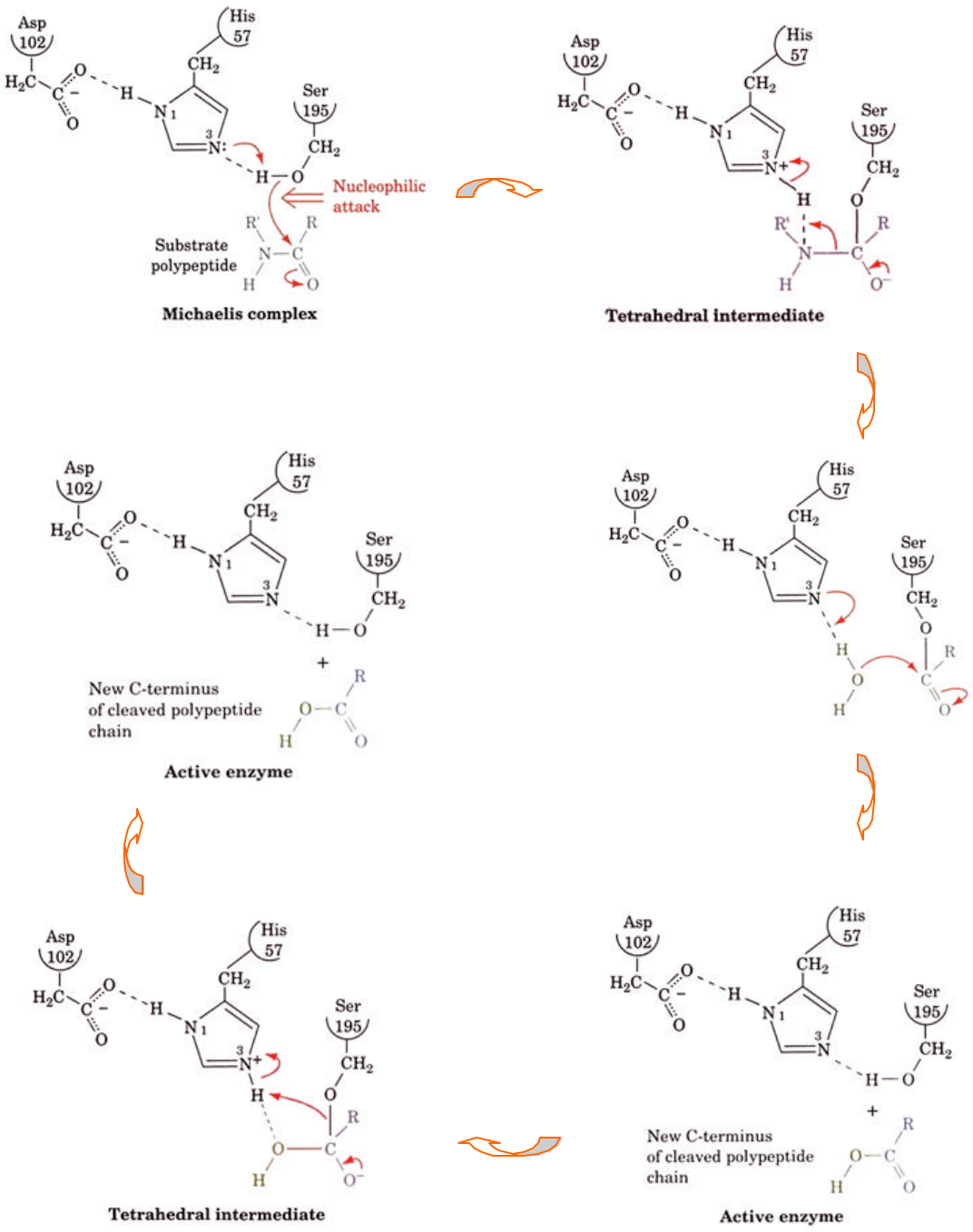


Fig.1.2 Mechanism of action of serine proteases.

(Ref.: http://info.bio.cmu.edu/courses/03231/Protease/SerPro.htm)

Serine proteases:

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 [25]. 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. The key steps in the hydrolysis have been illustrated in Fig. 1.2. The 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 P1 residue exclusively dictates the site of peptide bond cleavage. The primary specificity is affected only by the P1 residues; the residues at other positions affect the rate of cleavage. The subsite interactions are localized to specific amino acids around the P1 residue to a unique set of sequences on the enzyme. Some of the serine peptidases from the *Achromobacter* spp. are lysine-specific enzymes [26], whereas those from *Clostridium* spp. are arginine specific (clostripain) [27] and those from *Flavobacterium* spp. are post proline-specific [28]. Endopeptidases that are specific to glutamic acid and aspartic acid residues have also been found in *B. licheniformis* and *S. aureus* [29]. The recent studies based on the three-dimensional structures of proteases and comparisons of amino acid sequences near the primary substrate-binding site in trypsin-like proteases of viral and bacterial origin suggest a putative general substrate binding scheme for proteases with specificity towards glutamic acid involving a histidine residue and hydroxyl function. However, a few other serine proteases such as peptidase A from E. coli and the repressor LexA show distinctly different mechanism of action without the classic Ser- His- Asp triad [9]. Some of the glycine residues are conserved near the catalytic serine residue, but their exact postitions are variable [15]. A few of the serine proteases belonging to the subtilisin family show a catalytic triad composed of the same residues as in the chymotrypsin family; however, the residues occur in a different order (Asp-His-Ser). Some members of the subtilisin family from the yeasts *Tritirachium* and *Metarhizium* sp. require thiol for their actiity. The thiol dependance is attributable to Cys 173 near the active-site histidine [30].

The carboxypeptidases are unusual among the serine-dependent enzymes in that they are maximally active at acidic pH. These enzymes are known to possesss a Glu residue preceding the catalytic Ser, which is believed to be responsible for their acidic pH optimum. Although the majority of the serine proteases contain the catalytic triad of Ser-His-Asp, a few use the Ser-base catalytic dyad. The Glu-specific proteases display a pronounced preference for Glu-Xaa bonds over Asp-Xaa bonds [31].

Aspartic proteases

Crystallographic studies have shown that the enzymes of the pepsin family are bilobed molecules with the active-site cleft located between the lobes and each lobe contributing one of the pair of aspartic acid residues that is essential for the catalytic activity [22, 32]. In most of the enzymes from the pepsin family, the catalytic Asp residues are contained in an Asp-Thr-Gly-Xaa motif in both the N- and C-terminal lobes of the enzyme, where Xaa is Ser or Thr, whose side chains can hydrogen bond to Asp. However Xaa is Ala in most of the retropepsins. The pepsins and majority of the members of the family show specificity for the cleavage of bonds in peptides of at least six residues with hydrophobic amino acids in both the P1 and P1' positions [33]. The structural and kinetic studies have suggested that the mechanism involves general acid-base catalysis with lytic water molecule that directly participates in the reaction.

Metalloproteases

Metalloproteases depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. Most of these are enzymes containing His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form a part of the site for binding of the metal, usually zinc. For thermolysin, based on the X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that Glu 143 assists the necleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the Zn^{2+} ion [34].

Cysteine proteases

Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of the serine proteases. The initial step in the catalytic process involves the

noncovalent binding of the free enzyme and the substrate to form the complex followed by the acylation of the enzyme with formation and release of the first product, the amine R'-NH2. In the next deacylation step, the acyl-enzyme reacts with a water molecule to release the second product, with the regeneration of free enzyme. The plant peptidase papain can be considered the archetype of cysteine peptidases and constitutes a good model for this family of enzymes, which catalyze the hydolysis of peptide, amide, ester, thiol ester and thiono ester bonds [35].

PHYSIOLOGICAL FUNCTIONS OF PROTEASES:

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. 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. Some of the major activities in which the proteases participate are described below.

Catabolism of proteins provides a ready pool of amino acids as precursors for the synthesis of proteins. Intracellular proteases such as ATP-dependent proteases in *E. coli* [36] and eukaryotes [37] are known to participate in executing the proper protein turnover for the cell. Formation of spores in bacteria [38], ascospores in yeast [39], fruiting bodies in slime moulds [40] and conidial discharge in fungi [41] involve intensive protein turnover. The alkaline serine protease of *Conidiobolus coronatus* was shown to be involved in forcible conidial discharge using a less conidia forming mutant. Formation of the less active protease by antoproteolysis represents a novel means of physiological regulation of protease activity in *Conidiobolus coronatus* [42]. Degradation of proteins in dormant spores by serine endoproteinases makes amino acids and nitrogen available for the biosynthesis of new proteins and nucleotides. Their activity is rapidly lost on germination of the spores [43]. Microconidal germination and hyphal fusion [44], also involves participation of a specific alkaline serine proteinase. Extracellular acid proteases are implied in the breakage of cell wall polypeptide linkages during germination of *Dictyostelium discoideum* spores [45] and *Polysphondylium pallidum* microcysts [46]. Activation of zymogenic forms of chitin

synthase by limited proteolysis has been observed in *Candida albicans*, *Mucor rouxii* and *Aspergillus nidulans*. Kex-2 protease catalyses the hydrolysis of prohormones, integral membrane proteins of the secretory pathway by specific cleavage at the carboxyl side of pairs of basic residues such as Lys-Arg or Arg-Arg [9]. Pepsin, trypsin and chymotrypsin occur as their inactive zymogenic forms, which are activated by the action of proteases. Proteinases A and B from yeast inactivate several enzymes in a two-step process involving covalent modification of proteins as a marking mechanism for proteolysis. Proteolytic modification of enzymes is known to result in a protein with altered physiological function e.g. leucyl-L-RNA synthetase from *E. coli* is converted into an enzyme which catalyzes leucine-dependent pyrophosphate exchange by removal of a small peptide from the native enzyme. The extracellular enzymes play a major role in nutrition due to their depolymerizing activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition. Modulation of gene expression mediated by protease has been demonstrated [47]. Proteolysis of a repressor by an ATP-requiring protease resulted in a derepression of the gene. Change in the transcriptional specificity of the B-subunit of RNA polymerase in *Bacillus thuringiensis* was correlated to its proteolytic modification [48]. Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation. Besides the general functions that are described so far, the proteases also mediate the degradation of a variety of regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophage [49] and programmed bacterial cell death [50]. Recently, a new physiological function has been attributed to the ATP-dependent proteases conserved between bacteria and eukaryotes. It is implied that they act as chaperones and mediate not only proteolysis but also the insertion of proteins into membranes and disassembly or oligomerization of protein complexes [51].

APPLICATIONS OF PROTEASES:

Proteases have a large variety of applications, major among them being in food, pharmaceutical, detergent and leather industries. Proteases that are used in food and

detergent industry are prepared in bulk quantities and utilized as crude preparations, whereas those that are employed in medicine are produced in low bulk but require high degree of purity prior to their applications.

1. Detergents: Proteases are one of the standard ingredients of all kinds of detergents ranging from household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in the laundry detergents accounts for approximately 25% (around 13 billion tons per year) of the total worldwide sales of enzymes. The ideal detergent protease should possess broad substrate specificity to facilitate removal of a large variety of stains due to blood, food and other body secretions. Activity and stability at high pH, temperature, compatibility with other chelating and oxidizing agents and high pI value added to the detergents are among the major prerequisites for the application of proteases in detergents. Esperase and Savinase T from Novo Industry produced by the alkalophilic *Bacillus* are the two commercial preparations, which have very high isoelectric points (pI 11) and hence can withstand higher pH ranges. A combination of lipase, amylase and cellulase is expected to enhance the performance of protease in the laundry detergents. All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of down-stream processing to prepare a microbe-free enzyme. An alkaline protease from the *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India and retained 43% of its activity at 50°C for 50 min in the presence of Ca^{2+} (25 mM) and glycine (1 M) [52].

2. Leather Industry: The use of proteases as alternatives to hazardous chemicals such as sodium sulphide has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and to remove non-fibrillar proteins such as albumins and globulins. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce time required for soaking. Alkaline proteases with hydrated lime and sodium chloride are used for dehairing resulting in significant reduction in wastewater. Trypsin in combination with other proteases of *Bacillus* and *Aspergillus* origin is used for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin and the amount of enzyme depends on the

type of leather (soft or hard) desired to be produced. Increased usage of enzymes for dehairing and bating not only prevents pollution problems but is also effective in saving energy. Novo Nordisk manufactures three different proteases viz. Aquaderm TM, NUE, and Pyrase for use in soaking, dehairing and bating respectively.

3. Food Industry: The use of proteases in food industry dates back to antiquity. The enzymes have been routinely used for various purposes such as cheesemaking, baking, preparation of soya hydrolysates and meat tenderization. In cheese making, the primary function of proteases is to hydrolyse the specific peptide bond viz. Phe105 - Met106 bond to generate parakappa-casein and macropeptides. Chymosin is preferred due to its highest specificity for casein, which is responsible for its excellent performance in cheese making. The proteases produced by GRAS (Genetically Regarded As Safe) cleared microbes such as *Mucor miehei*, *Bacillus subtilis*, *Endothia parasitica* are gradually replacing chymosin in cheese making. Genencor International increased its production in *Aspergillus niger var. awamori* to commercial levels. Whey is a byproduct of cheese manufacture. The insoluble heat denatured whey protein is solubilised by treatment with immobilised trypsin. Endo and exoproteases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough. Proteases have been used from ancient times to prepare soya sauce and other soya products. Proteolytic modification of soya proteins helps to improve their functional properties. Treatment of soya proteins with alcalase at pH 8 results in the soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks, and in the formulation of dietetic feeds. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering of protein hydrolysates. Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxy peptidase A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of endoprotease for the primary hydrolysis and the aminopeptidase in the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness. Immobilized preparation of thermolysin from *Bacillus*

thermoproteolyticus is used for the enzymatic synthesis of aspartame, which is used as a non-calorific artificial sweetener.

4. Pharmaceutical Industry: The wide diversity and specificity of proteases is gainfully utilized in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* (Luizym, Nortase) has been used as the digestive aids to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin in combination with broad-spectrum antibiotics is used in the treatment of burns and wounds. An asparginase isolated from *E. coli* is used to eliminate aspargine from circulating blood, which is found to be present in the various forms of lymphocytic leukemia.

5. Proteases in peptide mapping and sequencing: Limited proteolysis is necessary to study initial cleavage products, to monitor the time course of a reaction or to generate large peptide fragments. Examples of limited proteolysis in vivo are zymogen activation, prohormone processing or cleaving out peptides of a polypeptide chain. In most cases both peptide mapping and sequencing of proteins require a complete fragmentation of the protein, resulting in a strongly defined and reproducible peptide pattern. Proteases of lower specificity like chymotrypsin [53], thermolysin [54], subtilisin [55] or pepsin [56] cleave the protein adjacent to several amino acid residues, thereby yielding more and shorter peptide fragments. Coagulation factor Xa [57], thrombin [58] and enteropeptidase [59] are especially used during the isolation of recombinant proteins. Their sites are cloned into positions where they allow cleaving fragments or proteins of interest out of a construct or fusion protein. Exopeptidases are valuable tools for cleaving off N-terminal blocking groups, like N-acetyl or formyl groups or a pyroglutamate residue. Carboxypeptidases are used to get sequence information from the C-terminal end of the protein.

6. Proteases in the study of protein conformation: Limited proteolysis can be used as a classical biochemical method to probe structure and dynamics of proteins in solution, providing experimental results which are easy to obtain and well complement those derived from the use of other more classical physicochemical methods and approaches. The most suitable proteases for such a study are those displaying broad substrate specificity, such as subtilisin [60], thermolysin [61], proteinase K and pepsin [62]. The

protein fragment mixture generated by limited proteolysis can be analyzed by electrophoretic or chromatographic methods. Limited proteolysis can be used for monitoring the overall unfolding of a globular protein to the random-coil polypeptide chain [63] or local unfolding of a protein molecule when exposed to a denaturing environment. It can be used to dissect multidomain proteins into fragments capable of an independent folding since the peptides between the domains are usually flexible and serve as sites of preferential proteolysis compared to the individual domains of tight and rigid conformation [64]. The most classical cases being the dissection of immunoglobulin molecule into Fab and Fc pieces [65] and of calmodulin [66]. Limited proteolysis can be used to remove loose, flexible parts of a protein e.g. Hirudin [67] wherein a disordered C-terminal tail was separated from the well-structured, rigid N-terminal core domain using a variety of proteases (subtilisin, thermolysin, trypsin, and V8 protease). Recently, using the same rationale, the minimum size of a folded fragment of thermolysin was determined [68]. Recently, limited proteolysis has been used to probe the structural and dynamic differences between the holo and apo form of horse myoglobin (Mb) [69]. A variety of proteases (subtilisin, thermolysin, chymotrypsin and trypsin) cleave apoMb at the level of chain segment 89-96, whereas holoMb is fully resistant to proteolysis, thus showing that only the F-helix in apoMb is largely disrupted which was earlier inferred from spectroscopic measurements. Proteolytic enzymes can also be used as probes of structural features of partly folded states or molten globule states of proteins [70].

7. Proteases in the study of membrane proteins: Association of the glycoproteins with the cell surface and the types of these associations can be determined by treatment of intact cells or vesicular membranes with limited dilutions of proteolytic enzymes such as trypsin. When the inside-out membrane vesicles are treated with limited amounts of trypsin, a reduction in the apparent molecular weight on SDS-gels of angiotensin-converting enzyme [71] and the Lyt-2/3 (CD-8) antigen of cytotoxic T-lymphocytes [72] indicated the presence of cytoplasmic tails and hence of a transmembrane orientation of these proteins. The exposure of proteolytic sites is dependent on the specific conformation of the protein. Malfolded proeins are more susceptible to proteolytic digestion and in contrast to correctly folded proteins, can be easily degraded by proteolytic enzymes [73].

8. Proteases in tissue culture: Since the discovery of Rous and Johns (1916), proteases have been used extensively for the primary dissociation of tissues and for detaching cells in monolayers for subsequent replating [74]. Proteases with broad substrate specificity such as pronase, trypsin, collagenase, dispase, are used in tissue culture for various purposes. Large numbers of viable cells from several human tissues can be isolated by combining mechanical disintegration with 0.1mg/ml trypsin or 0.5mg/ml collagenase and 0.1mmol EGTA [75]. Proteolytic enzymes have been shown to adsorb to cell surfaces and persist in an active form as long as 24 h thereafter. They were found to prevent the formation of glycoprotein cell coat material at the surface and to interfere with the attachment, spreading and growth of cells on glass [76]. Ficin was found to be the most suitable enzyme compared to trypsin, papain and bromelain for isolation of bovine pulp cells due to its even rate of cell removal, good initial viability, subsequent growth of the separated cells in monolayer culture [77]. Overgrowth of cultured keratinocyte preparations by fibroblasts could be significantly reduced by utilizing thermolysin since this enzyme selectively digests the dermal-epidermal junctions [78]. Alkaline protease from *Conidiobolus coronatus* was able to replace trypsin in animal cell cultures [79].

9. Protease-catalyzed peptide synthesis: The use of proteases to perform selective transformations in peptide synthesis is advantageous since chemical ligation methods are prone to racemization and suffer from time-consuming side-chain protection/deprotection necessities. Important approaches towards suppressing competitive reaction in the reversal of proteolysis are: leaving group manipulations of the acyl donor ester in kinetically-controlled synthesis; peptide synthesis in frozen aqueous systems and zymogen-catalyzed peptide synthesis [80]. In addition, in both equilibrium-controlled synthesis and in the kinetic approach [81], kinetically controlled syntheses promise favorable results for use with immobilized proteases as only low concentrations of enzymes and organic solvents are needed to dissolve the reactants. The examples of immobilized proteases used for peptide synthesis are: α-chymotrypsin attached to macroporous silica [82], thermolysin immobilized on Enzacryl AH [83]. Cryoenzymatic synthesis is the upcoming branch of peptide synthesis. Freezing decreases the rate of proteolysis and enhances hydroxylaminolysis. α–Chymotrypsin is able to act as a reverse carboxypeptidase catalyzing coupling of free amino acids as amino components in frozen

aqueous systems and was used for the synthesis of the luteinizing hormone releasing hormone (LH RH). Frozen state enzymology opens completely new possibilities in enzymatic peptide synthesis. The simplest strategy of peptide bond formation, which cannot be performed by chemical methods, is using N-terminal free amino acid or peptide esters as acyl donors [84]. Water-based high density media can also lead to a high peptide yields e.g., the artificial low-calorie sweetener precursor Z-aspartame is synthesized in semi-preparative scale by thermolysin-catalyzed coupling of Z-Asp-OH and H-Phe-OMe.

GENETIC ENGINEERING OF MICROBIAL PROTEASES

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure-function relationship of genetic systems. It provides an excellent means for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms [85] with the aim of (i) enzyme overproduction by gene dosage effect (ii) studying the primary structure of the protease and its role in pathogenicity of the secreting microorganism and (iii) protein engineering to locate the active site residues and/or to alter the enzyme properties to suit its commercial applications. Protease genes from several bacteria, fungi and viruses have been cloned and sequenced. The objective of cloning bacterial and fungal protease genes has mainly been for the overproduction of enzymes for various commercial applications in food, detergent, and pharmaceutical industries. The cloning, sequencing, and/or expression of protease genes or cDNAs from microbial sources is given in Table 1.3.

PROTEIN ENGINEERING

Protein engineering allows introduction of predesigned changes in the gene for the synthesis of protein with altered desirable function. Recent advances in recombinant DNA technology and the ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the rapid progress of protein engineering. Identification of the gene and the knowledge of the three dimensional structure of the protein in question are the two main pre-requisites of protein engineering.

Table 1.3. Cloning, sequencing, and/or expression of protease genes or cDNAs from microbial sources.

Source of protease gene	Type of protease	Reference
Bacteria		
Bacilli		
B. subtilis 168		
apr	alkaline	86
npr	neutral	87
mpr	metallo	88
B. stearothermophilus MK232 and YG185-hyperproducing mutant of MK232*	highly thermostable, neutral	89
*B. stearothermophilus**	thermostable, metallo	90
*B. licheniformis**	alkaline and neutral	91
B. pumilus IFO 12092*	alkaline	92
*B. amyloliquefaciens**	subtilisin	93
Alkalophilic *Bacillus* strain	alkaline	94
B. alkalophilus PB92	alkaline	95
Pseudomonas		
P. aeruginosa IFO 3455	alkaline	96
Thermus aquaticus YT-1	alkaline	97
Thermomonospora fusca YX	alkaline	98
Fungi		
Filamentous fungi		
Mucor pusillus rennin (MPR)	acidic	99
Aspergillus		
A. oryzae M-9	acidic	100
A. oryzae ATCC 20386	alkaline	101
A. soya	alkaline	102
A. fumigatus	alkaline	103
A. nidulans	alkaline	104
Acremonium chrysogenium ATCC 11550	alkaline	105
Fusarium	alkaline	106
Tritirachium album limber	serine	
Proteinase K		107
Proteinase T		108
Yeasts		
S. fibuligera (PEP1)	acidic	109
Y. lipolytica (AEP) XRP2	alkaline	110
Others		
S. cerevisae PRC1	carboxypeptidase	111

Cloning of protease genes in *B. subtilis* is indicated by *

The three dimensional structure of proteases can give valuable insights into their function and specificity providing a framework to focus previous biochemical data and a sound basis for the design of new experiments and therefore, can be a rich source of information for the modern biochemist. Structural data on proteases can have a profound influence on the search and design of novel specific inhibitors (" rational drug design"). The x-ray crystallographic structures of many proteases have been determined [112-115]. The crystals of thrombin (which is important in the process of blood coagulation) in complex with its natural inhibitor hirudin revealed that it is a two component interaction, with extensive electrostatic interaction between the C-terminal tail of hirudin with the basic 'fibrinogen recognition exosite' of thrombin [116]. The crystal structure of human procathepsin X at 1.7A° resolution revealed several novel features compared to the enzymes of papain superfamily such as binding of short proregion of the enzyme with the aid of a covalent bond between the Cys 10 residue of the proregion and the active site Cys 31. This is the first example of a zymogen in which the inhibition of enzyme's proteolytic activity by the proregion is achieved through a reversible covalent modification of the active site nucleophile; it provides an explanation for its rather unusual carboxypeptidase activity and confirms the predictions based on homology modeling [117]. The crystal structure of duodenase, a serine protease from bovine duodenum mucosa at 2.4A° resolution revealed the presence of Ser 214 as the 4^{th} member of the catalytic triad of His 57, Asp 107 and Ser 195. The determination of structural features associated with the functional variation in the enzyme family may permit the design of enzymes with a specific ratio of trypsin/chymotrypsin activities [118]. The study of crystal structure of HIV-1 integrase (an enzyme that inserts the viral DNA into the host chromosome) complexed with its inhibitor will provide a platform for structure-based design of an additional class of inhibitors for antiviral therapy [119]. Crystal structure of the unliganded alkaline protease from *Pseudomonas aeruginosa* IFO3080 revealed that during enzymatic reaction, the structural changes around the active site may reflect the substrate-binding mode [120]. Some other examples of alkaline proteases which have been crystallized include those from *Serratia marcescens* [121], and *Bacillus lentus* [122].

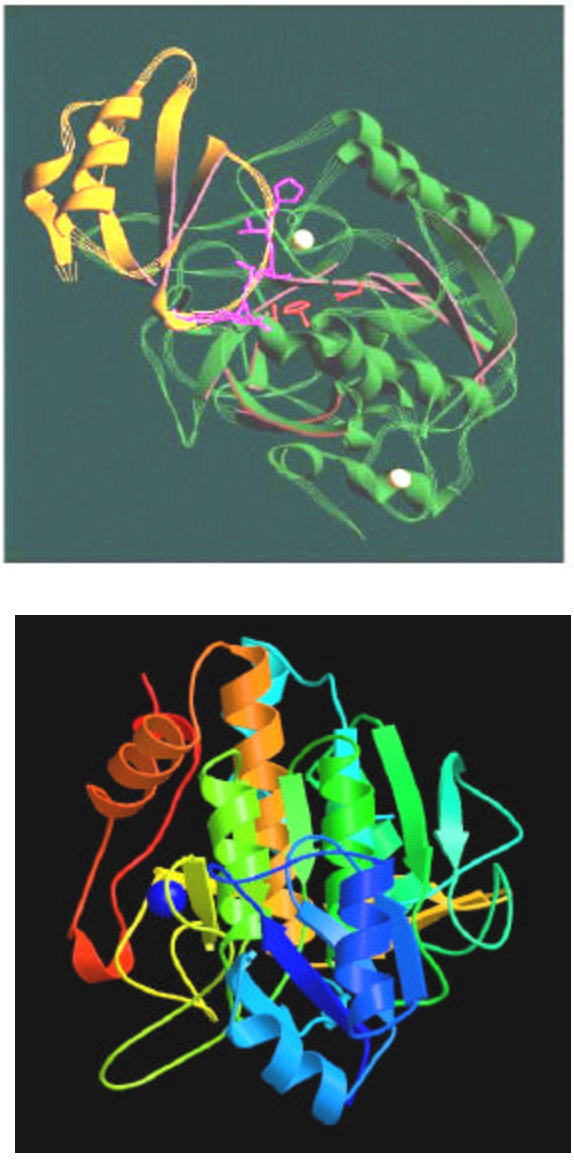


Fig. 1.3 Three-dimensional structure of proteases: (A) The crystal structure of the serine protease subtilisin Carlsberg (green) complexed with the specific protein inhibitor eglin C (yellow) at 2.3A resolution. The active site residues are seen in pink and red colour while the calcium ion is seen as a white ball (ref. 124 and http:\\www.

(B) Crystal structure of a specific 'foldase' (prosegment-subtilisin BPN' complex) (Ref. No. 125 and http:\\www….. The prosegment C terminus binds in the enzyme active site in a product-like manner and is required for efficient refolding of the subtilisin.

Protein engineering technique has been exploited successfully for obtaining proteases which show unique specificity and/or stability at high temperature and pH or in the presence of detergents. Protein engineering has also contributed substantially in understanding the structure-function relationship of proteases. Proteases from bacteria, fungi and viruses have been engineered to improve their properties to suit their particular applications.

1. Bacteria: Subtilisin has been chosen as a model system for protein engineering since a lot of basic information about this commercially important enzyme is available. Its pH dependance [125], catalytic activity [126, 127], stability against heat or denaturing agents [128, 129] and the substrate specificity [130-132] have been altered through SDM. Slightly reduced rate of thermal inactivation was observed for a subtilisin BPN' variant containing two cysteine residues (Cys22, Cys87) [133, 134]. Oxidation of Met222 adjacent to the Ser221 in the active site of subtilisin reduces the catalytic activity of subtilisin. Effect of substitution of Met222 with different amino acids revealed that small side chains yield the highest activity. The mutant enzymes Ser222, Ala222 and Leu222 were active and stable to peroxide for one hour. Probing of the specificity of the S binding site of Met222 Cys/Ser mutants of subtilisin from *B. lentus* with boronic acid inhibitors revealed similar binding trend for the mutant and the parent [135]. The disulphide bonds introduced in subtilisin away from its catalytic centre were shown to possess increased autolytic stability [18]. Higher thermostability of subtilisin E by introduction of disulfide bond engineered on the basis of structural comparison with thermophilic serine protease has been reported [136]. Strausberg et. al. have created the environment for stabilization of subtilisin by deleting the calcium binding loop from the protein [137]. Analysis of the structure and stability of the loop-deleted prototype followed by SDM resulted in a mutant with native-like proteolytic activity and exhibited 1000-times greater stability under strongly chelating conditions. Substitution of Asn241 burried in the neutral protease of *B. stearothermophilus* by Leu by SDM resulted in the increase in thermostability by 0.7+0.1 degree [138]. Thermostability of the neutral protease from *B. subtilis* was increased by 0.3 and 1.0°C by replacing Lys by Ser at positions 249 and 290 respectively, whereas the Asp249 and Asp290 mutants exhibited an increased stabilization by 0.6 and 1.2°C respectively [139]. A protein engineering

study was undertaken by Bruinenberg et. al. to determine the functions of one of the largest loop insertions (residues 205-219), predicted to be spatially close to the substrate binding region of the sk 11 protease from *L.delbrueckii* and susceptible to autoproteolysis [140]. Deletion/modification of this loop was shown to affect the activity and autoprocessing of the protease. Graham et al. showed that random mutagenesis of the substrate-binding site of α-lytic protease, a serine protease secreted by the soil bacterium *L. enzymogenes*, generated enzymes with increased activities and altered primary specifities [141]. Substitution of His120 with Ala in the Las A protease of *P. aeruginosa* yielded an enzyme devoid of staphylolytic activity. Thus, His120 was shown to be essential for Las A activity [142].

2. Fungi: Fungal aspartic proteases are able to cleave substrate with 'lys' in the P1 position. Sequencing and structural comparison suggest that two aspartic acid residues (Asp30 and Asp77) may be responsible for conferring this unique specificity. Lowther et al. worked on engineering the substrate specificity of rhizopuspepsin from *Rhizopus niveus* and showed the role of Asp 77 in the hydrolysis of the substrates with lysine in the P1 position [143]. Primary structure of aspergillopepsin I from *A. saitoi* ATCC 14332 (now designated as *A. phoenicis*) was deduced from nucleotide sequence of the gene [144]. To identify the residue responsible for determining the specificity of aspergillopepsin I towards the basic substrates in the substrate-binding pocket, Asp-76 was replaced with Ser residue by site-directed mutagenesis. The striking feature of this mutation was that only the trypsinogen activating activity of the enzyme was destroyed suggesting the importance of Asp-76 residue in binding to basic substrates. To elucidate whether the processing of proregion occurs by autoproteolysis or by involving a processing enzyme, Tatsumi et al. changed Ser228 to Ala228 by SDM [145]. S. cerevisiae cells harbouring recombinant plasmid with mutant Alp did not secrete active Alp into the culture medium. The yeast cells accumulated a protein of 44 kDa probably a precursor of Alp (34 kDa mature Alp + 10 kDa pro peptide), suggesting autoproteolytic processing of the pro region. Introduction of a disulfide bond by SDM is known to enhance the thermostability of a cysteine-free enzyme. Aqualysin I, thermostable subtilisin-type protease from *Thermus aquaticus* YT-1 contains four Cys residues forming two disulfide bonds [146]. The primary structure of Alp showed 44% homology

to that of aqualysin I and sites for Cys substitutions to form a disulfide bond were chosen in the Alp based on homology. Ser69, Gly101, Gly169 and Val200 were replaced by Cys in the mutant Alp. Both Cys-69/Cys-101 Cys-169/Cys 200, mutant Alps were expressed in *S. cerevisiae* and the enzymes were purified to homogeneity. The Cys-169/Cys-200 disulfide bond was shown increase the thermostability as well as the thermoresistance of *A. oryzae* Alp [147]. In vitro mutation of an aspartic acid residue predicted to be in the active site abolished the barrier activity of *S. cer*evisiae [148]. BAR1 possesses a carboxyl-terminal domain of an unknown function, deletion of 166 of 191 aa of this region had no significant effect on the barrier activity.

3. Viruses: The protease of HCMV was rendered stable by SDM of either of the three Val141, Val207 or Val254 reisudes to Gly substitutions [149]. The resulting stable proteases are useful as a screening tool for HCMV antivirals and as a diagnostic tool for diseases resulting from HCMV infection. Replacement of Asp64, a residue from the catalytic core sequence among aspartyl proteases, with Gly was shown to abolish correct processing of 53 k gag precursor by HTLV-I gag protease [150]. In poliovirus, the mutation of highly conserved residue viz. Cys147 or His161 produced an inactive enzyme while mutation of a nonconserved residue, Cys154 had a negligible effect on the proteolytic activity [151].

PROTEIN FOLDING

The principle of self-assembly states that the native three-dimensional structure of a protein is determined solely by its primary amino acid sequence and that folding of the peptide chain is a spontaneous process that does not require input of energy or accessory factors [152]. Another mechanism of assisted protein folding is seen in a variety of prokaryotic as well as eukaryotic proteases such as subtilisin [153], α-lytic protease [154], carboxypeptidase [155], cathepsin D [156], proteinase A [157], and aqualysin [158]. These proteins are unable to spontaneously refold when their denatured states are placed in conditions that favor the folded state [159] and share a common mechanism of folding into their bioactive conformations. Most of the studies concerning the structural origin of protease and their specificities have been carried out on subtilisins [160]. Subtilisin BPN' is synthesized as a precursor in the form of pre-pro-subtilisin [161]. The

30 amino acid pre-sequence serves as a singnal peptide for protein secretion across the membrane [162]. The prosequence, which consists of 77 amino acids, is only transiently connected to the 275 residue protease domain and is required for the correct folding of the enzyme [153]. During the folding reaction, the covalent linkage between the pro-sequence and subtilisin is not needed [156]. Further, the prosequence strongly inhibits the native enzyme [163], suggesting that it functions at a late step on the folding pathway. The propeptide of α–lytic protease (a serine protease not evolutionarily related to subtilisin) was also shown to be essential for proper activation of its protease domain [164]. There are now examples of all types of proteases viz., serine [165], aspartyl [166], cysteine [167] and metalloproteases [168] synthesized as precursor pro-proteases. Due to the covalent attachment of propeptides to the proteins which they help fold, they have been termed as 'intramolecular chaperones' [169] in contrast to the term 'molecular chaperones' which is used for a family of cellular proteins that mediate the folding of other cellular proteins [170]. In the absence of their propeptides, proteases fold into partially structured molten globule intermediates which can convert into active conformations upon addition of their propeptides [171,172], thereby suggesting that propeptides promote folding of their proteases by direct stabilization of the rate-limiting folding transition state [173]. Point mutations within the propeptides can affect their folding functions [174,175], which introduced the concept of 'protein memory' and suggests that propeptide domains function as steric chaperones [176]. It has been recently shown that propeptides continue their chaperoning function after its cleavage from subtilisin and that structural imprinting that results in conformational diversity originates during this reorganization stage (177). The only exception of folding of a protease in the absence of a propeptide is that of a proteolytically inactive mutant of subtilisin in which the high-affinity calcium-binding site was deleted from the protein. The folding rate of such a variant of subtilisin could be accelerated by high ionic strength (178).

IMMOBILIZATION:

Enzyme immobilization offers advantages over free enzymes in terms of multiple or repetitive use of a single batch of enzyme, rapid termination of reactions, controlled product formation, increased stability of the enzyme, predictable decay rates and

adaptibility to various engineering designs [179]. The interest in immobilized enzymes and their application to bioprocessing [180], analytical system [181] and enzymatic therapy [182] has grown in past decade. A few of the variety of applications of the immobillized enzymes are described here. The application of proteases for peptide synthesis has been stimulated to a considerable degree by the availability of immobilized enzymes. Covalently bound proteases catalyze peptide bond formation as effectively as the native enzymes since substrate specificity and stereospecificity remain unchanged. The peptides synthesized are free of contamination by proteolytic activities and denatured protein. Due to the increased stability of the immobilized enzymes to organic solvents, higher concentrations of such solvents can be used to influence the position of the thermodynamic equilibrium. α–Chymotrypsin attached to macroporous acrylic beads and thermolysin attached to 4% cross-linked beaded agarose are used for the synthesis of Ac-Phe-Ala-NH2 and Z-Phe-Leu-NH2, respectively [183]. α-Chymotrypsin (Chy) entrapped in polytetrafluoroethylene (PTFE) particles showed twofold catalytic activity for amino acid ester hydrolysis in aqueous solution than free enzyme. The Chy/PTFE particles also catalyzed the peptide synthesis in aqueous solution. with a yield of 14%. Both the synthetic and the hydrolytic activities of the entrapped enzyme were enhanced as compared with the free enzyme [184]. The crude protease produced by *P. aeruginosa* K-187 was covalently immobilized on a reversibly sol. polymeric support (hydroxypropylmethylcellulose acetate succinate). The utilization of this immobilized enzyme for the deproteinization of shrimp and crab shell wastes resulted in a 67% protein removal [185]. A novel enzyme biosensor for the detection of protein was made using a protease immobilized on an immunodyne membrane and by placing it on an electrode. The method was very effective for detecting a very low level of protein [186]. The effects of site-specific immobilization on the thermal stability of mutants of the thermolysin-like protease from *Bacillus stearothermophilus* (TLP-ste) showed that the protein stabilization by immobilization is most effective if the protein is attached to the carrier at that region where unfolding is initiated [187]. Milk clotting protease from Aspergillus niger MC4 immobilized on glycidyl methacrylate-pentaerythritol triacrylate copolymer GP4 was used for continuous production. of cheese using a packed bed reactor [188].

SEQUENCE HOMOLOGY

Studies on DNA and protein sequence homology are important for a variety of purposes and have, therefore, become a routine task in computational molecular biology. They serve as a prelude to phylogenetic analysis of proteins and assist in predicting the secondary structure of DNA and proteins. Proteases are a complex group of enzymes, which vary enormously in their physicochemical and catalytic properties. The nucleotide and amino acid sequences of a number of proteases have been determined and their comparison is useful for elucidating the structure-function relationship [11]. Homology of proteases with respect to the nature of the catalytic site has been studied [9,189]. It has been shown that the residues involved in the substrate and metal ion binding, catalysis, disulfide bond formation and those forming the active site are conserved. Accordingly, the enzymes have been allocated to evolutionary families and clans. It has been suggested that there may be as many as 60 evolutionary lines of peptidases with separate origins. Some of these contain members with quite diverse peptidase activities and yet there are some striking examples of convergence [12]. A number of reports are available regarding the homology of proteases. Takagi et al. found that the thermostable proteases of *B.stearothermophilus* and *B. thermoproteolyticus* are 85% homologous and the thermolabile proteases of *B. subtilis* and *B. amyloliquefaciens* are 82% homologous whereas the thermostable protease of *B. stearothermophilus* shares only 30% homology with the thermolabile protease of *B. subtilis* [190]. However, an amino acid sequence of 17 residues which also includes the active site histidine residue was found to be highly conserved in all the four neutral proteases suggesting that they share the same three dimensional structure around the active site despite the difference in their source and physicochemical properties such as thermostability. Koide and coworkers compared the amino acid sequences of intracellular serine proteases from *B. subtilis* with that of subtilisin Carlsberg and subtilisin BPN' and showed them to be 45% homologous [191]. The sequences around the catalytic triad of serine, aspartate and histidine are highly conserved suggesting that the genes for both the intracellular and extracellular proteases have evolved from a common ancestor by divergent evolution [192]. The amino acid sequence of an extracellular alkaline protease subtilisin J, is highly homologous to subtilisin E and shows 69% identity with subtilisin Carlsberg, 89% with subtilisin BPN'

and 70% with subtilisin DY. Amino acid sequence of subtilisin J is completely identical to that from *B. amylosacchariticus* except for two amino acid substitutions viz. Thr130 to Ser130 and Thr162 to Ser162 in addition to one amino acid substitution in the signal peptide and two in the propeptide region. Probable active site residues of subtilisin J,viz. Asp32, His64 and Ser221 are identical to those of other subtilisins from *Bacillus*. Therefore, it was concluded that the alkaline protease from *B. stearothermophilus* is a subtilisin. Similarly, the various serine alkaline proteases from *Bacillus* such as bacillopeptidase F, subtilisin, epr and ISP-1 show considerable homology and conserved amino acids around the active site residues viz. ser, asp and his [193].

The extracellular proteases of *B. subtilis* are synthesized as preproenzymes. Four neutral proteases from bacilli with known prosequences were compared and considerable homology within the propeptide region was observed [194]. Since the propeptide region mediates the folding of the protease, it would be interesting to learn about the residues essential for folding and to know whether the mechanism of folding is similar in these proteases. Sequences corresponding to the mature form of these enzymes were compared using thermolysin sequence as a reference. The zinc binding site (His142, His146, Glu166) and the residues participating in the catalytic reaction and positioning of substrate backbone in the active site (Asn112, Ala113, Glu143, Tyr157 and His231) were found to be conserved. Differences in these might lead to altered substrate specificities. Out of the four calcium binding sites in thermolysin, two sites viz. 3 and 4 are absent in thermolabile neutral proteases of *B. amyloliquefaciens* and *B. subtilis* (nprA) whereas in nprB, Asn187 in site 3 is replaced by arginine. Such changes are responsible for the loss of thermostability and can be detected by the sequence homology studies. Alkaline proteases from various species of *Aspergillus* also show a high degree of homology among themselves [195]. Alp, from *A. oryzi* shows considerable homology (29 to 44%) with the members of the subtilisin family with conserved active site residues [196]. However, Alp exhibits little homology with those of mammalian serine proteases such as trypsin and chymotrypsin. The deduced structure of the KEX1 protein, required for the production of killer toxin of *Kluyveromyces lactis* contains an internal domain with a striking homology to the sequences of subtilisin type proteases [197]. Therefore, it was deduced that the product of the gene KEX1 of *K. lactis* is a protease involved in the

processing of the toxin precursor.

The characteristic of trypsin related enzymes is the presence of disulfide bonds which are absent in all known subtilisins. Proteinase k, from *Tritirachium album limber* is a single chain protein of 277 amino acids with two disulfide bonds 34-124 and 179-248 and a free -SH group at position 73. Sequences around the active site residues correspond to those around the active site residues of subtilisins. Comparison of the proteinase k sequence with known subtilisins shows 35% homology and 44% sequence identity to thermitase which is indicative of relationship of proteinase k to subtilisin family. It is likely that these enzymes have evolved from a common ancestral precursor serine proteinase [198]. However, there is a distinct difference between the typical subtilisins and the proteinase k, the latter has two disulfide bonds, which are lacking in subtilisins. Therefore, it has been assumed that the two progenitors have diverged from an ancestral proteinase separating the subtilisin related enzymes into two subclasses (i) cysteine containing subtilisins e.g. proteinase k and thermitase and (ii) cysteine free subtilisins e.g. subtilisin novo, Carlsberg or DY.

The proteasome or multicatalytic endopeptidase complex is a high molecular mass multisubunit complex ubiquitous in eukaryotes and also found in the archaebacterium *Thermoplasma acidophilum* [199]. While eukaryotic proteasomes contain 15 to 20 different subunits, the archaebacterial proteasome is made of only two different subunits (α and β) and yet the complexes are almost identical in size and shape. The (233 aa) and (211 aa) subunits of *Thermoplasma* share a sequence identity of 24% and an overall similarity of 47%, indicating that the genes encoding the two subunits arose from a common ancestor. All the sequences of proteasomal subunits from eukaryotes available to date can be related to either the α or the β subunit of the *T. acidophilum* 'urproteasome' and they can be distinguished by means of a highly conserved N-terminal extention which is characteristic for α-type subunits. In terms of evolution the genes for these α and β subunits can be considered as paralogous (genes resulting from duplication and divergence of one gene within one genome) and therefore are able to acquire different functions. The subunit of the *Thermoplasma* proteasome shows sequence similarity to the *S. cerevisiae* wild type suppressor gene scl1 encoded polypeptide, which is probably identical to the subunit YC7- α of the yeast proteasome. This lends support to a putative

role of proteasomes in the regulation of gene expression [200]. The amino acid sequence of *Xenopus* proteasome subunit XC3 is highly homologous (95.3%) to those of rat the RC3 and human HC3 subunits [201]. The presence of an accessible nuclear targetting signal at the C terminus of the subunits suggests that it is probably involved in the regulation of the cellular distribution of the proteasome.

The secretable acid protease of the yeast *Saccharomycopsis fibuligera* carries a hydrophobic amino terminal segment of about 20 amino acids which resembles signal sequences found in a wide variety of secretary protein precursors [202]. Alignment of this sequence with the aspartyl protease family showed significant homologies, especially in the regions surrounding the two active site aspartate residues. These results suggest that the gene PEP1 is a structural gene for the secretable acid protease of *S. fibuligera*. The aspartic protease from *Rhizopus niveus* (RNAP) shows 76% homology with rhizopuspepsin, 42% with penicillopepsin, and 41% with human pepsin [203, 204]. The homology between RNAP and rhizopuspepsin is found throughout their structures. Based on this homology, an intron within the coding region and a prepro-enzyme sequence of 66 aa upstream of the mature enzyme were detected in RNAP. Studies of the homology of proteases have shown that the residues involved in the substrate and metal ion binding, catalysis, disulfide bond formation and active-site formation are conserved. Analysis of sequence homology is used in deciphering the structure-function relationship.

EVOLUTIONARY RELATIONSHIP OF PROTEASES

Proteases are present in all living organisms and are considered to have arisen in the earliest phases of biological evolution, some billion years ago. Comparisons of amino acid sequences, three dimensional structures and mechanism of action of proteases assist in deciphering of their course of evolution. Changes in molecular structure have accompanied the demands for altered functions of proteases during evolution. We have compiled the amino acid sequences of proteases from diverse origins such as microbes, plants and animals and have arranged them in three different groups based on the pH of their action. These sequences which have been selected from SWISS-PROT and PIR entries are of comparable length and have been aligned using the CLUSTAL W programme for multiple alignments [205].

Acidic proteases: The proteases selected here for comparison of amino acid sequences are active between pH 2 and 6. They include mostly aspartic proteases and also some of the cysteine and metalloproteases. They are about 380 to 420 aa residues in length and have different amino acid residues constituting the active site as shown in Table 1.4 [1]. The homology between these acidic proteases is shown in Fig 1.3A. The sequences belonging to pepsin family (A1) are grouped together and are aligned below the other sequences. As expected, there is a considerable homology among these five acidic proteases. The sequences around the two aspartic residues (D97 and D258, residues numbered according to the bajra protease) constituting the active site are conserved. Among these five, the rat and monkey proteases show maximum homology (68.4%) and are related to the mosquito lysosomal aspartic protease. When four monkey pepsinogens which show development-dependent expression were compared, a very high homology was observed [206]). Pepsinogens A-1 and A-2\3 differed only in seven amino acids and only in five amino acids when pepsin moiety alone was examined. The mosquito lysosomal protease is very closely related to human cathepsin D exhibiting 92% homology [207]. The amino acid sequences of *C. tropicalis* and *Saccharomycopsis fibuligera* show considerable homology (42.6%). High scores of similarity were obtained when the acid protease from *C. tropicalis* was compared with *Rhizopus* aspartic proteases, human pepsinogen A precursor, the protease A from yeast, the barrier protein from *S. cerevisiae* and an acidic protease from *S. fibuligera* [208]. The cysteine protease from *Hordeum vulgare* shows some homology to the snake venom metalloprotease from *Crotalus atrox*, which is not statistically significant whereas the gpr protease from *B. megaterium*, which plays a vital role in spore germination shows least homology to all other acidic proteases, but shares one of the active site aspartate residues (D258) with others. The gpr acidic proteases of *B. subtilis* and *B. megaterium* showed 68% identity in their sequences, but comparison of B. subtilis gpr amino acid sequence with its serine or metalloproteases revealed no significant homologies which also supports our observations [209]. This suggests that the genes for these proteases do not share common ancestor and if they do so, they have undergone much divergence. The lack of homology between the spore protease and other *B. subtilis* proteases can be explained by their different properties like number of subunits and sequence specificity for substrate. Thus

TABLE 6. Proteases selected for multiple alignment[a]

SWISS-PROT/PIR entry	No. of amino acid residues	Type of protease	Residue(s) at active site
Acidic proteases			
CYS2_HORVU	373	C1/papain (cysteine)	C158 H297, N318
HRTD_CROAT	414	M12B (metallo)	E311
GPR_BACME	371	U3 (aspartic)	D89, D258
ASPP_AEDAE	395	Lysosomal (aspartic)	D96, D258
CARP_CANTR	390	Candidapepsin (aspartic)	D96, D258
CARP_SACFI	390	Saccharopepsin (aspartic)	D96, D258
PEPC_RAT	382	Gastricsin (aspartic)	D96, D258
PEP2_MACFU	378	Pepsin A (aspartic)	D96, D258
Neutral proteases			
PRCA_THEAC	233	PS	U
PRC3_YEAST	250	PS	U
PRC6_SCHPO	259	PS	U
PRC2_ORYSA	270	PS	U
PRC6_ARATH	250	PS	U
PRC6_DICDI	250	PS	U
PRC8_CAEEL	259	PS	U
PRC6_DROME	249	PS	U
PRC3_XENLA	233	PS	U
CANS_BOVIN	263	C2/calpain (cysteine)	U
CANS_PIG	266	C2/calpain (cysteine)	U
TRYT_CANFA	275	S1/trypsin (serine)	H74, D121, S191
TRYB_HUMAN	275	S1/trypsin (serine)	H74, D121, S191
SNPA_STRLI	237	M7 (metallo)	E64
Alkaline proteases			
JC6052	355	Trypsin-like protease	H91, D126, S201
EYLA_BACAO	380	S8/subtilase	D120, H150, S302
SUBT_BACST	381	S8/subtilase	D120, H150, S302
PRTK_TRIAL	384	S8/subtilase	D120, H150, S302
ALP_TRIHA	409	S8/subtilase	D120, H150, S302
ALP_CEPAC	402	S8/subtilase	D120, H150, S302
ORYZ_ASPFL	403	S8/subtilase	D120, H150, S302
ORYZ_ASPFU	403	S8/subtilase	D120, H150, S302
I50494	410	Serine protease inhibitor	U

[a] Key to the entry names of acidic proteases: CYS2_HORVU, *Hordeum vulgare*; HRTD_CROAT, *Crotalus atrox*; GPR_BACME, *Bacillus megaterium*; ASPP_AEDAE, *Aedes aegyptii*; CARP_CANTR, *Candida tropicalis*; CARP_SACFI, *Saccharomycopsis fibuligera*; PEPC_RAT, *Rattus norvegicus*; PEP2_MACFU, *Macaca fuscata*. Sequences are numbered according to the *Hordeum vulgare* cysteine protease. Key to the neutral protease sequences: PRCA_THEAC, *Thermoplasma acidophilum*; PRC3_YEAST, *Saccharomycopsis fibuligera*; PRC6_SCHPO, *Schizosaccharomyces pombe*; PRC2_ORYSA, *Oryza sativa*; PRC6_ARATH, *Arabidopsis thaliana*; PRC6_DICDI, *Dictyostellium discoideum*; PRC8_CAEEL, *Caenorhabditis elegans*; PRC6_DROME, *Drosophila melanogaster*; PRC3_XENLA, *Xenopus laevis*; CANS_BOVIN, *Bos taurus*; CANS_PIG, *Sus scrofa*; TRYT_CANFA, *Canis familiaris*; TRYB_HUMAN, *Homo sapiens*; SNPA_STRLI, *Streptomyces lividans*. Sequences are numbered according to the *Thermoplasma* protease. PS, proteasome subunit; U, unknown. Key to the alkaline protease sequences: JC6052, *Escherichia coli*; ELYA_BACAO, *Bacillus amyloliquefaciens*; SUBT_BACST, *Bacillus subtilis*; PRTK_TRIAL, *Tritirachium album* Limber; ALP_TRIHA, *Tritirachium harzianum*; ALP_CEPAC, *Cephalosporium acremonium*; ORYZ_ASPFL, *Aspergillus flavus*; ORYZ_ASPFU, *Aspergillus fumigatus*; I50494, *Cyprinus carpio*. Residues are numbered according to the *E. coli* protease.

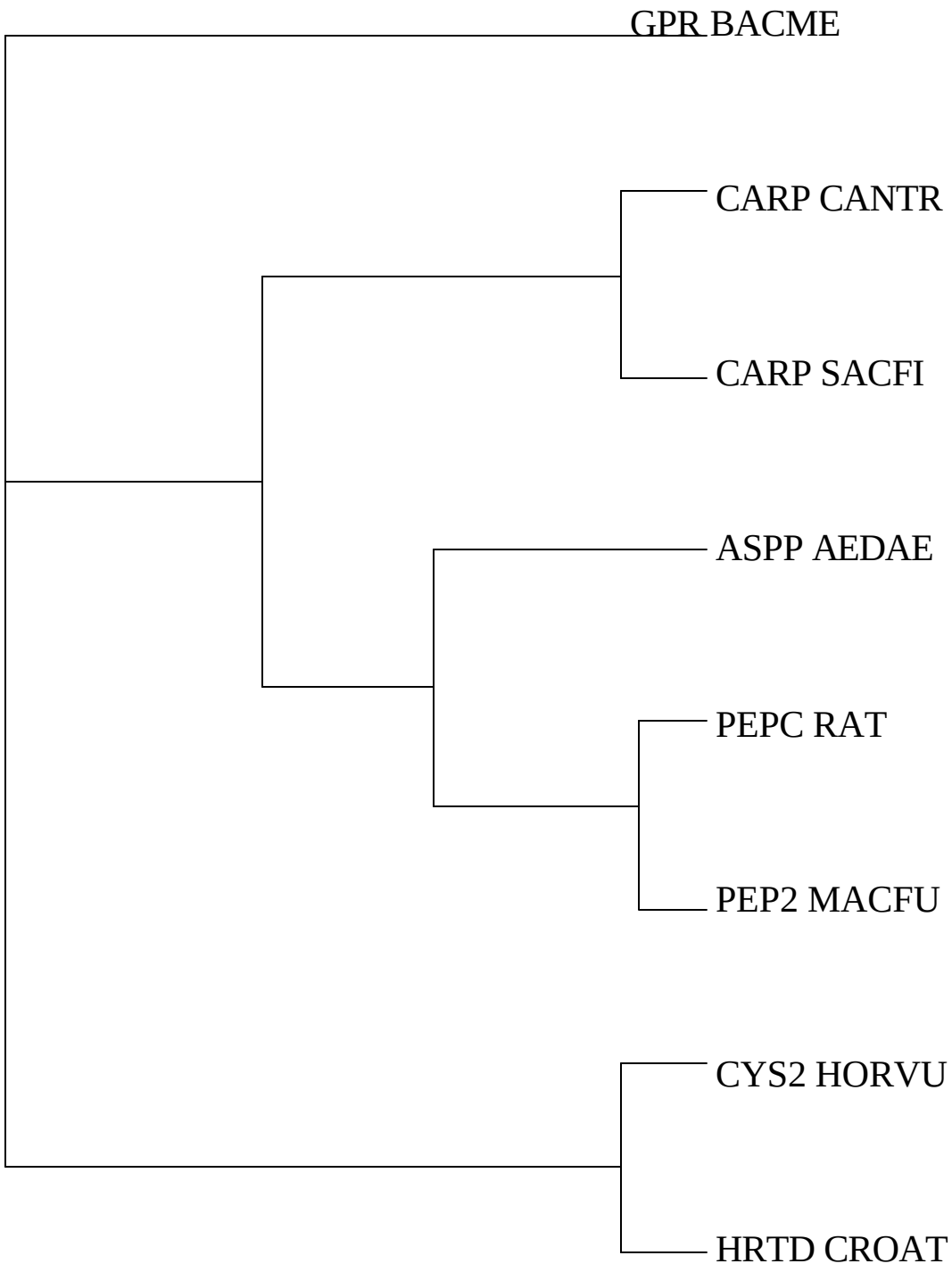


Fig. 1.3 B Dendrogram showing the relationships among the acidic proteases created by the TreeView Package. Abbreviations of the species described are those used in Table 1.4. The differences between the sequences are proportional to the length along the horizontal axis.

our results, in agreement with the previous reports, indicate that the extent of homology is more if the proteases belong to the same family and in the same family, the homology is more if the phylogenetic distance is less. A pairwise computer comparison also provides more information about the evolutionary relationships between the members of the different families. The dendrograms generated by this analysis, using the TreeView package [210] demonstrate the relationship among the proteins based on the similarity of the amino acid sequences (Fig.1.3B).

Neutral proteases: The neutral proteases selected for sequence analysis are active at neutral or weakly alkaline or weakly acidic pH. and are in the size range of 225 to 275 aa residues (Table 1.4). The homology between them is shown in Fig. 1.4A. Out of 14 proteases, 9 belong to the T1A or proteasome A family of the multicatalytic endopeptidase complex. The sequences of the proteasomal subunits aligned here can be related to the subunit of the *Thermoplasma* proteasome and show considerable homology. It is still not clear as to which family of proteases do the proteasomes belong to [211]. As in cysteine family of proteases, all 9 proteasome subunits show a conserved proline residue (P-17) which may serve to prevent unwanted N-terminal proteolysis [9]. Many residues at the N-terminus are highly conserved, which is a characteristic of the type subunits. The similarity decreases towards the C-terminus which appears to be rather variable [200]. Although the subunit shows no sequence motif characteristic for serine proteases, it contains all the essential amino acid residues forming the catalytic triad or the "charge relay system" (ser, asp, his). These residues are found to be conserved (ser16, his73, asp84) except for the histidine in the subunits of *Thermoplasma*, yeast (*S. cerevisiae*) and *Coenorrhabditis elegans* (residues numbered according to the *Thermoplasma* subunit sequence). Therefore, it is possible that the active site is shared between the α and β subunits [199]. The tyrosine autophosphorylation site at Tyr 123 is conserved in 6 out of the 9 sequences. The cAMP/cGMP dependent phosphorylation sites between amino acid residues 31 and 37 are found only in *Thermoplasma* and *Drosophila* [212] as is reported by Zwickl et al. [200]. A consensus nuclear location signal (NLS) between amino acid residues 49 and 56 [213] and a region complementary to the NLS consensus sequence [214] between amino acid residues 201 and 212 can be

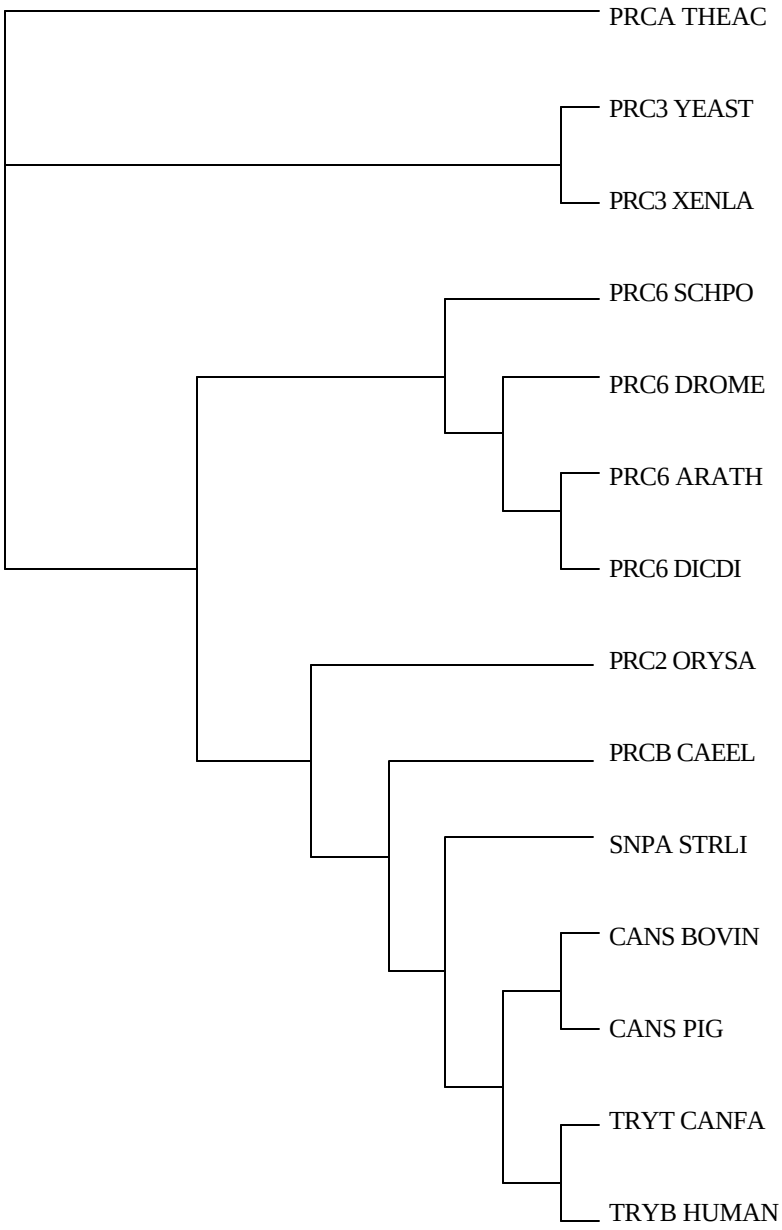


Fig. 1.4B Dendrogram showing the relationships among the neutral proteases created by the TreeView Package. Abbreviations of the species described are those used in Table 1.4. The differences between the sequences are proportional to the length along the horizontal axis.

identified in these sequences. Thus, the sequence comparison of various proteasome subunits from archaebacteria to mammals shows high homology. The bovine and porcine proteases which belong to the calpain or C2 family of cysteine proteases differ from each other in only six amino acid residues and thus show almost 99% homology with highly conserved calcium binding domains and N-terminal glycine rich hydrophobic region. The region rich in proline residues (76 to 81, aa numbered according to *Thermoplasma* protease) is also conserved except at position 79, where proline is replaced by valine. Tryptase precursors from human and dog [215] which belong to the S1 or trypsin family of serine proteases show 76% sequence identity. The signal sequence from residues 1 to 30 is 60% identical; the difference is only in the site of glycosylation which is Asn132 in dog and Asn233 in human. The sequences for active site and disulfide bond formation are highly conserved, and correspond to those of chymotrypsinogen [216]. The relationship between these neutral proteases is evident from the dendrogram shown in Fig 1. 4B.

Alkaline proteases: The alkaline proteases selected here are active in the pH range of 8 to 13 and are about 420 to 480 residues in length. Six of them belong to the S8 or subtilase family of serine proteases (Table 1.4). They are aligned in their phylogenetic order, as shown in Fig. 1.5A. Considerable homology within the same genus is observed for proteases from *Bacillus*, *Aspergillus* and other three fungal proteases. However, these proteases show comparatively less homology among themselves. The active site residues as well as the residues around the active site are highly conserved, suggesting that they may have been evolved from a common ancestor. The sequences of *E. coli* and *Cyprinus carpio* seem to be homologous to some extent but they do not share common active site residues and, therefore, a common ancestor. These two in turn show no significant homology to the other seven alkaline proteases. The overall homology among all these sequences can be represented by the dendrogram in Fig. 1.5B. The results of our analysis of the aa sequences of the acidic, neutral and alkaline proteases indicate that the members of the pepsin family of acidic proteases may have evolved from a common ancestor by convergent evolution. High homology between the sequences of the α subunits of proteasomes provides evidence for the presence of an evolutionarily conserved gene

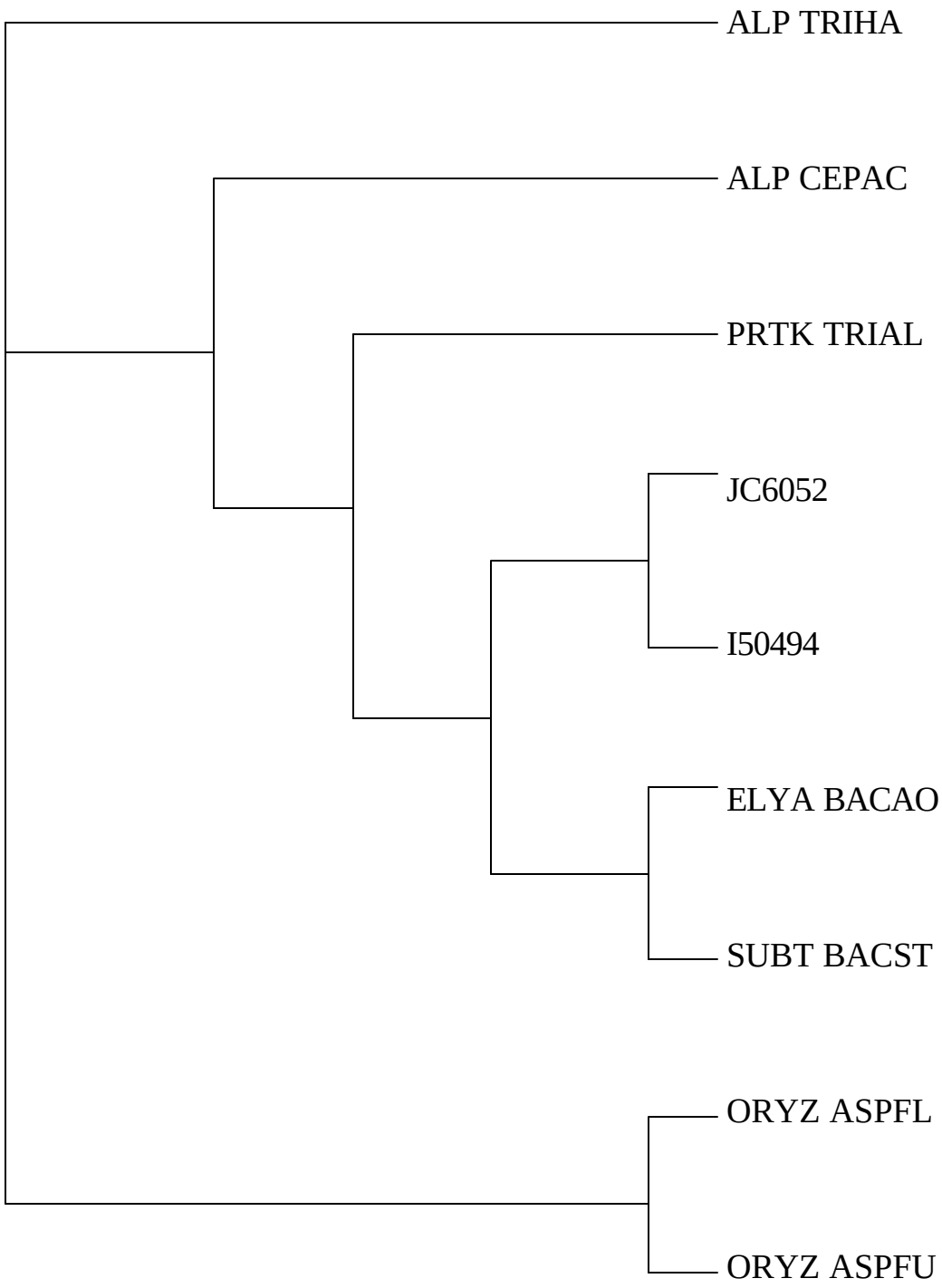


Fig. 1.5 B Dendrogram showing the relationships among the alkaline proteases created by the TreeView Package. Abbreviations of the species described are those used in Table 1.4. The differences between the sequences are proportional to the length along the horizontal axis.

family. No amino acid residues are observed which are conserved in all the acidic or neutral proteases except glycine. The alkaline serine proteases seem to have evolved from a common ancestor by divergent evolution. In general, the sequences belonging to the same family show more homology or they are more closely related. This criterion is currently used to assign a particular sequence to a particular family viz. serine, cysteine, aspartate or metalloprotease family. Within a family, the extent of homology is inversely proportional to the phylogenetic distance. The proteases from distantly related organisms show less homology or more diversity. However, this needs extensive sequence analysis of proteases as the homology depends on many parameters or factors such as structure, function, source, nature of catalytic/active site etc. Thus proteases are highly diverse enzymes having different active sites, metal binding regions, residues involved in disulfide bond formation, the positions of which vary in different proteases as can be detected by multiple alignments.

CURRENT LACUNAE AND POTENTIAL SOLUTIONS

Proteases are a complex group of enzymes, which differ in their properties such as substrate specificity, active site and catalytic mechanism. Their exquisite specificities provide a basis for their numerous physiological and commercial applications. Despite the extensive research on several aspects of proteases from ancient times, there are several lacunae in our knowledge about these enzymes and there is a tremendous scope for improving their properties to suit the projected applications. The future lines of development would include (i) genetic approaches to generate microbial strains for hyperproduction of the enzymes (ii) application of site-directed mutagenesis to design proteases with unique specificity and increased resistance to heat and alkaline pH (iii) synthesis of peptides (synzymes) to mimic proteases (iv) production of abzymes with specific protease activity and (v) understanding of structure-function relationship of the enzymes. Although the section on protein engineering describes in detail how SDM has been employed to alter the properties and functions of proteases of bacterial, fungal and viral origin, some of the important problems faced in their desired usages and the possible solutions to overcome these hurdles are discussed below.

Enhancement of thermostability: Industrial application of proteases in detergents or for leather processing demands stability of the enzyme at higher temperatures. One of the

common strategies to enhance the thermostability of the enzyme is to introduce disulfide bonds into the protease by SDM. Introduction of a disulfide bond in subtilisin E from *Bacillus subtilis* resulted in an increase of 4.5°C in T_m of the mutant enzyme without any change in its catalytic efficiency [217]. However, the properties of the mutant enzyme were found to revert to those of the wild type enzyme. Enhanced stability of subtilisin was observed by Asn109 and Asn218 to Ser mutations. The analog containing both the mutations showed an additive effect on thermal stability. Thermostability of the alkaline protease from *Aspergillus oryzae* is important due to its extensive use in the manufacture of soya sauce. The optimum temperature of the wild type was enhanced from 51°C to 56° by introduction of a disulfide (Cys 169-Cys 200) bond. Another strategy for improving the stability of the protease was by replacing the polar amino acid groups by hydrophobic groups. The presence of positively charged amino acids in the N terminal turn of an α-helix is undesirable in view of the possibility of an occurrence of the repulsive interactions with the helix dipole. Replacement of Lys by Ser or Asp showed increase in thermostability of the neutral protease from *B. subtilis* in the range of 0.3 to 1.2°C [139]. Although these approaches result in increased stability of proteases, the difference in the thermostability of the parent and the mutant enzyme is only marginal and further research using cassette mutagenesis etc. is necessary to yield an enzyme with substantially enhanced thermostability.

Prevention of autoproteolytic inactivation: Subtilisin, an extensively studied protease, finds an extensive application in detergent formulations due to its stability at alkaline pH. However, its autolytic digestion presents a major problem in its use in industry. It was deduced that there is a correlation between the autolytic and conformational stability. Computer modelling followed by an introduction of a Cys 24 or Cys 87 mutation resulted in destabilization of subtilisin from *Bacillus amyloliquefaciens* [218]. Introduction of a disulfide bond increased the stability of the mutant to a level less than or equal to that of the wild type enzyme. It appears logical that mutations in the amino acid involved at the site of autoproteolysis may prevent the inactivation of the proteases by their self-digestion.

Alteration of pH optimum: Different applications of proteases reqire specific optimum

pHs for the best performance of the enzyme e.g. the application of proteases in leather and detergent industries demands an enzyme with an alkaline pH optimum whereas that for cheese making opts for an acidic protease. Protein engineering enables to tailor the pH dependence of enzyme catalysis to optimize the industrial processes. Modifications in the overall surface charge of the proteins is known to alter the pH optimum of the enzyme. Substitution of Asp 99 to Ser in subtilisin from *Bacillus amyloliquefaciens* has demonstrated the potential of altering the pH optimum of the enzyme by systematic multiple mutations on the surface of the protein [219].

Apart from these, attempts are also being made for changing the substrate specificity of the proteases as desired for their industrial applications. Strategies like SDM, combinatorial random mutagenesis approach have been used to generate mutants that secrete proteases with functional properties different from those of the parent enzyme[141]. Improvement in the yield of proteases has been achieved by screening for hyperproducing strains and/or by optimization of fermentation medium. Strain improvement using either conventional mutagenesis or recombinant DNA technology have been useful in improving the production of proteases. The existing knowledge of the structure-function relationship of proteases, coupled with gene-shuffling techniques, promises a fair chance of success, in the near future, in evolving proteases that were ever made in nature and that would meet the requirements of the multitude of protease applications.

OBJECTIVES OF THE PRESENT INVESTIGATION:

Proteases are one of the highest value commercial enzymes as they have extensive applications in food, pharmaceutical, detergent, and dairy industries and serve as important tools in determination of structure of proteins and polypeptides. The biotechnological promise of proteases makes them an ideal candidate for structure-function relationship studies. Alkaline proteases hold a great potential for application in leather and detergent industries due to the increasing awareness of developing environmentally friendly technologies. Fungal alkaline proteases offer a distinct advantage over the currently used bacterial proteases in terms of the ease of preparation of microbe-free enzyme as against the cost-intensive filtration technology required for the isolation of a bacterial enzyme. A fungus *Conidiobolus macrosporus* (NCIM 1298) isolated in our laboratory produces high amounts (30U/ml) of an alkaline protease (APC). Its activity at highly alkaline pH, broad substrate specificity, and the ease of cell-free enzyme preparation suggest that it has a potential for industrial application. Higher thermostability is often a primary goal for improving the properties of an industrial enzyme. A comprehensive understanding of the active site of the enzyme and of the mechanism of its inactivation is essential for delineating its structure-function relationship. The present investigation, therefore, was undertaken to study some of the molecular and biochemical aspects of the protease.

The highlights of the work done are as follows:

1. Structure-function relationship of the APC: purification of APC and characterization of the amino acid residues at or near the active site.
2. Characterization of unfolding and refolding pathways of the protease.
3. Thermal stability of the alkaline protease:

   (i) Thermal inactivation of the alkaline protease and use of various additives to improve the thermal stability.

   (ii) Immobilization of alkaline protease for reuse and increased thermal stability.

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CHAPTER TWO

STRUCTURE-FUNCTION RELATIONSHIP OF AN ALKALINE PROTEASE (APC) FROM *CONIDIOBOLUS MACROSPORUS* (NCIM 1298)

SUMMARY

Conidiobolus macrosporus (NCIM 1298) produces about five electrophoretically distinct extracellular alkaline proteases. In the present studies, one of the major proteases (APC) having highest electrophoretic mobility and activity has been purified to homogeneity using gel-X-ray film contact print technique followed by DEAE-cellulose chromatography. The purified APC showed a single band on SDS-PAGE and eluted as a single peak upon gel filtration on HPLC as well as on isoelectric focussing confirming homogeneity of the preparation. The optimum pH of the enzyme was pH 10.0 and it exhibited maximum activity at 40°C. The enzyme was stable over a wide range of pH (5.0-12.0) and at 45°C for 1 h at pH 7.5. APC showed a molecular weight of 28,500 and 27,000 by SDS-PAGE and gel-filtration respectively and a pI of 9.8. The molecular weight was further confirmed by amino acid analysis, which revealed that the protease consists of 263 amino acid residues. The protein was 11% glycosylated. It was inhibited by *Streptomyces* subtilisin inhibitor (SSI) but not by soybean-trypsin inhibitor. APC was able to hydrolyse substrates such as casein, haemoglobin, gelatin, myoglobin, ovalbumin, and BSA indicating its broad substrate specificity. The enzyme was inhibited by metal ions such as Cu^{2+}, Hg^{2+}, and $Fe3^{+}$.

Inhibition of the enzyme by Woodward's reagent K (WRK) (10 mM), diethylpyrocarbonate (DEPC) (5 mM) and phenylmethylsulfonyfluoride (PMSF) (10 μM) indicated the presence of Asp, His and Ser respectively in the active site of the alkaline protease. There was no effect of EDTA (10 mM) on the enzyme activity. Inactivation of the enzyme by PMSF was not reversed by treatment with β-mercaptoethanol. The enzyme was inhibited by PHMB (10 μM) and the inhibition was reversed by treatment with thiol containing compounds. These results confirm that APC is a serine alkaline protease and not a cysteine or metalloprotease. In addition, the APC was also inhibited by N-bromosuccinimide (NBS) (10μM) pointing to the presence of Trp residue in the active site. The presence, microenvironment and proximity of the essential Trp with the essential His and Cys residues in the active site of the alkaline protease were deciphered for the first time using chemical modification, chemo-affinity labeling and fluorescence spectroscopy. Kinetics of inactivation by N-bromosuccinimide (NBS) and p-hydroxymercuribenzoate (PHMB) revealed that a single Trp and Cys are essential for the activity besides the Asp, His and Ser residues of the catalytic triad. Full protection by casein against inactivation of the enzyme by NBS and quenching of Trp

fluorescence upon binding of the enzyme with NBS, substrate (sAAPF-pNA) or *Streptomyces* subtilisin inhibitor (SSI) confirmed the participation of the Trp residues at the substrate/inhibitor binding site of the alkaline protease. The microenvironment around the Trp residues was probed by studying the quenching of fluorescence by various solute quenchers. Comparison of the K_{sv} values for the charged quenchers CsCI (1.66) and KI (7.0) suggested that overall Trp microenvironment in the protease is electropositive. The proximity of Trp with His was revealed by sigmoidal shape of the fluorimetric titration curve with a pK_a of 6.1. Both protonated and modified form (DEPC treated) of histidine could quench the Trp fluorescence but quenching by the protonated form was greater. The vicinity of essential Cys with the Trp was demonstrated by the resonance energy transfer between the intrinsic fluorophore (Trp) and the 5-iodoacetamide-fluorescein labeled Cys (extrinsic fluorophore). Our results on the proximity of Trp with essential His and Cys thus confirm the presence of Trp in the active site of the *Conidiobolus* alkaline protease.

INTRODUCTION

The biotechnological promise of proteases makes them an important candidate for undertaking structure-function relationship studies. A zygospore-forming fungus *Conidiobolus macrosporus* (NCIM 1298) produces high yields (30U/ml) of alkaline protease (APC) in a short fermentation cycle (48h) comparable to that of the bacterial enzymes. Its activity at high alkaline pH and the ease of cell-free enzyme preparation are advantageous features for its potential industrial application. Purification of an enzyme is a prerequisite for studying its properties and structure-function relationship. Proteases in general are both extracellular and intracellular and can be purified by conventional procedures. For intracellular proteases, the cells can be lysed by detergents or by sonication followed by usual purification procedures employed for the extracellular enzymes. Majority of the procedures involve concentration of the culture filtrate either with salt or solvent precipitation or by ultrafiltration. The concentrated culture filtrate is then further purified by a combination of chromatographic procedures such as affinity chromatography (using a bacitracin-sepharose column or sepharose bound to inhibitor or substrate or CNBr-activated sepharose), ion-exchange chromatography (such as CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-sepharose, and gel filtration (such as sephadex). Apart from these conventional procedures, more sensitive and advanced procedures such as FPLC [1], converging-diverging foam fractionation [2], crystallization [3] and preparative PAGE [4] have been used for the purification of the proteases. The homogeneity of the enzyme preparation is checked by polyacrylamide gel electrophoresis or by gel-filtration or by IEF and the pure enzyme is then used for further characterization. The purification, biochemical and physicochemical properties of some of the proteases have been summarized in Table 2.1.

A comprehensive understanding of the active site of the enzyme and of the mechanism of its inactivation are essential for delineating the structure-function relationship of the enzyme. The knowledge of amino acid residues involved at or near the active site of an enzyme is one of the important pre-requisites for manipulating the protein by site-directed mutagenesis. X-ray crystallography is generally used to demonstrate spatial arrangement of amino acid residues in the presence and absence of the substrate of an enzyme. However, protein crystallography has its limitations, such as its inapplicability to the dynamics of molecular interactions in solution, difficulties in obtaining pure crystals and the laborious nature of the

Table 2.1 Characteristics of proteases from various species.

Organism	Purification procedure applied	Mr (kD)	Opt. pH	Opt. Temp. (°C)	Inl
Pseudomonas aeruginosa	Gel-filtration, ion-exchange chromatography	32	8.0	60	EDTA, Cu^{2+}
Xanthomonas sp. YL-37	Amm. Sulphate fractionation, CM cellulose, sephadex G-100	62	11.0	50	EDTA, Zn^{2+}, Hg
Thermoactinomyces sp. E 79	DEAE-column chromatography, ammonium sulphate, DEAE-sepharose chromatography	36	9.0	70	PMSF,
Alkalophilic *Thermoactinomyces* sp. HS 682	Butyl-Toyopearl 650M and SP-Toyopearl 650S chromatography	25	11.5-13.0	70	DFP, Pl Cu^{2+}
Thermoactinomyces sp. E 79	DEAE-sepharose CL-6B, Butyl-Toyopearl 650M column chromatography	31	11	85	PMSF
Bacillus licheniformis NS-70	Ammonium sulfate fractionation, DEAE-, CM- and phenylsepharose column chromatography	32	12	65	PMSF, Hg^{2+}, C
Organism	Purification procedure applied	Mr (kD)	Opt. pH	Opt. Temp. (°C)	Inl
Alkalophilic *Bacillus* sp. K8M-K16	Column chromatography	28	12.3	55	PMSF, chymost
B. subtilis RM 615	80% acetone precipitation, DEAE- and CM-cellulose chromatography	28	---	60	PMSF, trypsin i
Bacillus stearothermophilus	Heat treatment, ultrafiltration, gel-filtration chromatography	33.5	8.0-10.0	85	Co^{2+}, H

Oerskovia xanthineolytica TK-1	Phenyl-sepharose CL-4B, DEAE-sephacel	20	9.5-11	50	PMSF,
Alkalophilic bacteria	CM-sephadex	25	10.5	55	PMSF,
AT Bacillus sp. B18	DEAE- and CM-Toyopearl 650M column chromatography	30	85	12.0-13	DFP
Penicillium charlessii	sephacryl S-200, DEAE-cellulose, ion-exchange chromatography, FPLC on superose S2	44	7.0-9.0	---	PMSF
Streptomyces griseus HC – 1141	Ammonium sulfate, DEAE-cellulose, sephadex G-150	31	---	---	---
Bacillus sp. 1121	Ammonium sulfate, sephadex G-150, DEAE-cellulose	55	---	---	---
Bacillus macerans	Fractional precipitation with acetone, ethanol or ammonium sulfate, DEAE-sepharose	24	---	---	---
Alkaline protease associated with nuclear polyhedrosis virus	Detergent-treated virus paritcles, affinity column chromatography CNBr-activated sepharose	---	9.6	30-40	Hg^{2+}, C
Nonlysosomal alkaline protease from rat and mouse liver acetone powders (High mol. wt)	---	300	---	---	-SH gro reagents aprotinir leupepti chymost
Rat chromatin	Affinity chromatography, sepharose bound to casein	18	9.5-10	---	---
Neurospora crassa	Heat-treatment, ion-exchange, gel-filtration chromatography	30.5	---	---	---
Aspergillus clavatus	Ultrafiltration, alcohol precipitation, DEAE-cellulose	---	7.8	37	---

	and Sephadex G-200				
Shewanella strain AC10	---	44	9.0	5-15	---
Conidiobolus sp.	Ammonium sulfate saturation, alcohol precipitation, DEAE-cellulose and sephadex G-200	---	7.0-9.0	50C at pH 8.0, 40C at pH 9.0	Ag^{2+}, E KmnO4 hydroqu
Conidiobolus lampragues	Ammonium sulfate precipitation, CM-sephadex, conA-sepharose, sephadex G-75	20	---	---	---
Conidiobolus coronatus (lowest Mr)	---	6.8	9.7	45	---
Conidiobolus coronatus	Ammonium sulfate precipitation, preparative gel electrophoresis, CM-cellulose chromatography	24	9.7-10.0	6.0-8.0	Zn^{2+}, M

procedure. Other methods that can precede or complement X-ray analysis are, therefore, needed for studying the conformational changes in proteins. Spectroscopic approaches such as absorption and fluorescence spectroscopy are simple to apply and can frequently detect changes in the microenvironment of specific amino acid residues.

Alkaline proteases belong to the class of serine proteases in the dan S_8 of subtilases [5] and are shown to possess a catalytic triad of Asp32/His64/Ser221 at their active site. In this section, we describe the purification and characterization of the *Conidiobolus* alkaline protease and report for the first time, the evidence for the presence of Trp residue besides the well-known Asp-His-Ser triad in the active site of the alkaline protease. The microenvironment of the Trp has been probed by studying the quenching of fluorescence in the presence of substrate, inhibitor or various solute quenchers. The proximity of Trp with His has been demonstrated by quenching of the Trp fluorescence by the protonated form of His. The vicinity of the essential Cys with the Trp has been shown by the fluorescence energy transfer between the initrinsic fluorophore and the fluorescently labelled Cys.

MATERIALS AND METHODS

Malt extract, yeast extract, peptone and agar (all from Difco laboratories, USA), glycerol and D-glucose (Qualigens ExcelaR, India), ultrafiltration membranes (Amicon, USA), ampholines (LKB, Sweden), Hammerstein casein (Merck, Germany), 5-IAF (Pierce, USA), NBS, DEPC, PHMB, PG, NAI, OPTA, PMSF, WRK, DTNB, TNBS, sAAPF-pNA, molecular mass markers, Coomassie blue G-250 and R-250, DEAE-cellulose, acrylamide, ammonium persulphate, SDS, β-ME, GH, TEMED, L-cysteine, acrylamide, CsCl, KI, and DTT were purchased from Sigma Chemical Company (USA). All other chemicals used were of analytical grade.

2.1 MICROORGANISM AND ENZYME PRODUCTION:

Conidiobolus sp. (NCIM 1298) is an isolate from our laboratory and has been deposited in the National Collection of Industrial Microorganisms, Pune, India. It was maintained on MGYP agar (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3%; peptone, 0.5% and agar, 2%) slants at 15°C. Enzyme was produced in 500 ml Erlenmeyer flasks containing 100 ml MGYP medium with soybean meal (2%) as an inducer of the protease. Vegetative inoculum (10%) grown in the same medium for 24 h was used to inoculate the fermentation media. The flasks were incubated at 28°C on a rotary shaker at 200 rpm. The experimental flasks were harvested after 48 h. The clear supernatant after centrifugation at 13,000×g was used as the source of the enzyme.

2.2 ENZYME ACTIVITY AND PROTEIN ESTIMATION:

Kunitz's modified spectrophotometric method was used for determining protease activity [32]. Reaction mixture (2.0 ml) contained appropriately diluted enzyme in carbonate-bicarbonate buffer (0.1 M), pH 10.0 and casein (10 mg) as a substrate. After incubation at 40°C for 20 min, 3 ml of acidified TCA was added to stop the reaction. The reaction mixture was allowed to stand at room temperature for at least 30 min before the precipitate was filtered through Whatman No. 1 filter paper and the 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 of one absorbance unit per ml of reaction mixture per min at 40°C. The protease was

appropriately diluted in 1 ml of 0.1 M Tris-Cl buffer, pH 8.0 at 25°C when sAAPF-pNA (60 µg) was used as the substrate [33] and incubated for 5 min. 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 protein content was determined by the dye binding method of Bradford using BSA as a standard [34].

2.3 ENZYME PURIFICATION AND PROPERTIES:

All purification steps were performed at 4°C unless stated otherwise. Culture supernatant was concentrated to $1/20^{th}$ of its volume by ultrafiltration through a membrane of 10,000 cut off and concentrated culture filtrate (20 mg protein) was subjected to preparative polyacrylamide gel electrophoresis (PAGE) in a column (15×4.8 cm) using cationic system C described by Zuidweg *et al* [35] with slight modifications. The separation gel contained KOH- borate buffer (KOH, 0.1 M; and boric acid, 0.5 M), pH 7.6, acrylamide (12%), bisacrylamide (0.2%), TEMED (0.125%), and APS (0.07%). Electrode buffer- Tris-borate, contained Tris (0.13 M) and boric acid (0.2 M) at pH 7.8. Electrophoresis was carried out at 200 V and 50 mA with basic fuchsin as a tracking dye. After electrophoresis, a vertical strip of the gel was cut and incubated in the assay buffer (0.1 M carbonate-bicarbonate, pH 10.0) for 10 min. It was then overlaid by an equal sized X-ray film for detection of the enzyme activity in the gel by contact print technique [36] for different time period (from 5 to 15 min) at 37°C. The X-ray film was removed and washed with hot water (60-70°C) until the protease bands were visible. The enzyme band which showed highest mobility on the gel as detected by the gel-X-ray film contact print technique was excised, homogenized and eluted in 0.1 M potassium phosphate buffer, pH 7.5. The eluate was concentrated by ultrafiltration and further subjected to purification by batchwise chromatography on DEAE-cellulose equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The protease activity was located in the unadsorbed fraction. The purity of the enzyme was determined by analytical SDS-PAGE [37] and by gel filtration on HPLC using TSK G 2000 SW prepacked column (7.5×600 mm). The pure enzyme thus obtained was used for studying the properties of the enzyme.

Molecular mass of the APC was determined by SDS-PAGE according to the method of Laemmli using BSA (66 kD), ovalbumin (45 kD), pepsin (34 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), β-lactoglobulin (18 kD) and lysozyme (14 kD) as reference proteins followed by silver staining [38]. The molecular weight was also determined by gel

filtration of the enzyme on HPLC using TSK-G-2000 SW column which was calibrated using the above marker proteins and potassium phosphate buffer (50 mM), pH 7.5 as the mobile phase. The isoelectric point of the APC was determined by the modified straight tube method [39] using ampholines in the range of pH 3.0-10.0. Amino acid analysis of the APC was carried out on Pharmacia LKB alpha plus amino acid analyzer. Samples were hydrolyzed by standard acid hydrolysis conditions using 6 N HCL at 110°C for 22 h. Cys, Met, and Tyr were protected using proper protecting reagents. Trps were determined by spectrophotometric method of Spande and Witkop [40].

The APC was anlayzed for their carbohydrate content by the phenol-sulphuric acid method for estimation of total carbohydrate [41].

The optimum pH for the enzyme activity was determined by assaying the protease at pH values ranging from 2.0-12.0 in universal buffers at 40°C. The optimum temperature for enzyme activity was determined by assaying the protease at pH 10.0 at different temperatures (10-55°C).

The pH and temperature stabilities were determined by pre-incubating the protease in universal buffers of various pH values (pH 2.0-12.0) at different temperatures (10-60°C) for 1 h after which residual enzyme activities were determined at pH 10.0 and 40°C.

APC was incubated with different amounts of casein (0-10 mg) and sAAPF-pNA and TAME (0.02-0.2 mM) under assay conditions and the kinetic parameters were calculated from the Lineweaver-Burk plots.

The effect of different metal ions was determined by pre-incubating the APC with various metal salts (10 mM) for 1h and then assaying the enzyme for residual activity. Inhibition of the enzyme by various proteinaceous inhibitors was determined by incubating the protease with the inhibitor in various inhibitor to enzyme molar ratios (1:0 to 1:20 for soybean-trypsin inhibitor and 1:0 to 1:5 for *Streptomyces* subtilisin inhibitor) at 25°C for 20 min. The residual protease activity was determined by comparison with the controls of the enzyme incubated without inhibitor under similar conditions.

2.4 CHEMICAL MODIFICATION STUDIES:

Suitably diluted APC (1-2 μM) was incubated with various chemical modifiers (10 mM) in appropriate buffers in a final volume of 1.0 ml for 20 min at 25°C. Control tubes containing

enzyme alone were incubated under identical conditions and the residual activity was determined.

The APC (3 μM) in 50 mM potassium phosphate buffer, pH 7.5 was incubated with varying concentrations of PMSF (1-10 μM), WRK (1-10 mM) or DEPC (1-10 mM) in a reaction volume of 1ml at 25°C for 20 min. Aliquots were removed at different time intervals and the residual activities were determined under standard assay conditions. The modification of the enzyme (1.3 μM) upon the addition of DEPC was monitored spectrophotometrically by measuring the change in the absorbance at 240 nm as described by Ovadi et al. [42].

2.4.1 Modification of tryptophan residues:

2.4.1.1 Reaction with N-bromosuccinimide (NBS)

Kinetics of enzyme inactivation by NBS was carried out by incubating protease (2.4 μg) with varying concentrations of the NBS in 0.1 M potassium phosphate buffer, pH 7.5 in a volume of 1 ml at room temperature. Aliquots were withdrawn periodically and the residual activity was determined. The results were analyzed according to Levy *et al* [43].

[A]*Quantitation of the Trp residues:*

Spectrophotometric titration of Trp residues by NBS was carried out in two cuvettes, one containing protease (50 μg) in 0.1M potassium phosphate buffer, pH 7.5 and another containing buffer. Successive 5 μl aliquots of NBS (0.5 mM) were added to the sample as well as to the reference cuvette and decrease in absorbance at 280 nm was monitored. Simultaneously, the drop in the enzyme activity was also assayed. The number of Trp residues oxidized per mole of enzyme was calculated by the method of Spande and Witkop [40].

[B] *Monitoring changes in Trp environment:*

[B.1] Circular dichroism (CD):

The far UV-CD spectra of the native and NBS modified enzyme (0.17×10^{-4}mM) were recorded on a JASCO J715 spectropolarimeter attached with a Julabo water bath.

[B.2] Fluorescence spectroscopy:

Fluorescence measurements were performed with a Perkin-Elmer LS-50B spectrofluorimeter

equipped with a Julabo F-20 water-bath. The samples were excited at 295 nm and an excitation and an emission bandwidth of 7.5 nm was used.

[B.2.1] Enzyme-substrate/inhibitor interaction:

The effect of NBS, sAAPF-pNA or SSI on the fluorescence of the APC was determined after incubation of the enzyme (0.3 μM) with increasing concentrations of substrate/inhibitor at 15°C for 10 min. The spectrum of only SSI was subtracted from the corresponding spectrum of APC plus SSI.

[B.2.2] pH dependence of the fluorescence intensity:

Fluorimetric titration was carried out by gradual addition of 0.04 N HCl to the native (8×10^{-4} mM) or modified enzyme (treated with 10 mM DEPC) solution in 0.1 M NaCl, the pH of which was adjusted to pH 11.0 by the addition of 10 μl of 0.05 N NaOH. The absolute quantum yield calculated according to Shinitzky et.al. [44] was plotted against pH and the pK_F was determined.

[B.2.3] Quenching of Trp fluorescence:

Relative fluorescence intensity of the APC (10 μg) in 0.1 M potassium phosphate buffer, pH 7.5 was recorded in the absence and presence of various concentrations of acrylamide (0-0.1 M), cesium chloride (0-0.3 M) and potassium iodide (0-0.3 M). The quenching data were analyzed using the Stern-Volmer equation [45],

$$F_o/ F = K_{sv} (Q)$$

Where F_o is the fluorescence of the protein in the absence of quencher and F is the observed fluorescence at the concentration (Q) of the quencher. K_{sv} is the collisional quenching constant, which was determined from the slope of the Stern-Volmer plot at lower concentrations of the quencher. The enzyme was incubated with Gdn. HCl (3 M) and the quenching studies were repeated.

2.4.2 Modification of cysteine residues:

2.4.2.1 Reaction with p-hydroxymercuribenzoate (PHMB)

Kinetics of enzyme inactivation by PHMB was carried out by incubating APC (2.4 μg) with varying concentrations of the inhibitor in 0.1 M carbonate-bicarbonate buffer, pH 10.0 in a reaction volume of 1ml at room temperature. Aliquots were withdrawn periodically and the residual activity was determined. The results were analyzed according to Levy *et al* [43].

Effect of thiol containing compounds on reversal of PHMB inhibition was checked by incubating the PHMB (1 μM)-treated enzyme in presence of L-cysteine (10 mM), DTT (50 mM) or β-ME (140 mM) for 30 min and by assaying the residual activity.

2.4.2.2 Reaction with DTNB (5,5, dithiobis (2-nitrobenzoate))

Protease (10 μM) in 1 ml 0.05M potassium phosphate buffer, pH 7.5 was treated with DTNB (0.2 mM) at room temperature and the increase in absorbance at 412 nm was observed. The number of sulfhydryl groups modified was calculated by using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} [46].

2.4.2.3 Labelling of protease with 5-iodoacetamidofluorescein (5-IAF)

APC (10 μM) was modified by treatment with 100 fold excess of 5-IAF in 0.05 M potassium phosphate buffer, pH 7.5 for 24 h in the dark. At pH 7.5, iodoacetamide specifically reacts with free –SH groups [47]. The labelled protein was separated from the free reagent by passage through a Sephadex G-10 column equilibrated with the same buffer. Fractions showing absorption maxima at both 280 nm and 492 nm were pooled and concentrated by lyophilization. Covalent modification of the protein was confirmed by SDS-PAGE.

Resonance energy transfer:

Fluorescence spectrum for 5-IAF labelled APC was monitored at an excitation wavelength of 295 nm. Appearance of an additional peak at 520 nm besides that at 340 nm was recorded. 5-IAF labelled protein was further modified by NBS (10 μM) and decrease in fluorescence intensity at both 340 nm and 520 nm was recorded.

2.4.3 Substrate protection:

The effect of substrate protection was monitored by incubating the enzyme with casein (10 mg) before treating with the modifier.

RESULTS

Characteristics of the strain and production of enzyme:

The isolate *Conidiobolus macrosporus* (NCIM 1298) is a fast growing aerobic fungus, which forms whitish mycelium on MGYP/agar slants after incubation for 5-7days at 28°C. When the organism is grown on agar plates containing skimmed milk (1%), a distinct clearance zone can be observed indicating the hydrolysis of milk proteins due to the extracellular secretion of proteases. Production of several proteases (five) distinguishes this species from *Conidiobolus coronatus*, which produces only two extracellular alkaline proteases. *Conidiobolus macrosporus* also produces considerable amounts of intracellular proteases, but comparatively in much lower mount than the extracellular enzyme. Another interesting and distinguishing feature is the formation of the zygospores (Fig.2.1). Production of the enzyme was accompanied by an increase in pH of the culture from 7 to 9 and was maximum (25 to 30 U/ml) after 48 h of cultivation at 28°C (Fig.2.2).

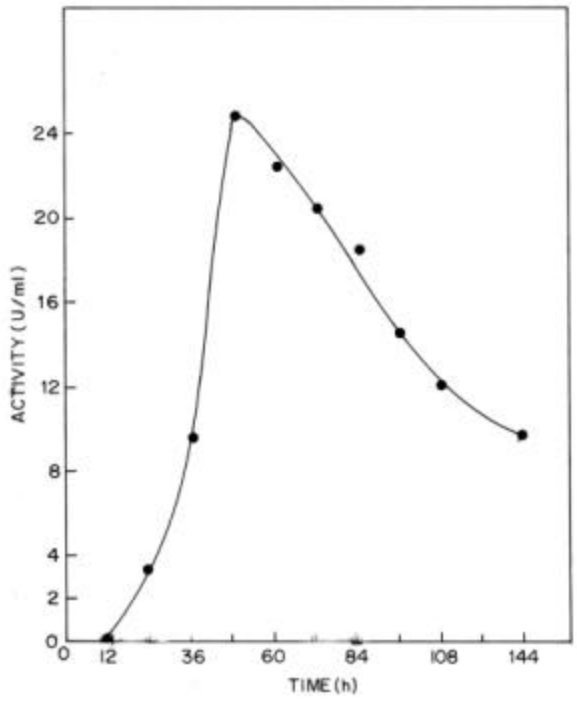


Fig. 2.2 Time course for the production of the alkaline proteases by *Conidiobolus macrosporus*

The organism was grown in MGYP medium containing soybean meal (2%) as the inducer. Aliquots of the culture broth were removed after every 12 h and assayed for protease activity.

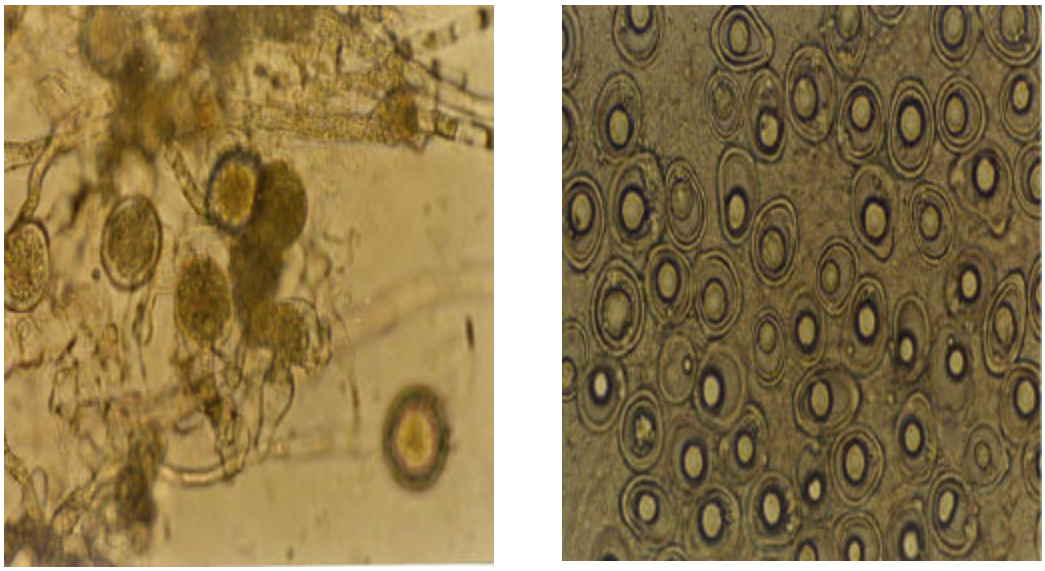


Fig. 2.1 The mycelium and the zygospores of *Conidiobolus macrosporus*

Conidiobolus macrosporus was grown on MGYP-agar slants for 3-6 days at 28°C. the mycelium and the zygospores were observed under the Leica epifluorescence binocular research microscope (magnification 100X).

Lane 1 Lane 2

Fig.2.3 Detection of the *Conidiobolus* alkaline proteases by gel-X-ray film contact print technique

Activity bands of the protease were visualized by overlaying the gel on X-ray film as described in materials and methods. Exposure time of the gel to X-ray film was 5 min. Lane 1, proteases from crude culture filtrate; lane 2, the purified alkaline protease.

Enzyme purification:

The crude extracellular broth of the *Conidiobolus macrosporus* contains five alkaline proteases as detected by the gel-X-ray film contact print technique (Fig.2.3). In the present work, the protease having the highest cationic mobility was purified to homogeneity using preparative cationic gel electrophoresis followed by batchwise DEAE-cellulose chromatography. The flow diagram for purification of the protease is illustrated in Fig. 2.4. The purified enzyme showed a single band on gel electrophoresis in the presence of SDS (Fig. 2.5) indicating homogeneity and monomeric nature of the protease. It eluted as a single peak upon gel filtration on HPLC (Fig. 2.6) and on isoelectric focussing (Fig.2.7) confirming purity of the preparation. The specific activity of the enzyme increased from 31 U/mg to 222 U/mg, thereby indicating a seven-fold purification of the protease with a 2% yield. Table 2.2 summarizes the results of the purification of the *Conidiobolus* protease.

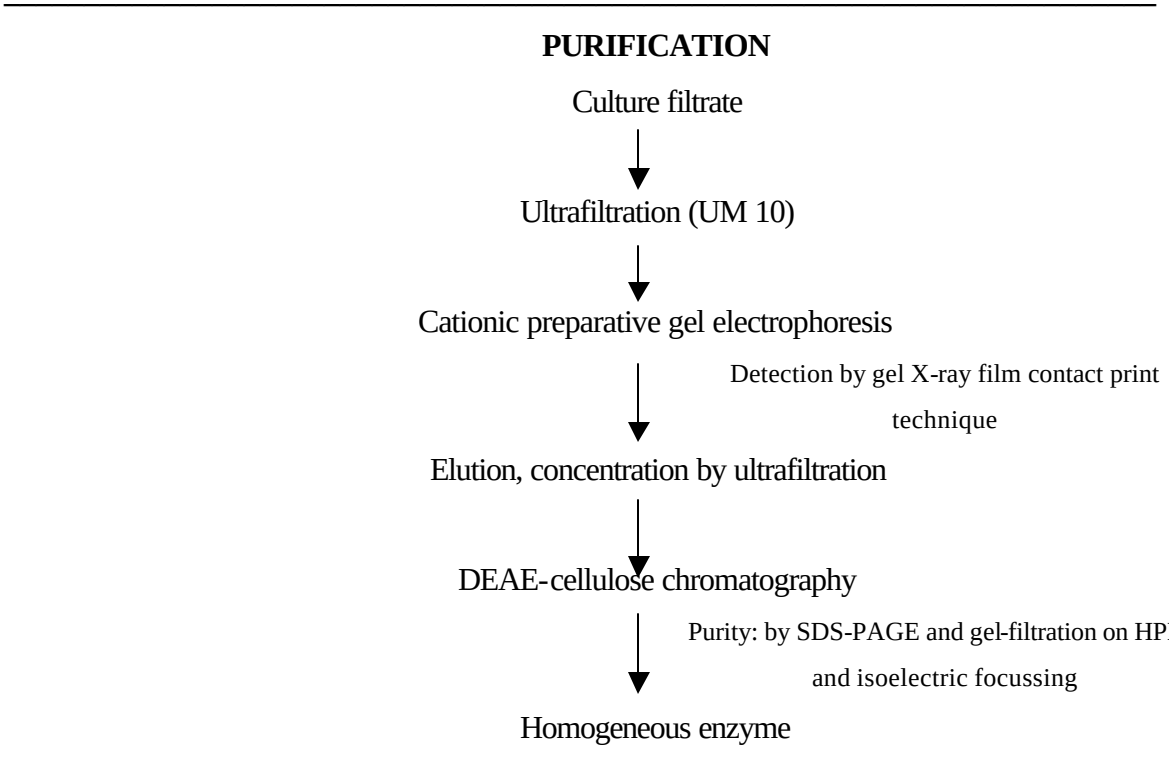


Fig. 2.4 Summary of the purification procedure employed for APC.

Table 2.2. Purification of *Conidiobolus* alkaline protease

Step	Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)
1.	Culture filtrate	800	680	20,800	31	1.0
2.	Ultrafiltration	40	256	10,140	40	1.3
3.	Electrophoresis	20	2	408	204	7.0
4.	DEAE-cellulose Chromatography	18	1.8	400	222	7.3

Properties of the enzyme:

Table 2.3 summarizes the physicochemical properties of the enzyme.

Table 2.3 Physicochemical properties of the purified enzyme

Property of the enzyme	Value obtained
Mr	
By SDS-PAGE	28.5
By gel-filtration on HPLC	27.0
By amino acid composition	28.0
pI	9.8
Optimum pH	10.0
pH stability	5.0-12.0
Optimum temperature	40°C
Temprature stability	Upto 45°C at pH 7.5 for 1 h.
Carbohydrate content	10-11%

The value of Mr (28.5 kD) obtained by SDS-PAGE was close to that obtained by gel-filtration on HPLC (27 kD) and revealed the monomeric nature of APC. The amino acid composition of the APC is given in Table 2.4. The protease had an optimum pH of 10.00 and showed 90% and 30% of the optimum activity at pH 12.0 and pH 6 respectively.

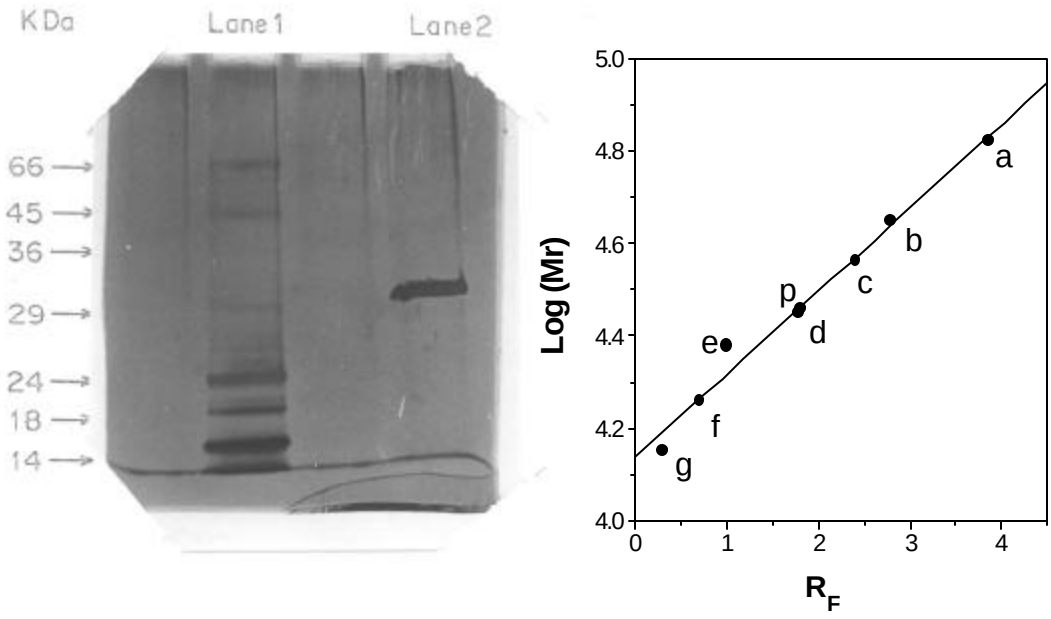


Fig 2.5 SDS-PAGE of purified APC

Lane 1, molecular weight markers: a, BSA (66 kD); b, ovalbumin (45 kD); c, trypsinogen (36 kD); d, carbonic anhydrase (29 kD); e, pepsin (24 kD); f, β-lactalbumin (18 kD) and g, lysozyme (14 kD); lane 2, p, purified APC (10 µg). Acrylamide (12%) was used for the gel electrophoresis. The gel was stained with silver nitrate.

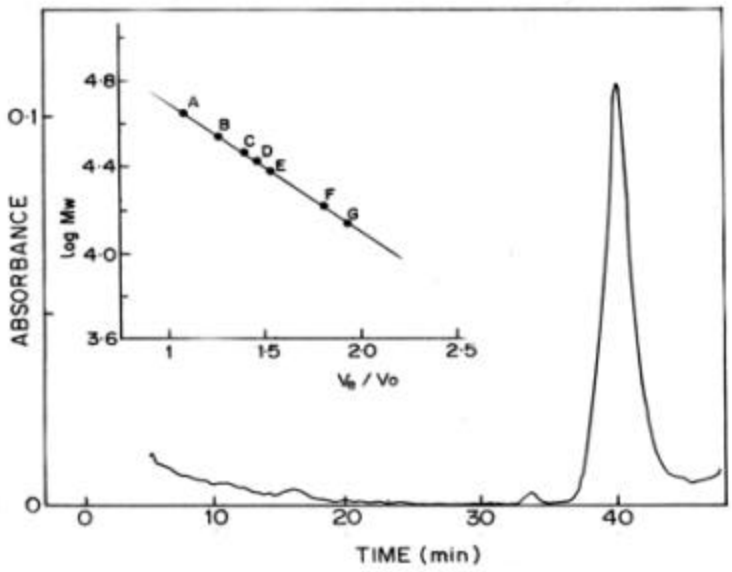


Fig. 2.6 Determination of Mr of alkaline protease by gel filtration on HPLC

Elution profile of purified APC upon HPLC. Inset: Molecular weight of the alkaline protease (D) was determined using the markers (A) ovalbumin, (B) trypsinogen, (C) carbonic anhydrase, (E) pepsin, (F) β-lactalbumin and (G) lysozyme.

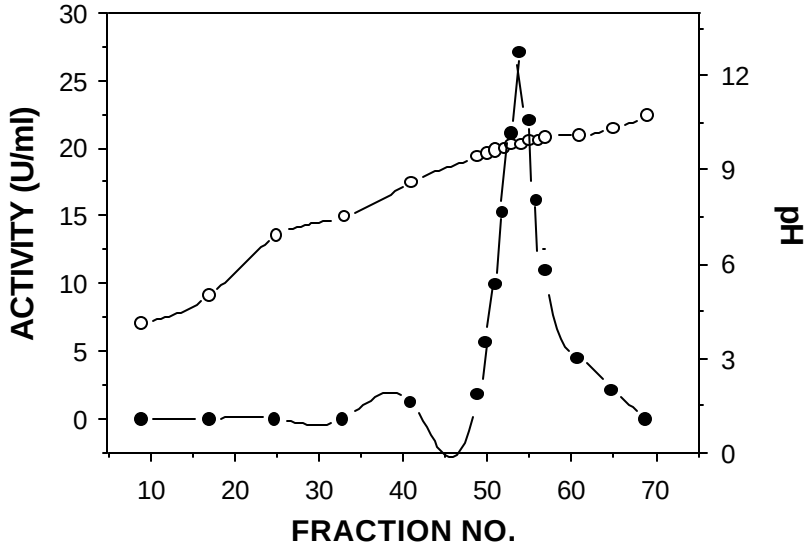


Fig. 2.7 Isoelctric focussing of the purified APC. The pI was determined using the ampholytes in the range of pH 3.0 to 10.0. The fractions were assayed for protease activity (λ) and checked for pH (○).

Table 2.3. Amino acid composition of the *Conidiobolus* alkaline protease.

Amino acid residue	*C. macrosporus* **No. of residues**	*C. coronatus** **No. of residues**
Aspartic acid	31	23
Threonine	21	19
Serine	24	21
Glutamic acid	13	3
Proline	13	5
Glycine	37	34
Alanine	35	40
Cysteine	1	1
Valine	24	18
Methionine	1	1
Isoleucine	10	7
Leucine	12	8
Tyrosine	5	3
Phenylalanine	5	4
Histidine	6	9
Lysine	17	23
Arginine	5	4
Tryptophan	3	3

The Trp residues were determined according to the method of Spande and Witcop [40].

The Cys residues were determined from titration with DTNB [46].

***** The data for the protease I from *C. coronatus* has been taken from the ref. [47].

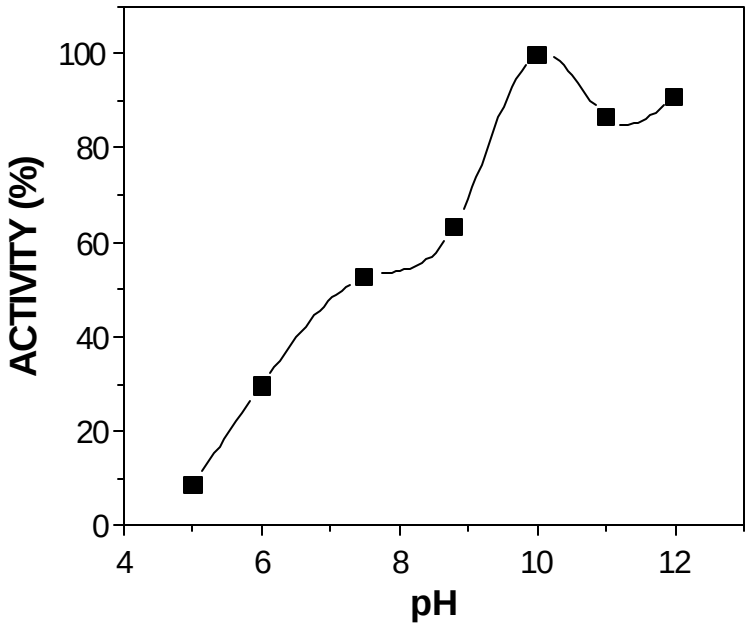


Fig. 2.8 Optimum pH of the APC.

The protease activity was determined at 40°C at different pHs (5.0-12.0) by the caseinolytic assay. The maximum activity obtained was taken as 100%.

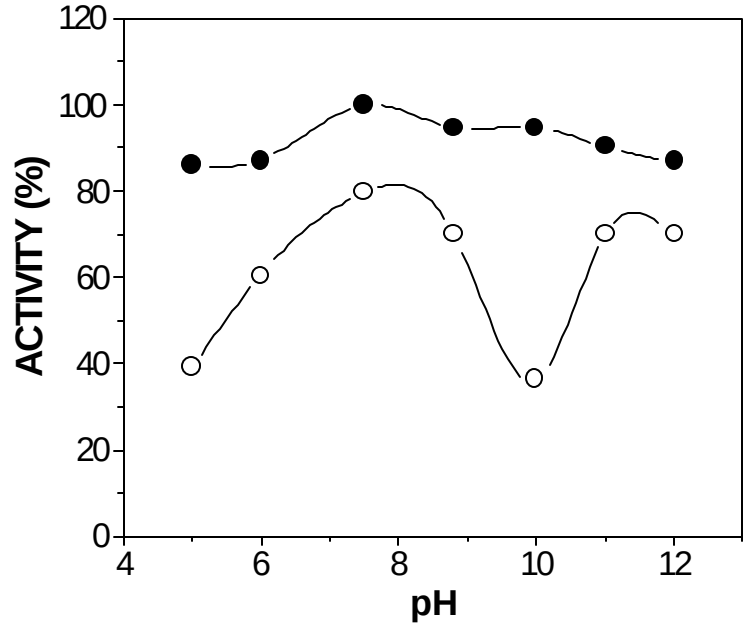


Fig. 2.9 pH stability of the APC

The enzyme samples (15U) were incubated in various buffers (pH 5.0-12.0) at 25°C for different time period (1-228h). After the incubation, residual activity in each sample was determined by caseinolytic assay. Activity of the control sample incubated at 4°C, at pH 7.5 was taken as 100%. Residual activity after 6 h (λ), after 228 h (○).

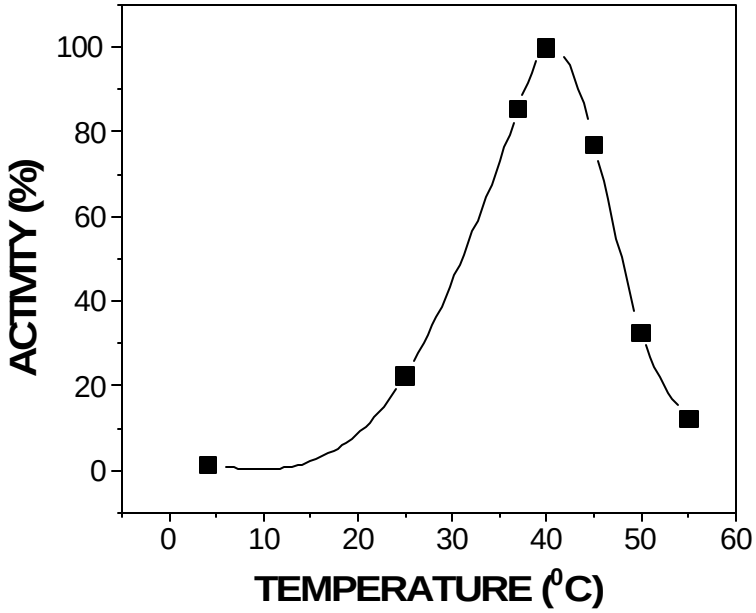


Fig. 2.10 Optimum temperature of the APC

The protease activity was determined at pH 10.0 with casein as substrate at different tempreatures (25-55°C). The maximum activity obtained was taken as 100%.

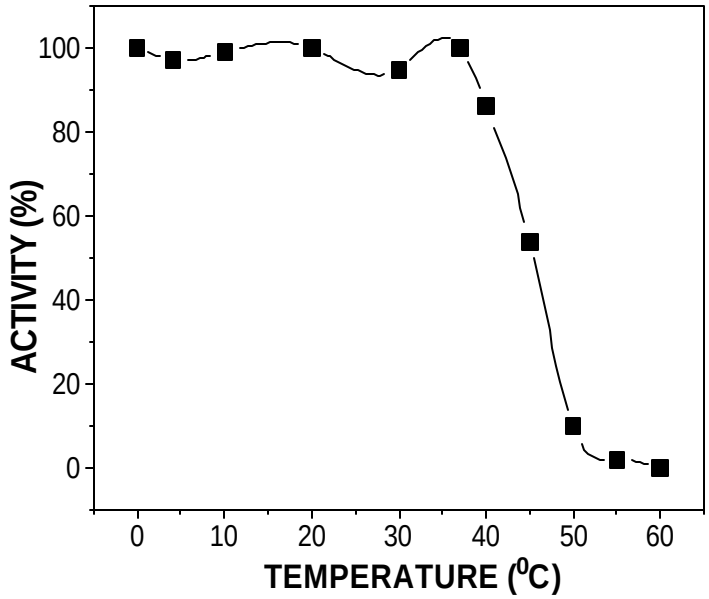


Fig. 2.11 Thermal stability of the APC

The enzyme aliquots (0.3U) were incubated at various temperatures ranging from 0 to 60°C, at pH 7.5 for 1h, and the residual activity was determined. Activity of control sample kept at 4°C at pH 7.5 was taken as 100%.

significant amount of its activity (> 50%) between pH 5 to 12 for 228 h at 25°C (Fig. 2.9) except at its optimum pH i.e. pH 10.0. The temperature optimum of the enzyme was 40°C and was stable upto 45°C for 1 h at pH 7.5. Temperature stability of the enzyme has been described in section A of Chapter 4. The kinetic parameters like K_m, and turnover number (K_{cat}) are listed in Table 2.5.

Table 2.5 Kinetic parameters for the APC

Substrate	Km	Kcat
Casein	2.9 mg/ml	4.77×10^{5} min^{-1}
SAAPF-pNA	5.28 mM	1.64×10^{6} min^{-1}
TAME	50 mM	28 min^{-1}

Among the metal ions tested, heavy metal ions such as Hg^{2+}, Cu^{2+}, Fe^{2+} completely inhibited the enzyme activity (Table 2.8). EDTA (10 mM) had no effect on the protease activity.

Table 2.6 Effect of metal ions (10 mM) on protease activity

Metal ion	Residual activity (%)
None	100
K^{+}	95
Mg^{2+}	122
Mn^{2+}	113
Ca^{2+}	92
Na^{2+}	94
Ba^{2+}	102
Mo^{2+}	66
Hg^{2+}	0
Zn^{2+}	72
Co^{2+}	100
Fe^{3+}	0
Cu^{2+}	0

APC was inhibited by subtilisin inhibitor (SSI) at the protease: inhibitor molar ratio of 2:1 but not by soybean-trypsin inhibitor indicating its subtilisin-like nature. The APC exhibited a broad substrate specificity, as evidenced by its ability to hydrolyze a large variety of proteins such as casein, hemoglobin, myoglobin, bovine serum albumin, gelatin and ovalbumin. It was also able to hydrolyze the synthetic substrates such as sAAPF-pNA, TAME. However, it was unable to hydrolyze BAPNA.

Table 2.7 Protease activities with different substrates

Substrate	Protease activity (U/ml)
Proteinaceous	
Casein	19.2
Hemoglobin	36.2
Myoglobin	18.45
Bovine serum albumin	4.35
Ovalbumin	1.85
Synthetic	
TAME	3.2
BAEE	1.8
BAPNA	0
SAAPF-pNA	113.6

The enzyme was incubated with proteins (10 mg/ml) and synthetic substrates (2 mM) as described in materials and methods and the protease activity was determined.

Amino acid residues at or near the active site of the alkaline protease:

The effect of various chemical modifiers on the activity of the *Conidiobolus* alkaline protease is shown in Table 2.8. Activity of the enzyme was completely lost upon treatment with PMSF (10 μM) or WRK (5 mM) or DEPC (10 mM). There was an increase in the absorbance at 240 nm upon addition of DEPC to the protease without any change in absorbance at 278nm. These results indicate the presence of Ser, Asp, and His residues respectively in the active site of the purified APC. Casein (10 mg) offered complete protection to the enzyme against inactivation by PMSF, DEPC, and WRK suggesting the presence of these residues at the substrate-binding

site of the protease. These residues constitute the characteristic active-site triad of the serine proteases. Incubation of the *Conidiobolus* alkaline protease with NAI (10 mM), PG (10 mM) or TNBS (1 mM) had no effect on the enzyme activity indicating the absence of Tyr, Arg and Lys respectively in the active site.

Table 2.8 Effect of various chemical modifiers on protease activity

Inhibitor	Conc. mM	Incubation buffer Salt, mM, pH	Inhibition (%)
PMSF	0.005	Phosphate, 50, 7.5	100
DEPC	10	Phosphate, 50, 6.0	94
WRK	10	Phosphate, 50, 6.0	100
TNBS	1	Phosphate, 50, 7.5	0
NAI	10	Phosphate, 50, 7.5	0
IA	10	Phosphate, 50, 7.5	0
PG	10	Carbonate, 50, 8.0	0
NBS	0.01	Phosphate, 50, 6.0	100
HNBB	10	Phosphate, 50, 7.5	100
DTNB	0.2	Phosphate, 50, 7.5	100
PHMB	0.01	Carbonate, 100, 10.0	100
OPTA	1	Phosphate, 50, 7.5	100
5-IAF	1	Phosphate, 50, 7.5	100
EDTA	10	Phosphate, 50, 7.5	0

Tryptophan is essential for the *Conidiobolus* alkaline protease activity:

Spectrophotometric titration of the alkaline protease with NBS revealed that there was a progressive decrease in absorption at 280 nm accompanied by simultaneous inactivation of the enzyme activity after successive addition of NBS (Fig.2.12a). 10 μM NBS was required for the total loss of activity. Decrease in the absorption at 280 nm and inability of NAI (Tyr specific modifier) eliminates the possibility of thiol and Tyr group modification respectively by NBS. The number of Trp residues oxidized per mole of the enzyme was found to be 3.15. However, the plot of number of Trp residues oxidized against loss in activity demonstrated

(a)

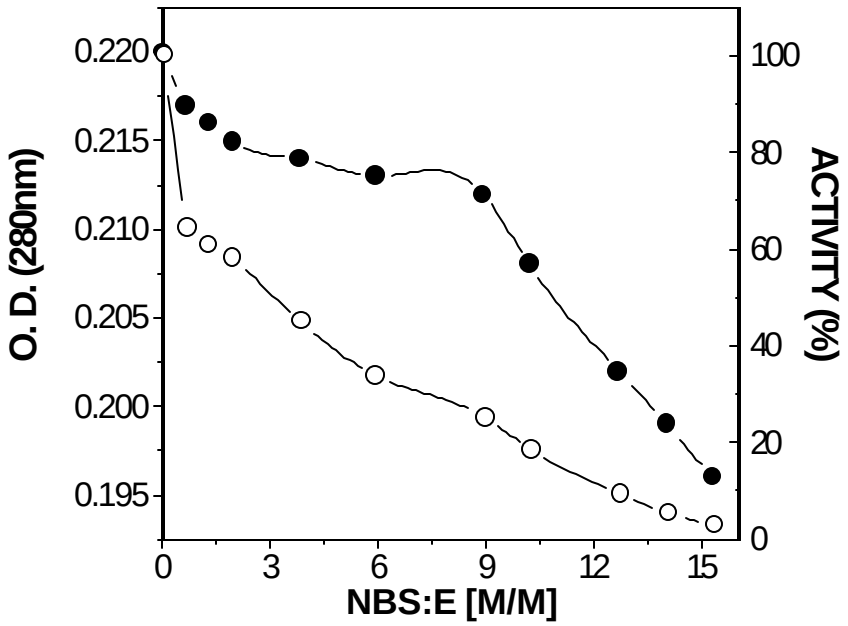


Fig. 2.12 (a) Titration of APC with NBS

Aliquot (5μl) of NBS (5 x 10^{-4} M) were added successively to the enzyme (50μg). After each addition, the residual activity (O), and the absorption at 280nm (λ) were monitored.

(b)

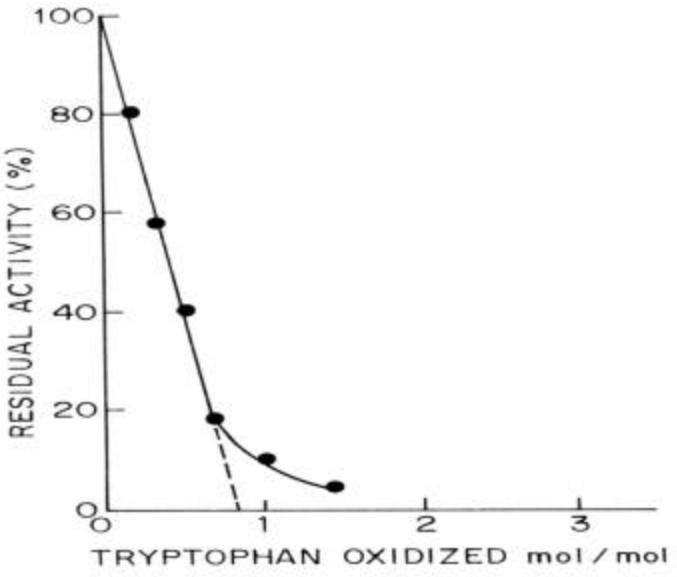


Fig.2.12 (b) Determination of number of Trp residues oxidized by NBS

Percentage residual activity plotted against the number of Trp residues oxidized as deduced by spectroscopic studies.

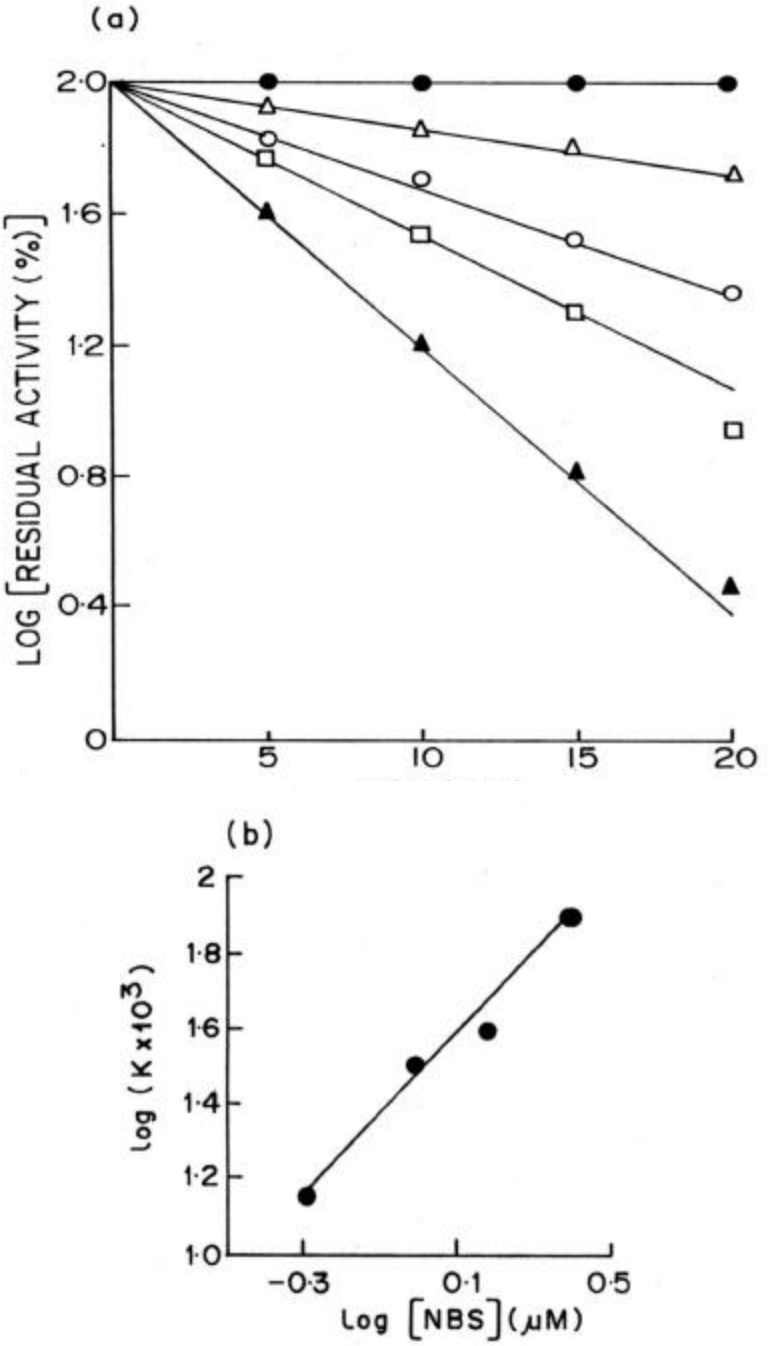


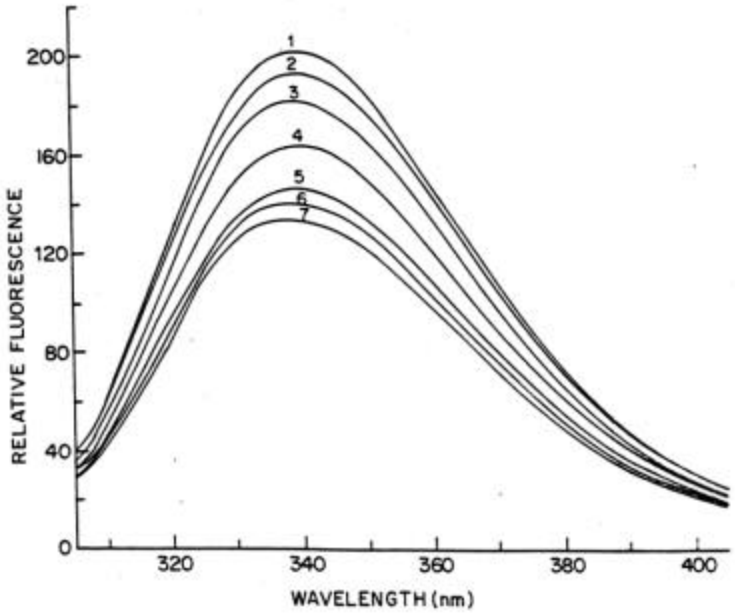
Fig.2.13 Kinetics of inactivation of APC by NBS:

(a)The enzyme (7×10^{-4}mM) was incubated with NBS (0μM, λ; 0.5μM, Δ; 1μM, μ; 1.5μM, ; and 2μM, σ) for different time intervals. (b) Apparent order of the reaction with respect to NBS concentration.

that complete inactivation of the enzyme occurred due to oxidation of a single Trp residue (Fig. 2.12b). Semilogarithmic plots of residual activity as a function of time for various concentrations of NBS (Fig. 2.13) were linear indicating that the inactivation follows pseudo-first-order kinetics. Plot of logarithm of pseudo-first-order rate constants as a function of logarithm of the NBS concentration yielded a slope of 1.0 for NBS. The loss of enzyme activity can thus be attributed to the inactivation of single Trp residue. The second order rate constant was determined to be 3.45×10^{3} $M^{-1}min^{-1}$. The K_m value of the NBS-modified enzyme (30%inactivation) was higher (3.6mg/ml) than that of the native enzyme (2.9mg/ml). The K_{cat} values for the native and modified enzyme were 21,260 and 17,575 min^{-1} respectively. Casein (1mg) was able to offer 100% protection to the enzyme against NBS-inactivation suggesting the presence of Trp residues at or near the substrate-binding site of the *Conidiobolus* alkaline protease. The enzyme was also completely inactivated by HNBB (10mM) confirming the participation of Trp at the active site of the protease.

Monitoring perturbance in the environment of essential Trp residues:

Alkaline protease from *Conidiobolus* sp. exhibits an emission maximum at 340 nm when excited at 295 nm. The titration of the native enzyme with increasing concentrations of NBS led to the progressive quenching of the tryptophanyl fluorescence due to oxidation by NBS (Fig. 2.14a). No shift in the emission maximum (340 nm) was observed upon treatment with NBS suggesting that there is no change in the conformation of the protein. The far UV-CD spectrum of the APC exhibits two minima at 219nm and 209nm, characteristic of a protein having an α-helix. The far UV-CD spectra of the native and NBS-treated enzyme were similar (Fig.2.14b) revealing no change in the secondary structure of the enzyme. These results also confirm that the inactivation of the enzyme by NBS is not due to the gross change in its conformation but is due to the reaction of NBS specifically with Trp. A progressive quenching in the fluorescence of the native enzyme at 340 nm when excited at 295 nm was observed concomitant to the binding of substrate, sAAPF-pNA or inhibitor, SSI (Fig. 2.15), indicating the change in the environment of Trp residues and confirming their presence at or near the substrate/inhibitor binding site.



(b)

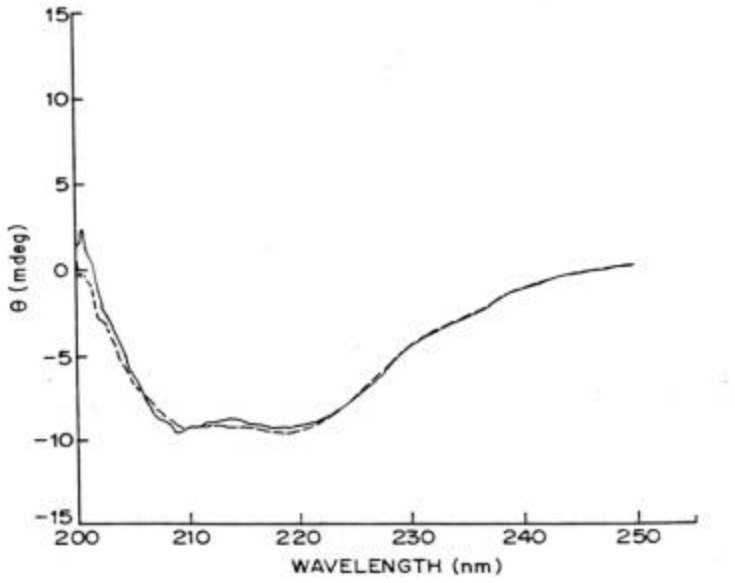


Fig. 2.14 Monitoring conformational changes in the NBS-modified enzyme

(a) Fluorescence spectra of the enzyme at E: NBS molar ratio of 1:0, 1:1, 1:3.3, 1:3.5, 1:8, 1: 10.7 and 1: 13.3 (b) Far UV-CD spectra of the native (—) and the modified enzyme (E:NBS molar ratio, 1:10) (----)..

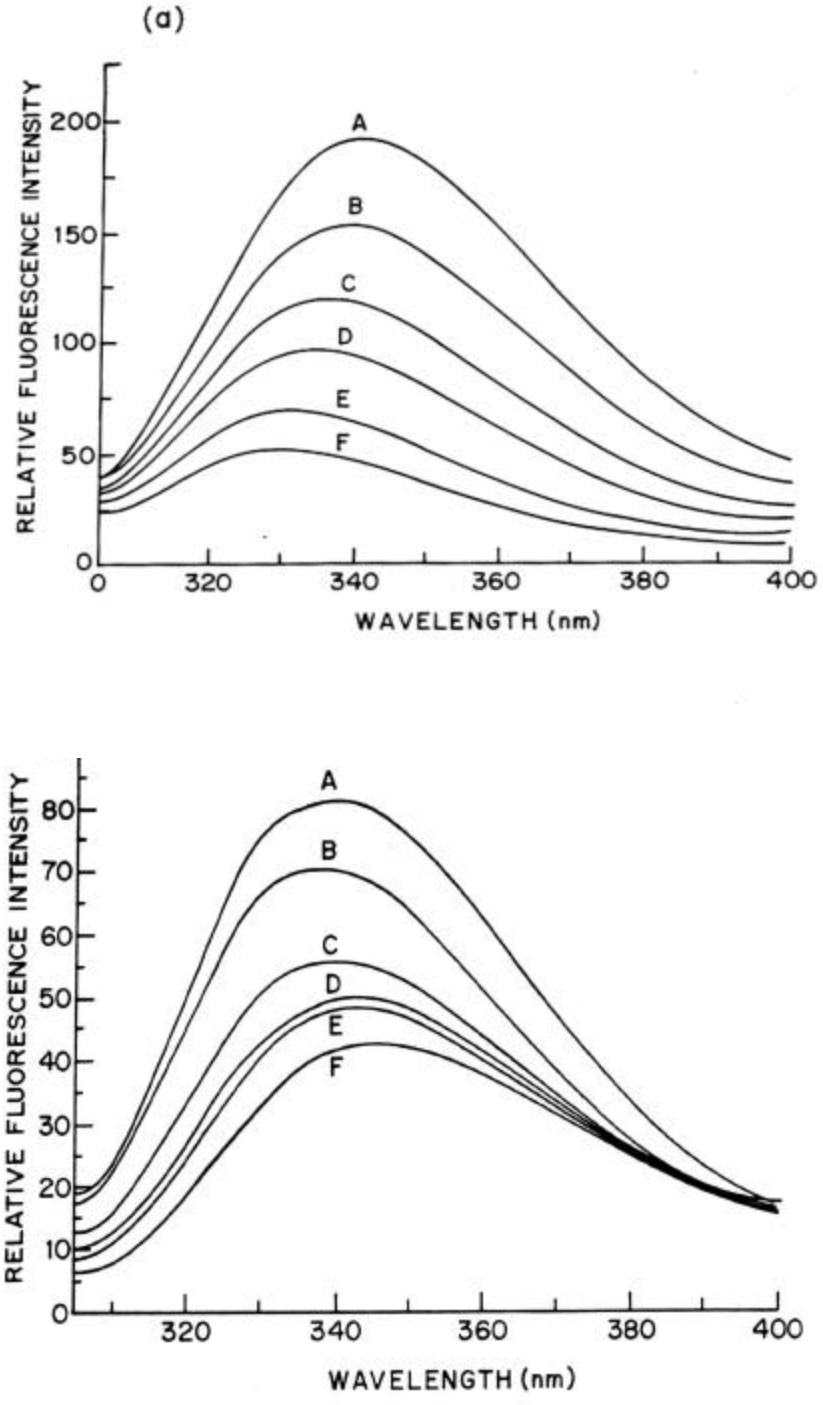


Fig. 2.15 Effect of substrat or inhibitor on the intrinsic fluorescence of the APC.

The enzyme (1.75×10^{-4} mM) was treated with increasing concentrations of (a) sAAPF-pNA (A, 0μM; B, 10μM; C, 25μM; D, 40μM; E, 60μM and F, 80μM) or (b) SSI (A, 0μM; B, 0.14μM; C, 1μM; D, 1.43μM; E, 1.79μM and F, 2.86μM). The excitation wavelength was 295nm.

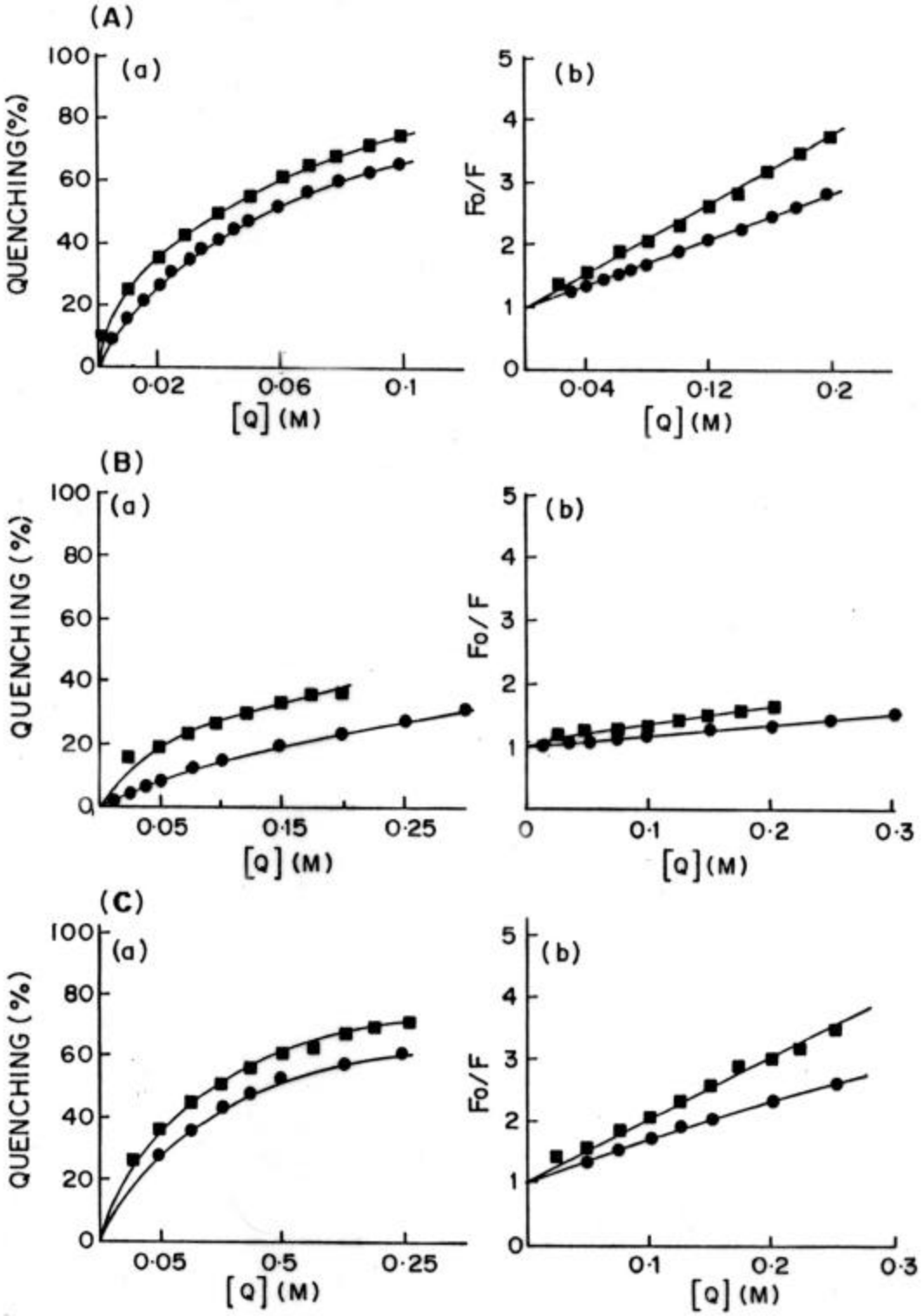


Fig. 2.16 Microenvironment of Trp residues of the APC

Effect of acrylamide (A), CsCl (B), and KI (C) on (a) the fluorescence of the native enzyme (λ) and in the presence of 3M Gdn.HCl (ν). (b) Corresponding Stern-Volmer plots.

Solute quenching of tryptophanyl fluorescence:

Solute quenching of protein fluorescence is used to gain information regarding the localization and polarity of fluorophores in proteins. In our study, quenching of tryptophanyl fluorescence of the alkaline protease with neutral (acrylamide), positively charged (Cs^+) and negatively charged (I^-) solutes was studied. 25% quenching was obtained with 20 mM acrylamide, 225 mM CsCl and 42.5 mM KI, indicating that among the quenchers used, acrylamide is the most efficient quencher (Fig.2.16). The enzyme retained full activity in the presence of acrylamide (0.1 M), CsCl (0.3 M) and KI (0.3 M) and as there was no shift in the emission maximum of the enzyme at 340 nm, the observed quenching of fluorescence was not due to the change in conformation of the protein. The Stern-Volmer plots for all the three quenchers were linear (Fig.2.16) indicative of a single class of fluorophores, all equally accessible to the quencher. Protein unfolding in presence of 3 M Gdn.HCl led to an increase in the efficiency of quenching in case of all the three quenchers. There was an overall (approximately 3 fold) increase in the collisional quenching constants (Ksv) in the presence of Gdn.HCl (Table 2.9).

Table 2.9. Quenching parameters of *Conidiobolus* alkaline protease.

Quencher concentration	**Gdn.HCl. (3M)**	**K_{sv}**
Acrylamide (5-100mM)	-	18.0
Acrylamide (5-100mM)	+	55.0
CsCl (12.5-300mM)	-	1.7
CsCl (12.5-300mM)	+	4.0
KI (50-250mM)	-	7.0
KI (50-250mM)	+	22.0

Fluorimetric detection of Trp-His interaction:

When the emission fluorescence intensity of APC at a fixed wavelength (350 nm) was plotted as a function of pH (Fig. 2.17), a sigmoidal curve with a pKa of 6.1 was obtained. The emission spectrum is mainly due to Trp residues, as the protein was excited at 295 nm. The pH dependence of fluorescence intensity, therefore, suggests the presence of an ionizable group with a pKa of 6.1 (His) that affects a Trp residue in alkaline protease. The effect of modification of His by DEPC on the titration curve of fluorescence intensity as a function of pH was also studied.

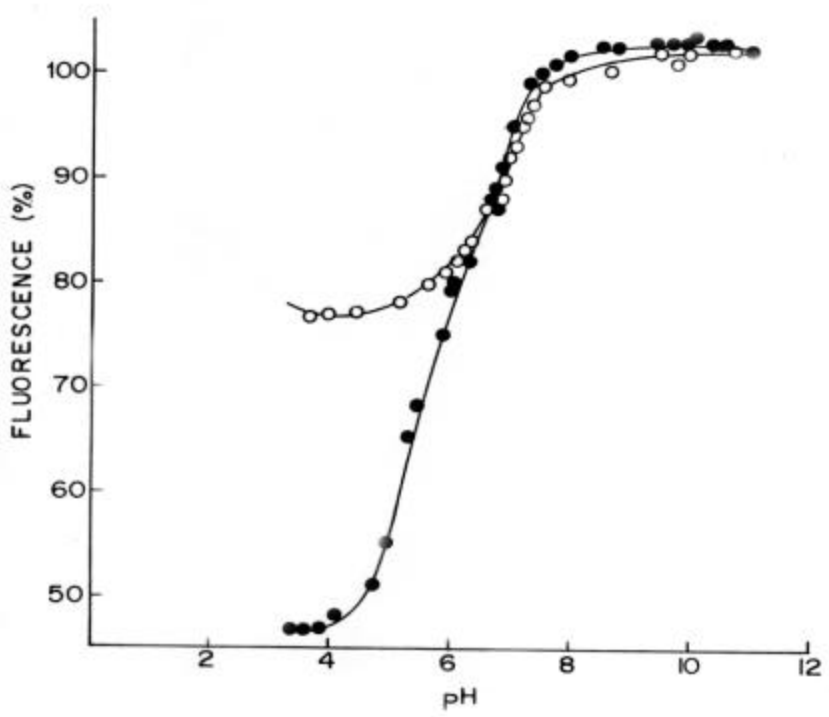


Fig. 2.17 pH dependence of fluorescence intensity of alkaline protease.

Effect of gradual addition of HCl (0.04N) on the native (▲) and DEPC (10mM) treated (○) enzyme (8×10^{-4}mM) in 0.1M NaCl buffer, pH 11.0, as observed by monitoring the fluorescence intensity at 350nm.

No significant quenching of the Trp fluorescence was observed in the acidic limb of the sigmoidal curve upon treatment of the enzyme with DEPC (10mM) and the pKa increased to 6.65. Although both protonated and modified forms of His can quench the fluorescence of Trp, the quenching by the protonated form is greater.

Cysteine is essential for the *Conidiobolus* alkaline protease activity:

Inhibition of the enzyme by Hg^{2+} or PHMB (10µM) indicated that the thiol containing amino acids, particularly Cys may be present in the active site. The enzyme was inhibited by OPTA (1 mM) but not by TNBS (1mM) supporting the presence of a thiol group in the active site of the enzyme. There was no significant change in fluorescence spectrum of the PHMB treated enzyme revealing that the observed inactivation was due to the modification of specific amino acid residues rather than change in the conformation of the enzyme. Semilogarithmic plots of residual activity as a function of time for various concentrations of PHMB (Fig. 2.18) were linear indicating that the inactivation follows pseudo-first-order kinetics. Plot of logarithm of pseudo-first-order rate constants as a function of logarithm of the inhibitor concentration yielded a slope of 1.0 for PHMB. The loss of enzyme activity can thus be attributed to inactivation of a single Cys residue. Modification of the enzyme with DTNB was accompanied by an increase in the absorbance of the modified protein at 412 nm, which corresponded, to one mole of free -SH per mole of enzyme. Complete protection against inactivation by PHMB was offered by casein (10 mg) suggesting the presence of Cys residues at or near the substrate-binding site of *Conidiobolus* alkaline proteases. Inactivation of the enzyme by PMSF was not reversed by incubation with β-ME (0.14 M) confirming that it is a serine and not a cysteine protease. L-Cysteine, DTT or β-ME exhibited reversal against inactivation by PHMB confirming the presence of essential thiol group in the active site of enzyme. Protection of enzyme inactivation against both PHMB and OPTA by these thiol compounds provides further support for the involvement of Cys at the active site.

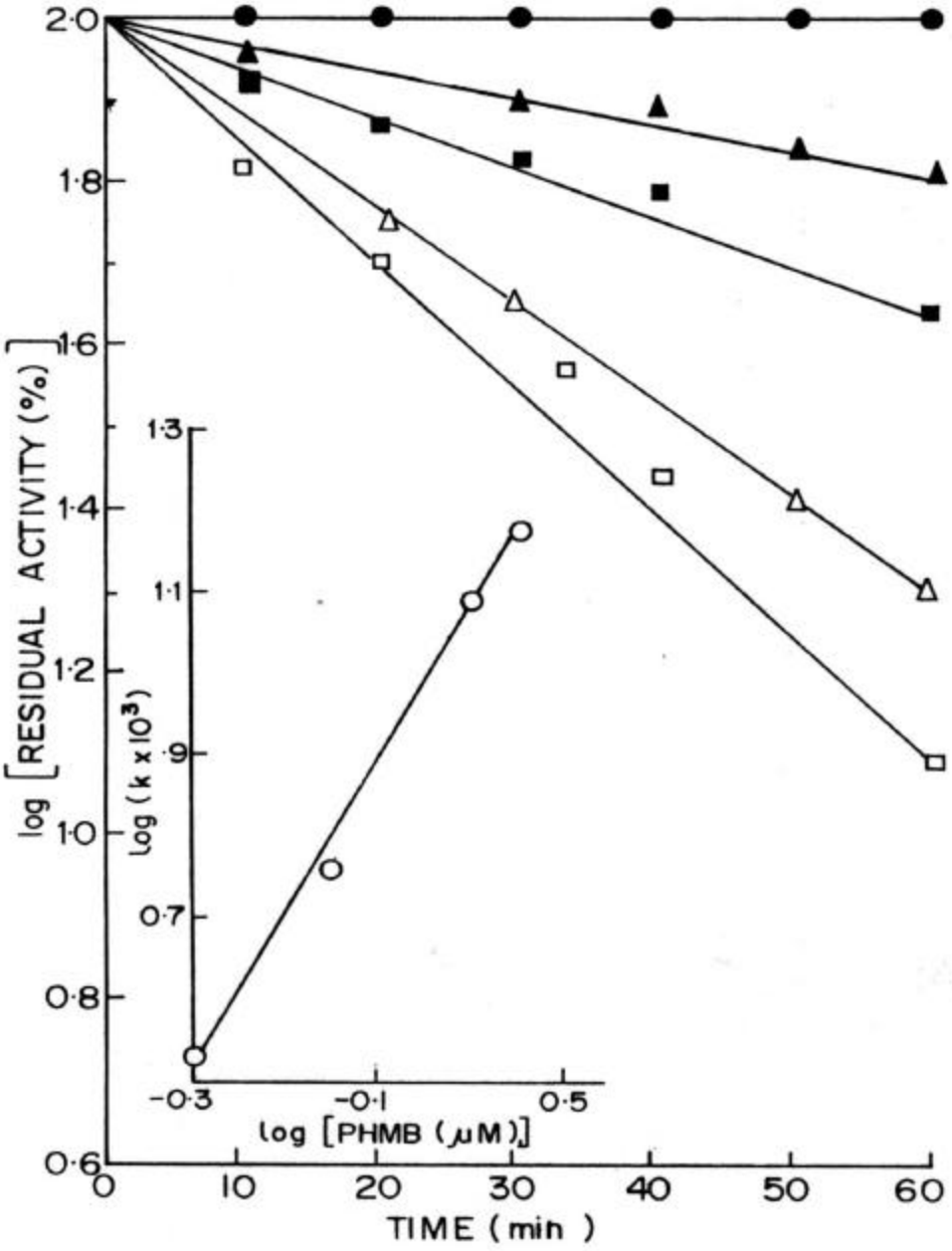


Fig. 2.18 Kinetics of inactivation of the APC by PHMB

The enzyme (7×10^{-4}mM) was incubated with PHMB (0μM, λ; 0.5μM, σ; 1.0μM, ν; 2.0μM, Δ; and 2.5μM,). The logarithm of observed first-order rate constant (k) was plotted against the logarithm of concentration of PHMB.

Fluorescence energy transfer between Trp and Cys:

In order to assess the proximity of the Trp and Cys residues at the active site of the enzyme by fluorescence energy transfer, fluorescent label was introduced at the -SH group of the enzyme by its reaction with 5-IAF. A single absorbance peak was detected at both 280 nm and 492 nm for the fluorescein labelled protein after elution from Sephadex G-10 column. SDS-PAGE of the labelled protein showed a single fluorescent band confirming the covalent binding of fluorescein probe to the enzyme (data not shown). For the 5-IAF bound enzyme, the emission maximum (340 nm) of the intrinsic fluorophore Trp (excitation at 295 nm) was the same as that of the unbound enzyme, suggesting no conformational change in the modified enzyme (Fig. 2.19).

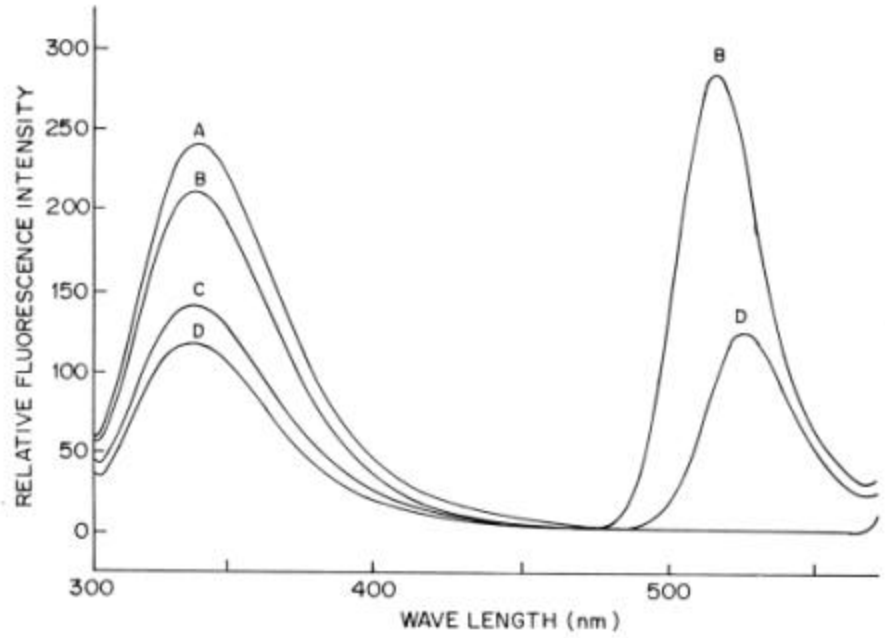


Fig. 2.19. Resonance energy transfer between Trp and fluorescein labelled Cys

Fluorescence of native enzyme (1.75×10^{-4}mM) (A), treated with NBS (10μM) (C) and 5-IAF labelled enzyme (10μg) (B), treated with NBS (10μM) (D).

Resonance energy transfer between Trp and fluorescein labelled Cys (extrinsic fluorophore) in the alkaline protease was demonstrated by (a) the partial quenching of the intrinsic fluorescence of the enzyme upon binding to the probe and concomittant appearance of an additional emission peak at 520 nm , characteristic of bound 5-IAF and (b) by decrease in emission peak at 520 nm upon treatment of fluorescein-labelled alkaline protease with NBS. The results of the experiment suggest that the single fluorescein- labelled Cys is within 10- 20 $^{\circ}$A from the Trp residue present in the active site.

DISCUSSION

The little investigated fungi, *Conidiobolus* and *Entomophthora* are the members of the order Entomophthorales and exhibit an unusual phenomenon of forcibly discharging mature conidia from a conidiophore. *Conidiobolus macrosporus* (NCIM 1298) exhibits an interesting feature of zygospore formation which serves as an additional means of propagation besides its asexual reproduction by conidia formation. It produces about five elecrophoretically distinct extracellular proteases. In the present work, one of the major proteases from *Conidiobolus macrosporus* (NCIM1298) having highest electrophoretic mobility has been purified. Since a large amount of the enzyme is required for structural studies, initial attempts were directed towards the development of a simple and efficient procedure to obtain a homogeneous preparation in good yield. Purification of alkaline proteases from different sources has been achieved by several chromatographic procedures (Table 2.1). However, the use of preparative cationic gel electrophoresis followed by gel-x-ray film contact print technique offers a simple, rapid and sensitive method for the purification of the alkaline protease. Several methods are available for the detection of electrophoretically separable proteinase isoforms based either on the co-polymerization of the substrate with the polyacrylamide gel [48] or on the post-electrophoretic incubation of the gel with the substrate [49]. However, they are tedious, time consuming and require the staining and destaining of the gel. The use of gelatin coating present on the X-ray film as a substrate for the off-gel detection of proteinase facilitated a single step, 7-fold purification of the *Conidiobolus* protease.

The purified APC exhibited maximum activity at pH 10.0 as shown by most of the alkaline proteases (Table 2.1). An alkaline protease from *Streptomyces* sp. and extremophiles such as alkalophilic *Thermoactinomyces* sp. HS 682 have an optimum pH of 13.0 [50,8]. The APC is stable over a wide range of pH (pH 5-12) even after 132 h, which is important for its industrial importance. Most of the fungal proteases are stable in the pH range of 5.0 to 10.0, especially at neutral pH and at temperatures below 55-60°C. Alkaline proteases from various fungi exhibit optimum temperature of 40-50°C as is observed for APC (40°C), while those from bacteria have higher temperature optima (as high as 85°C [14] and 115°C [51]. The fungal alkaline proteases are generally stable upto 50-60°C whereas the bacterial alkaline proteases exhibit higher thermal stabilities (eg *Thermoactinomyces* sp. E79 [8] secretes an alkaline protease, which is stable upto 75°C; stability increased upto 90°C in presence of

Ca^{2+}). Higher thermal stabilities of bacterial proteases as compared to fungal proteases make them more preferred as 'detergent enzymes'. However, the high pH stability of the fungal alkaline proteases and their ease of isolation compared to the bacterial enzymes are attracting more attention for their use as additives in detergents.

The isoelectric point of the APC was determined to be 9.8. Most alkaline proteases from microbial origin have pIs in the range of 6.0-11.0, although there are a few reports of alkaline proteases having acidic pIs as low as 3.8 [52, 53], and 2.8 [54].

The molecular mass of the APC was found to be 28.5kD by SDS-PAGE, whereas by gel-filtration chromatography it was found to be 27.0 kD. It has been suggested that ionic or hydrophobic interactions with the gel-matrices could lead to increased retention times and thereby leading to underestimation of mass [55]. The molecular mass calculated according to amino acid composition of the APC is consistent with that determined by SDS-PAGE. The molecular weight of most of the alkaline proteases is in the range of 15-40kD, however, there are reports of an alkaline protease from *Conidiobolus* sp. [31] having an Mr of 6.8 kD (the lowest Mr protease reported so far) and from *B. subtilis* [56] having an Mr of 540 kD. The nonlysosomal alkaline protease from rat and mouse liver has been reported to have an Mr of 300 kD as detected by Sephacryl S-300 and 650 kD as detected by gel filtration [23].

Analysis of amino acid composition of the APC and its comparison with that of protease I from *Conidiobolus coronatus* revealed that both show predominance of basic amino acids over acidic ones which is also revealed by their high pI values (9.8 and 9.9 respectively). Presence of cysteine residues is uncommon in case of many alkaline proteases, however it is observed in few alkaline proteases [57,58].

The carbohydrate content of the APC was 11%. Alkaline proteases having small amount of carbohydrate are reported from *A. flavus* [59], *Thermus* Tok3 [60] and *B. subtilis* [61].

Comparison of the Kcat values of APC for the substrates indicate that among the various substrates, the synthetic substrate sAAPF-pNA and casein are more efficiently hydrolyzed. The APC was also able to hydrolyze a large variety of proteins and synthetic substrates (Table 2.7), thereby indicating its broad substrate specificity. It was able to hydrolyze esters as well as anilides (except BAPNA), thereby exhibiting esterase and amidase activities.

Properties of the APC such as a high pI value of 9.8, activity at high alkaline pH (10.0) and at 40°C, and broad substrate specificity are ideal for its application in detergent and leather industries.

Trp at the active site of the Conidiobolus alkaline protease:

Conidiobolus alkaline protease exhibits properties such as alkaline pH optimum, Mr of 28,000 and inhibition by SSI which are typical of a subtilisin-type protease. However, besides the characteristic residues of the catalytic triad (Asp, His, Ser), reported to be present in the subtilisins [62], an essential Trp and thiol residue were detected in the active site of *Conidiobolus* alkaline protease. The enzyme was completely inhibited by very low concentrations of NBS (5 μM). The progressive decrease in the absorption at 280nm upon titration of the enzyme with NBS indicated modification of the Trp and/or Tyr residues. Decrease in the absorption at 280 nm eliminates the possibility of thiol group modification by NBS. Complete inhibition of the enzyme by NBS but not by NAI supports the viewpoint that modification of Trp rather than Tyr residues is responsible for the observed loss of activity. Inactivation of the enzyme by NBS was found to be dependent on both time and inhibitor concentration and followed first order kinetics (Fig.2.13a). Although three Trp residues were oxidized per mole of the enzyme, modification of only one Trp residue was responsible for the complete inactivation of the protease (Fig.2.12b). The high K_m and low K_{cat} of the modified protease compared to that of the native enzyme indicated decreased affinity of the modified enzyme for the substrate and its reduced catalytic efficiency respectively. Substrate protection studies on the NBS modified enzyme showed that the inactivation could be prevented to a considerable extent by preincubating the enzyme with excess amount of casein. The titration of native enzyme with increasing concentration of NBS led to a progressive quenching of tryptophanyl fluorescence due to oxidation by NBS (Fig. 2.14a) but did not alter the fluorescence profile (λ_{ex}, 295nm; λ_{em} 340nm) indicating no shift of Trp residues from polar to nonpolar environment. The far UV-CD spectra revealed no change in the secondary structure of the enzyme molecule (Fig. 2.14b). Therefore, NBS-induced inactivation of the enzyme was a result of direct chemical modification of an essential Trp and cannot be attributed to disruption of the protein structure. In addition to NBS, the enzyme was also completely inactivated by HNBB.

SSI binds to the *Conidiobolus* alkaline protease as evident from the subsequent loss in protease activity. The interaction of the *Conidiobolus* protease with inhibitor (SSI) or substrate (sAAPF-pNA) led to a perturbance in the microenvironment of the Trp residues as demonstrated by the quenching of the Trp fluorescence of the enzyme upon binding with

either substrate or inhibitor. These results confirm the presence of Trp in substrate or inhibitor binding site of the alkaline protease from *Conidiobolus* sp. Earlier, two separate antiparallel β-sheet interactions have been reported in subtilisin-SSI complex viz. (i) between the P1-P3 residues of SSI and the Ser 125-Gly 127 chain segment (the "S1-S3 site") of subtilisin and (ii) between the P4-P6 residues of SSI and the Gly 102-Tyr 104 chain segment (the "S4-S6 site") of the enzyme [63]. The latter interaction is unique to subtilisin. The structures of complexes of serine proteinases with their proteinaceous inhibitors only subtly differ from those of the complexes with true substrates.

Assessment of the Trp microenvironment:

Fluorescence of proteins and peptides is attributed to the presence of natural fluorophores viz. Tyr and Trp and is generally dominated by the Trp residues. Indole, Trp and their derivatives are highly sensitive to solvent polarity and to a specific interaction between the solvent and the indole ring. Thus, the emission spectra of these residues can reflect the polarity of their surrounding environment. The tryptophanyl emission of *Conidiobolus* alkaline protease (340 nm) occurs at a shorter wavelength, relative to the emission of Trp in water (348 nm). This blue shift is a result of shielding of the Trp residue from water by the protein matrix. Trps appear to be uniquely sensitive to quenching by a variety of substances, as a result of a propensity of the excited indole nucleus to donate electrons while in the excited state and can be gainfully utilized in determination of the accessibility of the Trp residues in proteins by quenching measurements. This phenomenon can be gainfully utilized in determination of accessibility of the Trp residues, which act as intrinsic fluorophores in proteins. Acrylamide is an efficient uncharged quencher of Trp fluorescence while KI and CsCl are considered as companion probes since they bear opposite charges and can be effectively used in determining the nature of the Trp microenvironment. In our study, 25% quenching in the fluorescence of the alkaline protease was obtained with 20mM acrylamide, 225 mM CsCl and 42.5 mM KI, indicating that acrylamide is the most efficient quencher. No shift in the emission maximum of the enzyme at 340 nm and full activity in presence of acrylamide (0.1 M), CsCl (0.3 M) and KI (0.3 M) revealed the quenching to be a physical process. The Stern-Volmer plots for all the three quenchers were linear (Fig. 2.16) indicative of a single class of fluorophores, all equally accessible to the quencher. Unfolding of the protein upon addition of 3M Gdn.HCl led to a red shift of 9 nm and an overall (approximately 3 fold) increase in the collisional quenching constants (K_{sv}) (Table II) indicating increased efficiency of quenching. KI and

CsCl bear opposite charges and thus, their relative quenching efficiencies depend not only on the accessibility of the protein fluorophore but also on the net charge in the vicinity of the fluorophore. The K_{sv} value for CsCl is lower (1.66) than that for KI (7.0). Since CsCl is a positively charged quencher, this situation will arise if the microenvironment of Trp is electropositive, thereby not allowing the quencher to come in the active volume element of the fluorophore due to charge repulsion. Therefore, higher efficiency of quenching by KI as compared with CsCl indicates the presence of an electropositive environment around Trp residues in the *Conidiobolus* protease.

Cys at the active site of the APC:

Inhibition of the APC by Hg2+ , PHMB, OPTA but not by TNBS indicated the presence of Cys in the active site of APC. L-cysteine, DTT or β-ME exhibited reversal of inactivation by PHMB confirming the presence of the essential thiol group at the active site. PMSF at pH 7.0 is known to react with and inactivate papain [64]. However,PMS-papain was completely reactivated by treatment with thiol compounds. In case of APC, inactivation of the enzyme by PMSF was not reversed by treatment with β-ME, whereas the PHMB inhibition was reversed by thiol containing compounds confirming that APC is a serine alkaline protease. There are several serine peptidases that show significant thiol dependence i.e. they are activated by thiol compounds such as DTT and/or inhibited by thiol-blocking reagents [65]. Among such serine peptidases are prolyl oligopeptidase, yeast carboxypeptidase together with other members of the carboxypeptidase C family and an important subset of subtilisin family. APC, can, therefore be categorized under a sub group of subtilisins containing thiol at the active site.

Proximity relationship between the essential Trp, His and Cys residues:

The stabilization energy of the folded state of a protein is mainly a result of non-covalent interactions between constituent amino acids. Interaction between charged groups such as that of His and aromatic amino acid like Trp is one of them. Determination of the changes in the fluorescence intensity in response to the alterations in pH, especially in the pH range of 4-9 provides a simple means of detecting the presence of intramolecular Trp-His complexes in the protein. In the present studies, kinetics of enzyme inactivation by group specific modifiers has shown the presence of His and Trp in the active site of *Conidiobolus* alkaline protease. The plot of absolute quantum yield versus pH ensures the calculation of pK_F, which is pKb of the imidazole side chain as determined fluorometrically [44] and is similar to the plot of relative fluorescence intensity versus pH (Fig.2.17). As the protein is excited at 295

nm, the emission spectrum is mainly due to Trp groups. The sigmoidal curve, therefore, suggests the presence of an ionizable group with a pK_F of 6.1(His) affecting a Trp residue in the alkaline protease. No significant quenching of the Trp fluorescence was observed in the acidic limb of the sigmoidal curve upon treatment of the enzyme with DEPC (10mM) and the pK_F of the enzyme increased to 6.65. Although protonated and modified forms of His could quench Trp fluorescence, quenching by the protonated form was greater. Such interactions between the protonated imidazole ring of His and the indole ring of Trp are shown to occur in barnase [66] as well as in some cysteine peptidases such as bromelain, ficin, chymotrypsin and carboxypeptidase [44]. The Trp - His interaction in barnase has been shown to elevate the pKa of His thereby stabilizing the protonated form of His [67]. The His-Trp pairing in cysteine peptidases is known to be important in the catalytic activity of the enzyme through its combined effect of increasing the pK_a of the active site His and shielding the His 159 - Asn 175 hydrogen bond from external solvent to facilitate different steps in the catalytic mechanism of the enzyme [68]. His-Trp proximity, influencing Trp fluorescence intensity also supports our results on solute quenching of Trp fluorescence suggesting the microenvironment of Trp residues to be electropositive.

Extrinsic fluorescent probes such as 5-IAF are used to label proteins in order to monitor conformational changes, ligand interactions, and to estimate intermolecular and intramolecular distances [69]. In *Conidiobolus* alkaline protease, the proximity relationship among the Trp residues (intrinsic fluorophore) and the fluorescein labelled Cys residue (extrinsic fluorophore) was demonstrated by the fluorescence energy transfer experiment.

Our foregoing results have suggested that Trp is proximal to His and Cys in the active site of *Conidiobolus* alkaline protease. By corollary, it would be legitimate to speculate that His and Cys are also in close proximity with each other. Presence of Cys in the vicinity of essential His 68 in the proteases belonging to a subset of subtilisin family has been reported (Fig. 2.19). A free Cys (Cys 72) is found in thermitase, proteinase K, Kex 1 and Kex 2 proteases, and protease B [70-74]. No Cys is present in the primary structure of subtilisin BPN', subtilisin Carlsberg and in the alkaline proteases from *Acremonium chrysogenum* and *Aspergillus* oryzae [75-78]. Instead, Val 72 is close to His in the catalytic site. Thus, the presence of Cys in alkaline proteinases cannot be generalized.

Fig.2.19 Conservation of sequence around the catalytic histidine residue in the subtilisin family

Enzyme	Source	Sequence	Ref.
		κ	
		64 65 66 67 68 69 70	
Thermitase	*Thermoactinomyces vulgaris*	H G T H C A G	70
Proteinase	*Tritirachium album limber*	H G T H C A G	71
KEX 1 protein	*Kluyveromyces lactis*	H G T R C A G	72
KEX 2 protein	*Saccharomyces cerevisae*	H G T R C A G	73
Protease B	*Saccharomyces cerevisae*	H G T H C A G	74
Subtilisin BPN’	*Bacillus amyloliquefaciens*	H G T H V A G	75
Subtilisin carlsberg	*Bacillus licheniformis*	H G T H V A G	76
Alp	*Acremonium crysogenum*	H G T H V A G	77
Alp	*Aspergillus oryzae*	H G T H V A G	78

Free Cys was located below the functional His using X-ray crystallographic studies in both thermitase and proteinase K and appeared to be practically inaccessible to solvent [79]. Its possible positive effect on activity has been discussed but not established [80]. The replacement of Met 222 close to the active site Ser by Cys 222 in subtilisin BPN' resulted in 38% increase in activity indicating that the nearby -SH group has a positive influence on the activity of subtilisin BPN'. The mechanism of this phenomenon observed in mutant BPN' and the influence of free Cys close to functional His in thermitase are not established hitherto. An unblocked Cys 341 is found in the proximity of His 397 of yeast carboxypeptidase Y. It appears to be involved in correct positioning of the side chain of His 397 and in shielding of Asp 338 from solvent. Val 341 in the wheat enzyme plays a similar role [81]. Owing to the similarity in geometry of the catalytic triad (Asp/His/Ser) of serine carboxypeptidases to the trypsin and subtilisin families of enzymes, a similar role can be attributed to the essential Cys encountered in the active site of *Conidiobolus* alkaline protease. To summarise, our results demonstrate for the first time the presence of Trp in the active site of the alkaline protease and its proximity with the essential His and Cys residues.

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CHAPTER THREE

CHARACTERIZATION OF INTERMEDIATES ON THE UNFOLDING AND REFOLDING PATHWAYS OF APC

SECTION I

INTERACTION OF THE MOLTEN-GLOBULE STATE OF APC WITH THE MOLECULAR CHAPERONE, a -CRYSTALLIN

SUMMARY

α-Crystallin, the major eye lens protein with sequence homology to small heat shock proteins, acts like a molecular chaperone by suppressing the aggregation of damaged crystallins and proteins. To gain an insight into its chaperoning ability, we used a protease as the model system that is known to require a propeptide (intramolecular chaperone) for its proper folding. Under the conditions of low ionic strength (0.05 M), the alkaline protease from *Conidiobolus macrosporus* (NCIM 1298) unfolds at pH 2.0 through a partially unfolded 'I' state at pH 3.5 which undergoes transition to a molten-globule like 'I_A' state in the presence of 0.5 M sodium sulfate. At higher temperature, the 'I_A' state showed a complete loss of structure and was prone to aggregation. α-Crystallin bound to this 'thermally stressed I_A state' and suppressed its irreversible denaturation due to aggregation. α-Crystallin-bound enzyme exhibited native-like secondary and tertiary structure demonstrating for the first time the interaction of α-crystallin with the molten-globule state of the protease. ANS-binding studies revealed the involvement of hydrophobic interactions in the formation of the complex of α-crystallin and protease. Unfolding of the protease in presence of α-crystallin and its subsequent refolding by dilution to pH 7.5 resulted in an intermediate state having partial tertiary and secondary structure. α-Crystallin added to the already unfolded protease was unable to refold the protein pointing to a clear difference in the mechanism of chaperoning action of propeptide and α-crystallin. Our results show that α-crystallin blocks the unfavorable pathways that lead to irreversible denaturation of the alkaline protease and keeps it in a near-native, folding-competent intermediate state.

INTRODUCTION

The detailed mechanism of polypeptide chain synthesis is well established but it remains to "crack the second half of the genetic code" which ensures that these chains attain their functional native conformation [1] which exists in solution as a very compact, highly ordered structure. To date, a plethora of literature exists to support Anfinsen's hypothesis of self-assembly, which states that all the information necessary for folding of a protein into an active conformation resides in the amino acid sequence of the protein [2]. Many proteins, even in the absence of other cellular factors and without any input of energy have been successfully shown to achieve their correct native structure, which is the global free energy minimum [3]. This principle of self-assembly is qualified by the need in many cases for assistance by molecular chaperones which are currently defined as a family of unrelated classes of proteins that mediate the correct assembly of other polypeptides, but are not themselves components of the final functional structures [4]. Molecular chaperones function by binding to specific structural features that are exposed only in the early stages of assembly, thereby inhibiting unproductive assembly pathways that otherwise would act as kinetic dead-end traps and produce incorrect structures. Gro-EL and Gro-ES from *E. coli* and heat shock proteins (HSPs) such as HSP25 and HSP27 are among the well-studied chaperones [5,6].

Apart from molecular chaperones and some other accessory proteins like protein isomerases catalyzing cis-trans isomerization of peptide bonds or disulfide exchange [7,8], there is one more mechanism of assisted protein folding which was initially demonstrated in serine proteases such as subtilisin [9], α-lytic protease [10] and in carboxypeptidase Y [11]. These proteases when denatured are unable to spontaneously refold even when placed in conditions that favor folding and thus conflict with the self-assembly hypothesis. They are synthesized as precursors containing an amino-terminal propeptide usually preceded by a presequence or a signal peptide [12]. Propeptide is required for the formation of the active enzyme [9,13] while the signal peptide is essential for its secretion across the membrane. In addition to mediating folding,

prosequence strongly inhibits the native enzyme [14] suggesting that it functions at a late step on the folding pathway by helping overcome a kinetic barrier. However, requirement of a prosequence for proper folding facilitates the isolation and characterization of a stable folding intermediate [15]. As propeptides perform a function similar to that of a large family of heat-shock proteins [16], they had been broadly classified as 'molecular chaperones'. However, they differ from the latter in their highly specific nature and the absolute requirement for folding of the protein to which they are 'covalently' attached and hence are further classified as 'intramolecular chaperones' [17]. However, *in vitro* studies have demonstrated that covalent linkage between the propeptide and subtilisin is not needed during the folding reaction [9]. The resemblance of the propeptide with HSPs and its functioning despite the covalent linkage with the target protein prompted us to investigate the interaction of a protease with α-crystallin, which has sequence homologies with the HSPs and has been assigned the function of a chaperone [18].

α-Crystallin is a multimeric structural protein made up of αA (acidic) and αB (basic) subunits [19]. It plays a vital role in maintaining the transparency of the lens by refracting light and by suppressing the aggregation of other crystallins thereby preventing cataract formation. The important discovery by Ingolia and Craig [20] of homology of *Drosophila* heat-shock protein with α-crystallin and later research suggested that α-crystallin originated from the family of small heat-shock proteins [21]. α-Crystallin is heat stable [22] and its expression can be induced by thermal [23] or hypertonic stress [24]. The presence of α-crystallin in a multitude of cells and tissues [23-25] and its association with various diseases [26-28] indicates that it must be playing a major physiological role. The present investigation was undertaken to study the role of α-crystallin in the folding and unfolding of an alkaline protease from *Conidiobolus macrosporus* (NCIM 1298) (APC). The intermediates on the unfolding pathways of proteases are less studied compared to those on the folding pathways. Moreover, there are no reports on the binding of proteases to the chaperones other than the propeptide, which is a highly specific intramolecular chaperone. Therefore, the aim of our study was to investigate whether under conditions favoring unfolding, a molecular chaperone such as α-crystallin would interact with the protease to prevent its irreversible denaturation and would assist its refolding.

In this study, we have, for the first time, characterized an acid-induced molten globule state of the protease and demonstrated its interaction with α-crystallin both on the unfolding and refolding pathway. α-Crystallin binds to the aggregation-prone APC and keeps it in a folding-competent state by preventing its aggregation.

MATERIALS AND METHODS

α-Crystallin, 8-anilino-naphthalene sulfonic acid (ANS), and isatoic anhydride were purchased from Sigma. All other reagents used were of analytical grade.

Enzyme purification and assay: APC was purified and assayed as described in the Chapter 2.

Equilibrium unfolding and refolding of the APC: All the denaturation and renaturation experiments were carried out in 50 mM citrate-phosphate buffer of varying pH (2.0-7.5), in presence or absence of salt (0.5 M sodium sulfate). For complete unfolding, APC was incubated in 100 mM citrate-phosphate buffer of pH 2.0 for 6 h at 25°C. Refolding of the unfolded protease was initiated either by dialysis or by dilution under various conditions. Unfolded protease was dialyzed at 4°C against 25 mM citrate-phosphate buffer, pH 3.5 or 50 mM sodium phosphate buffer, pH 7.5 in absence or presence of salt. Refolding initiated by dilution was carried out by neutralizing the acid-unfolded protein to pH 7.5 by addition of dibasic sodium phosphate from a 1 M stock solution and was supplemented with sodium sulfate to a final concentration of 0.5 M.

Fluorescence studies: Fluorescence spectra were recorded on a Perkin-Elmer LS-50B spectrofluorimeter equipped with a JulaboF20 water bath. Trp fluorescence was recorded at an excitation wavelength of 295 nm and a slit width of 7.5 nm. The protein concentration was 0.3 μM. The temperature-dependent structural changes at various pHs were studied by incubating the protein in the temperature range from 10°C to 70°C for 30min. The salt-dependent conformational transitions at various pHs were monitored by recording the Trp fluorescence in the presence of 0.5 M sodium sulfate. Ionic strength of the buffer component was 50 mM. ANS (20 μM) was used as a fluorescent probe to detect the exposure of the hydrophobic surfaces at an excitation wavelength of 369 nm.

Fluorescent labeling of the alkaline protease: The alkaline protease (18 μM) was treated overnight with 50-fold molar excess of isatoic anhydride 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 [29].

Assay for aggregation: Protein aggregation was assessed either by monitoring the absorption at 340 nm or by Rayleigh light scattering experiments using the fluorimeter. Both the excitation and emission wavelengths were set to 475 nm and the change in scattering intensity of the protein (0.3 μM) was monitored.

Circular dichroism measurements: The CD measurements were carried out on a Jasco J715 spectropolarimeter fitted with xenon lamp. Changes in secondary and tertiary structure induced by pH and/or salt and/or temperature were monitored in the far-UV (190-250 nm) and near-UV (250-300 nm) region respectively using a 10 mm path length cell. The protein concentrations used for far-UV and near-UV spectra were 0.3 μM and 5.5 μM respectively. The spectra were averaged over five accumulations.

Second-derivative absorption spectroscopy: Absorption spectra were recorded for APC (5.5 μM) incubated for 6 h under various denaturing conditions in the wavelength range of 250-300 nm on a Shimadzu UV-VIS spectrophotometer UV1601PC. The spectra were derivatized keeping a wavelength difference of 0.5 nm. To determine the extent of exposure of aromatic amino acid residues (Tyr/Trp), the relative change of amplitude between the peak at 288.8 nm and the trough at 284.8 nm (referred to as 'a') and between the peak at 296 nm and the trough at 291.5 nm (referred to as 'b') was measured [30].

Size-exclusion chromatography: Analytical gel-filtration experiments were performed using Pharmacia TSKG2000SW column connected to Pharmacia-LKB HPLC system. APC (1.5 μM) was incubated at varying pH for 6 h in presence or absence of salt. The column was equilibrated for each sample by passing at least three bed volumes of buffer used for incubation of samples and 50 μl sample was injected to the column. The flow rate was maintained at 1ml/min.

Interaction of a-crystallin with APC: The complex of α-crystallin and I_A state of APC (in a molar ratio of 2:1) was prepared by incubating the mixture of the enzyme and α-crystallin in 50 mM citrate-phosphate buffer, pH 3.5 at 58°C for 20 min and then by gradually cooling to room temperature. Near and far UV-CD spectra of the complex and of α-crystallin incubated under the similar conditions were recorded. The spectrum of

APC bound to α-crystallin was obtained by subtracting the spectrum of α-crystallin from that of the complex. Fluorescence spectra of the above samples were recorded at an excitation wavelength of 295 nm. ANS (20 μM) was added to the complex of APC and α-crystallin, which was cooled to room temperature and fluorescence spectra were recorded using the excitation wavelength of 369 nm. During refolding, α-crystallin was added along with salt either to the unfolded protein before dialysis/dilution or before unfolding of the protease (in a molar ratio of 2:1). Equal amounts of α-crystallin or protease were treated under identical conditions and used as controls. Fluorescence and CD spectra of these samples were recorded after 3h.

RESULTS

Acid -induced unfolding of the APC:

The APC exhibits maximum activity and stability at pH 10.0 and pH 7.5 respectively. The pH-activity profile showed that the enzyme was stable upto pH 5.0. There was a rapid loss in activity with decrease in pH and complete inactivation of the enzyme occurred at pH 3.5 (Fig.3.1.1).

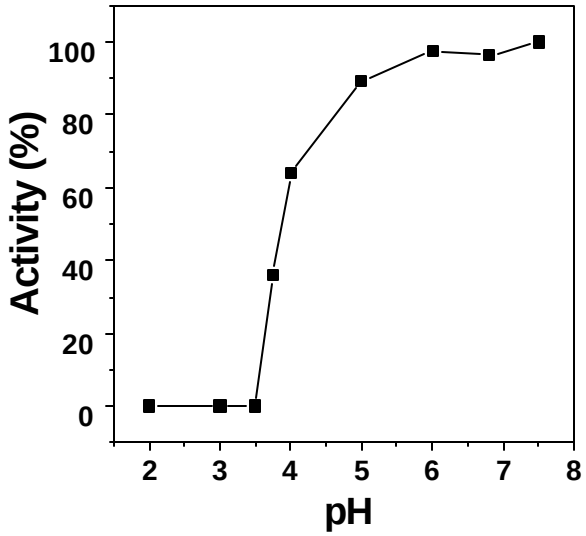

Fig. 3.1.1 pH-activity profile of the APC

APC (0.3 μM) was incubated with 50 mM citrate-phosphate buffer (pH 2.0 to 7.0) or sodium phosphate buffer (pH 7.5) at 25°C for 60 min and the residual activity was determined.

The CD spectra of the native enzyme at pH 7.5 (N state) in the aromatic region exhibited a sharp negative peak at 274 nm (Fig.3.1.2a) revealing an asymmetric environment around the Trp and Tyr side chains. The negative ellipticity decreased considerably at and below pH 3.5 indicating loss of tertiary structure. The spectrum of the N state in the far UV region exhibited two minima at 212 and 219 nm, which are characteristic of a protein having an α-helix (Fig.3.1.2b). There was negligible change in ellipticity at 220 nm upto pH 4.0. The ellipticity of the enzyme decreased at pH 3.5 (I state), but considerable native like secondary structure was observed. No secondary structure was observed at pH

2.0 (U state), indicating complete unfolding of the protein. The acid- induced unfolding transition was thus complete at pH 2.0.

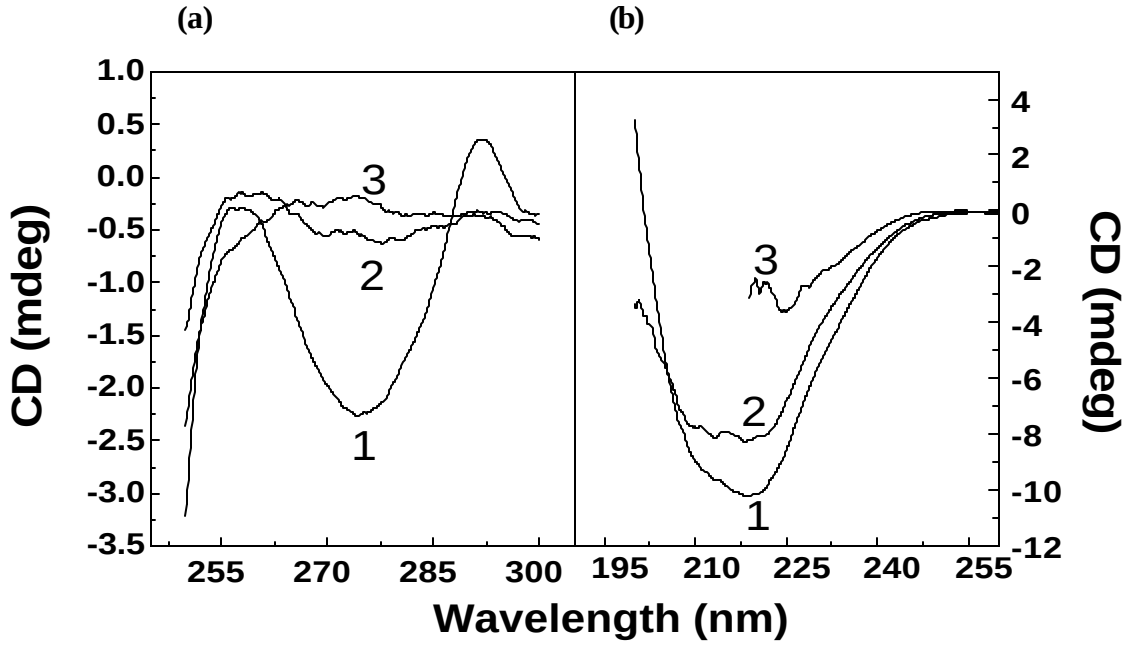

Fig. 3.1.2 CD spectra of the protease

(a) Near UV and **(b)** far UV-CD spectra of the protease at concentrations 5.5 and 0.6 μM respectively. (1) 'N' state, (2) 'I' state and (3) 'U' state.

Occurrence of the intermediate 'I_A' state:

APC is a monomeric protein with 263 amino acid residues with three Trp residues as revealed by amino acid analysis. The fluorescence spectrum of the 'N state' excited at 295 nm had an emission maximum at 340 nm indicating burial of Trp in the hydrophobic interior of the protein. The fluorescence intensity at the emission maximum (340 nm) remained unchanged upto pH 5.0 with a substantial decrease upon further lowering of pH (Fig. 3.1.3a). Fluorescence spectra revealed a red shift of 10-14 nm in the emission maximum of the enzyme below pH 4.0 (Fig. 3.1.3b) which indicated complete exposure of Trp residues to the solvent due to acid-induced unfolding of the protein. These spectra were similar to that of the protease denatured with 6 M guanidine hydrochloride. Addition of sodium sulfate (0.5 M) had no effect on the fluorescence intensity at pH 7.5 but at lower pHs, it gradually decreased without change in the emission maximum. However, a clear transition was observed at pH 3.5, with the formation of a new intermediate state having a fluorescence intensity lower (73%) than that of the N state

and about twice that of the U state. A blue shift of 10 nm accompanied by an increase in the fluorescence intensity was observed at pH 3.5 and 2.0 in presence of salt. The Trp residues in these states are more exposed to solvent than the N state but much more buried compared to the U state and are similar to the "A" state observed in case of many proteins [31]. This salt-induced state of the protease at pH 3.5 was termed as the 'I_A' state.

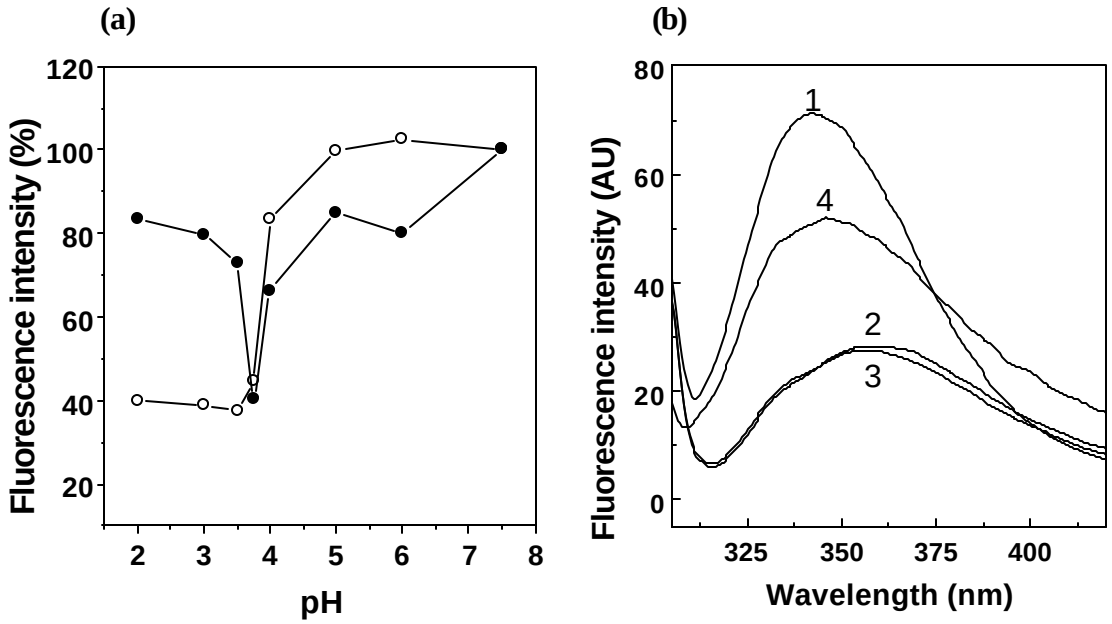

Fig. 3.1.3 pH dependence of the fluorescence intensity of APC

(a) Fluorescence intensity of APC (0.3 μM) was monitored at 340nm in absence (○) and presence (λ) of 0.5 M Na_2SO_4 at 25°C.

(b) Tryptophanyl fluorescence emission spectra of APC (1) 'N' state, (2) 'I' state (3) 'U' state and (4) 'I_A' state.

Characterization of the 'I_A' state:

ANS fluorescence

The extrinsic fluorophore ANS was used to determine the relative amount of exposed hydrophobic surfaces in the unfolding intermediates. ANS fluorescence of the N state was negligible and was not affected by the presence of salt (Fig.3.1.4a). A significant (four-fold) increase in ANS fluorescence was observed in case of the I_A state with a shift in the emission maximum from 510 to 478 nm (Fig. 3.1.4b) indicating burial of ANS molecule in a hydrophobic environment. Increased ANS binding is one of the important

characterisitics of the molten-globule (MG) state of a protein. Based on this criterion, I_A state represents the MG state of APC.

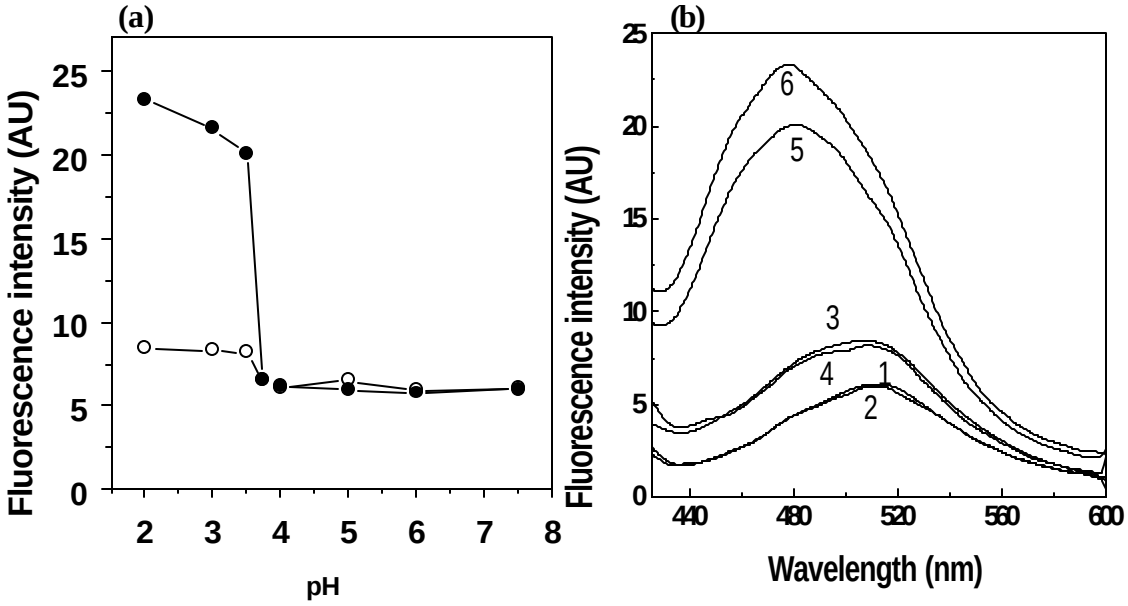

Fig. 3.1.4 Exposure of hydrophobic surfaces of APC detected by ANS-binding

(a) ANS (20 μM) was added to the enzyme (0.3 μM) and incubated for 20 min at 25°C in absence (○) and presence (λ) of 0.5 M Na_2SO_4.

(b) ANS fluorescence emission spectra of APC (1) 'N' state, (2) 'N' state in presence of 0.5 M Na_2SO_4, (3) 'I' state (4) 'U' state (5) 'I_A' state and (6) U state in presence of 0.5 M Na_2SO_4.

Size-exclusion chromatography

Further insight in the unfolding process was obtained by determining the relative hydrodynamic volumes of different conformational states of APC by gel-filtration chromatography (Fig.3.1.5). Native APC eluted from the column at a volume of 40 ml as a sharp peak indicating a defined structure whereas, the U and I states eluted at 32 ml as a broad peak, tailing considerably, suggesting a substantial increase in the hydrodynamic volume of the polypeptide chain upon unfolding due to acidification. The I_A state exhibited an additional peak at an elution volume of 36 ml. A comparison of elution volumes of these states indicated that the compactness of the I_A state was intermediate

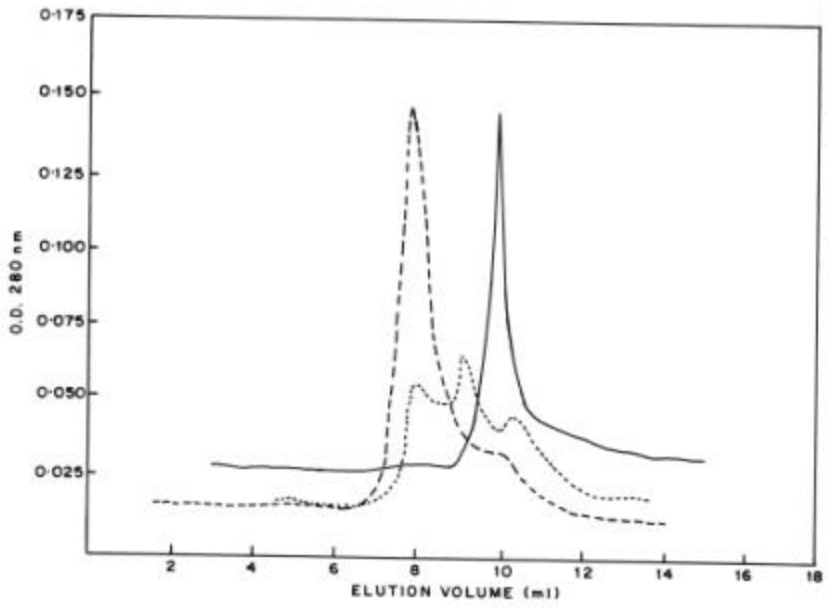

Fig. 3.1.5 Gel filtration profile of the different conformational states of APC
Nstate (—), I and U states (---) and I_A state (...).

between that of the N and U states.

Second derivative absorption spectroscopy

The relative exposure of Tyr and Trp residues of APC under various unfolding conditions was monitored by second derivative absorption spectroscopy. In the native enzyme, the maxima at 288.8 nm and 296 nm and minima at 284.8 nm and 291.5 nm are characteristic of the exposure of Tyr and Trp residues to the solvent (Fig.3.1.6). The aromatic residues of the enzyme were more exposed at pH 3.5 and 2.0, as compared to those of the native enzyme. The increase in exposure was also accompanied by a spectral shift indicating change in the polarity of the environment of these residues. I_A state of the enzyme showed a decreased Tyr/Trp exposure, which was intermediate between that of the native and denatured states of the enzyme. The a and b values of the I_A state (4 cm and 7.35 cm) were greater than those of the native state (3.25 cm and 5.2 cm) but were considerably lower than those of the unfolded states (6.25 cm and 8.6 cm) respectively.

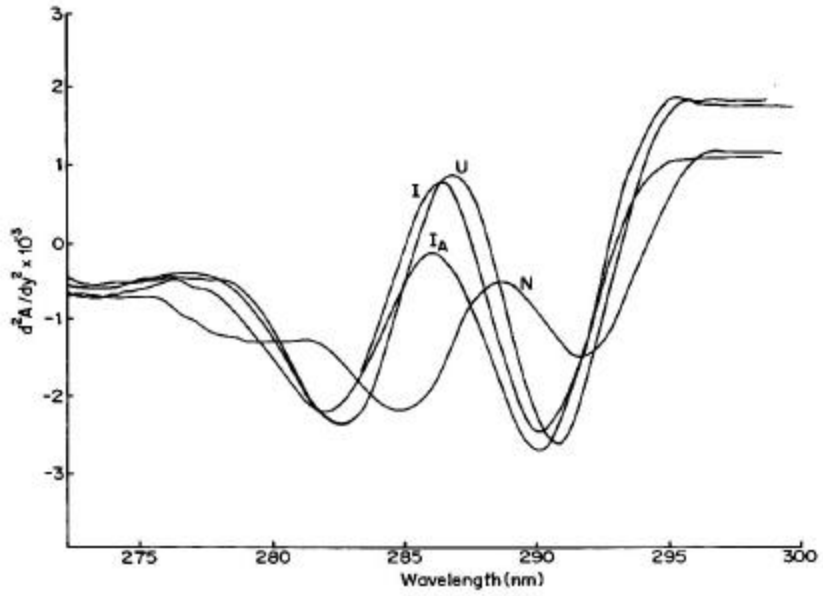

Fig.3.1.6 Second derivative absorption spectra of APC

The absorption spectra of the N, I, U and the I_A states of APC (5.5 µM) were derivatized at a wavelength difference of 0.5 nm.

Thermal cooperativity

The heat-induced denaturation of the APC at various pH values was studied by monitoring the changes in Trp fluorescence (excitation 295 nm), which is a parameter for determining qualitative differences in the polarity of the Trp environment (Fig.3.1.7). At higher temperatures (60°C and above), a red shift of 7.5-15 nm was observed at all pH values from 7.5-2.0, indicating complete exposure of Trp residues to the solvent. Presence of salt at pHs 7.5 to 3.7 did not alter the temperature transition. However, the I_A state was stable even at higher temperatures. In comparison to the large red shift observed at other pHs, very little change in the emission maximum (less than 4 nm) was observed at pH 3.5 in presence of salt indicating the stability of only this state at increasing temperatures. The cooperativity of thermal unfolding at different pHs was monitored [32] by plotting the ratio of relative fluorescence intensity at 330 and 350 nm as a function of temperature (Fig. 3.1.8).

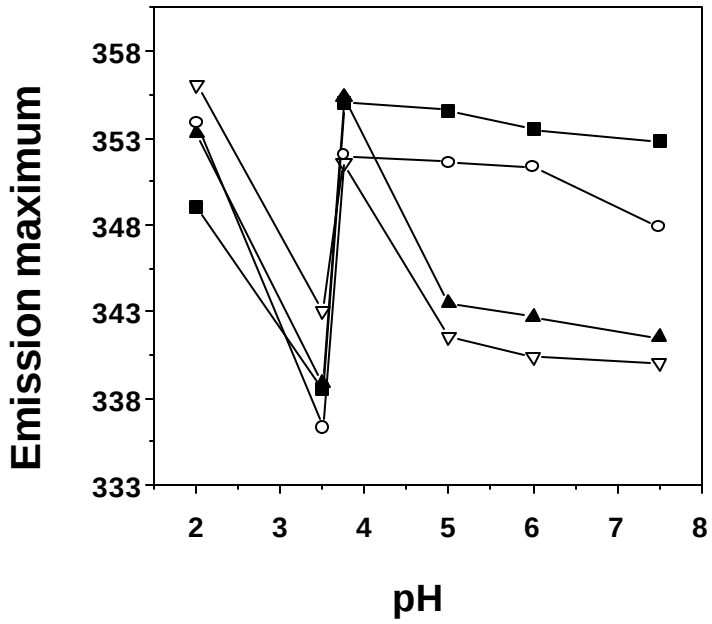

Fig. 3.1.7 Effect of pH on the fluorescence emission maxima of APC at different temperatures

The effect of increasing temperature (30 (λ), 40 ( ), 50 (σ), 60 (○) and 70°C (ν) on the emission maximum of APC. At pH 3.75 and 3.5 the effect was monitored in presence of 0.5 M Na_2SO_4. APC exists as 'I_A state' at pH 3.5 in presence of 0.5M Na_2SO_4.

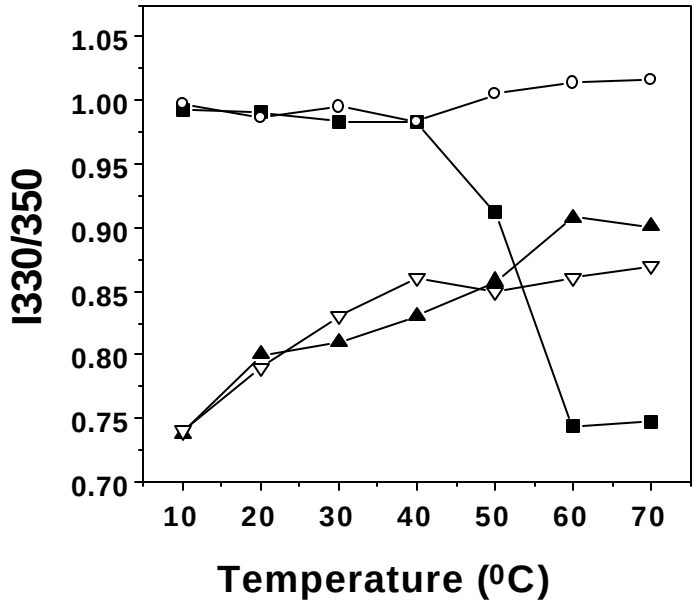

Fig. 3.1.8 Thermal cooperativity of APC at different pHs

The effect of increasing temperatures on shifts in the emission maximum of APC was assessed by monitoring the $I_{330/350}$ ratio at pH 7.5 (υ), pH 5.0 (ν), pH 3.5 ( ), pH 2.0 (σ) and pH 3.5 in presence of 0.5 M Na_2SO_4 (○).

The high and stable value of the $I_{330/350}$ ratio of the protease at pH 7.5 and 5.0 upto 40°C indicated that Trp residues at these pH values are in a highly hydrophobic environment. A decrease in $I_{330/350}$ value at 50°C and above indicated a red shift or increased exposure of Trp residues. Similarly, at and below pH 3.5, the Trp residues are completely exposed to the solvent resulting in low initial values of the $I_{330/350}$ ratio. However, the gradual decrease in fluorescence intensity at 350 nm with increase in temperature led to an increase in the $I_{330/350}$ ratio. In contrast, a negligible change in the ratio in case of the I_A state revealed a poor cooperativity of only this state to thermal unfolding. The enzyme in this state thus exists as a stable intermediate.

Interaction of the chaperone a -crystallin with the protease:

Rayleigh light scattering

The ability of α-crystallin to recognize non-native intermediates on the unfolding pathway of APC was probed by monitoring the effect of α-crystallin on the aggregation of the enzyme intermediate during thermal denaturation at 58°C. No aggregation was observed from pH values of 4 to 7.5 in the presence or absence of salt at 58°C. In marked contrast, there was a considerably large increase in the fluorescence intensity of the I_A state at 58°C with time (Fig.3.1.9). Complete prevention of aggregation occurred at a molar ratio of α-crystallin: APC of 2:1, showing that α-crystallin is very effective in recognizing and binding to non-native intermediates formed during the thermal denaturation of the proteins.

CD spectra

The conformation of the I_A state of APC bound to α-crystallin was studied by circular dichroism. The far UV-CD spectrum (Fig.3.1.10) exhibited considerable secondary structure. α-Crystallin, however, could not restore the secondary structure of the I state after heat shock confirming that it was able to recognize and bind to the aggregation-prone MG state of the APC and kept it in a folding-competent intermediate state by preventing its heat-induced aggregation.

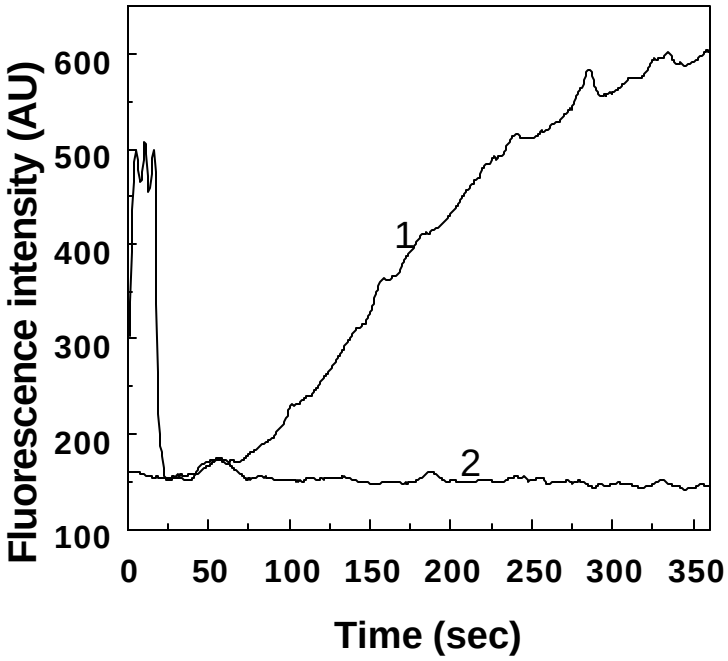

Fig. 3.1.9 Interaction of the chaperone a –crystallin with protease

Effect of α–crystallin on thermally induced aggregation of the I_A state. I_A state of APC (0.3 μM) at 58°C (1) in presence of α-crystallin (2).

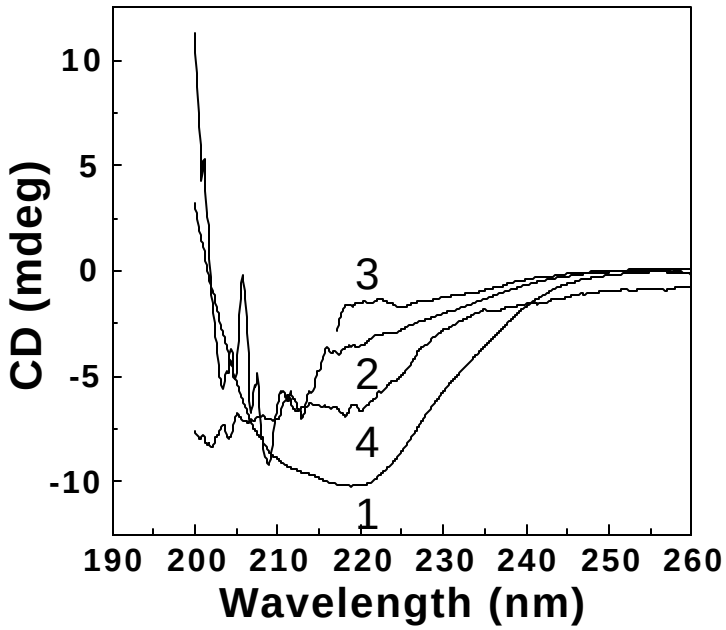

Fig. 3.1.10 Far UV-CD spectra of the APC

(1) N, (2) I_A state, (3) I_A state at 58°C and (4) α-crystallin-bound I_A state.

Tryptophan fluorescence

The emission maximum of the native enzyme was 340 nm while that of the aggregated I_A state was 336 nm (Fig.3.1.11). The completely unfolded enzyme emitted at 355 nm. The I_A state of APC bound to α-crystallin, however exhibited an emission maximum of 344 nm, thereby showing that the Trp residues in this state are more exposed to the solvent as compared to that of the N state but are less exposed than that of the U state. When the I state was heated in the presence of the chaperone, no change was observed in the fluorescence intensity as well as in the emission maximum of the enzyme, either at 58°C or upon cooling to room temperature (data not shown), demonstrating that α-crystallin does not interact with the I state of the enzyme at pH 3.5 even under thermal stress.

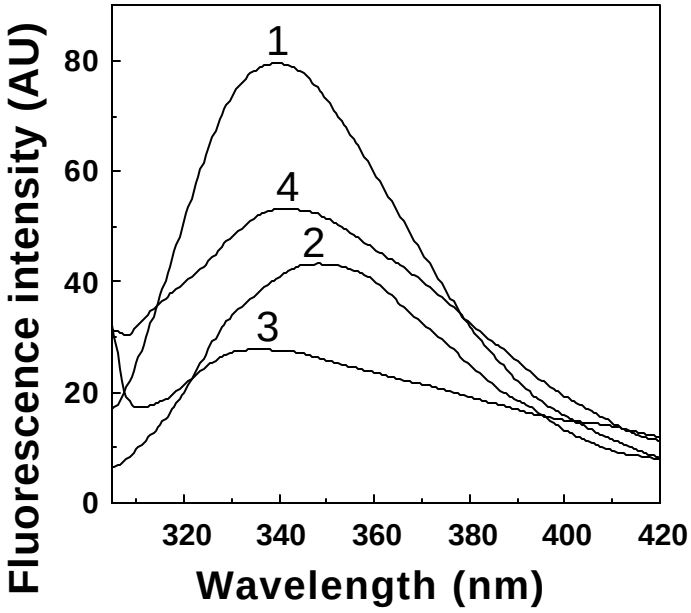

Fig. 3.1.11 Tryptophan fluorescence spectra of APC

(1) N state, (2) U state (3) I_A state at 58°C and (4) α-crystallin-bound I_A state.

Chemoaffinity labelling of the APC

Fluorescent chemoaffinity labeling using isatoic anhydride was further used to study the interaction of I_A state of APC and α-crystallin. Isatoic anhydride reacts with the nucleophilic groups of the proteins to yield o-aminobenzoyl protein conjugates [33]. The derivatized proteins exhibit an absorption band centered at 330nm together with an

emission band covering the spectral range of 360-500 nm. The emission maximum of the labeled APC (410nm) does not interfere with that of the unlabelled α-crystallin (336 nm) and is suitable for shedding some light on the conformation of APC in presence of α-crystallin after the heat shock. Heat treatment of the I_A state of the labeled protein led to quenching of fluorescence and a blue shift (λ_{max} 400 nm) due to aggregation (Fig.3.1.12). Mixing of APC with α-crystallin before heat treatment, however restored the emission maximum to 409nm that was similar to that of the native protein (λmax 410 nm). These results confirmed that α-crystallin prevents the denaturation of the protease during thermal stress and keeps it in a conformation similar to that of the N state.

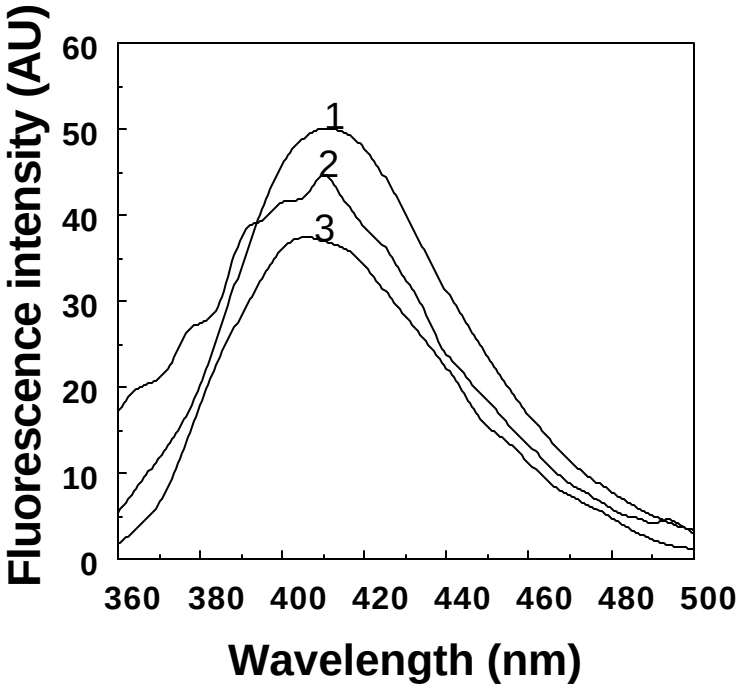

Fig. 3.1.12 Fluorescence spectra of the isoatoic anhydride-labeled APC

(1) Native enzyme, (2) α-crystallin bound I_A state, and (3) I_A state at 58°C.

ANS-binding

ANS fluorescence of the multimeric α-crystallin was enhanced upon heating to 58°C indicating exposure of hydrophobic surfaces. The complex of α-crystallin and I_A state of APC was detected using ANS fluorescence. Quenching in the ANS fluorescence of α-crystallin at 58°C upon the addition of I_A state of APC instead of additive increase in the

emission intensity indicated binding of α-crystallin to APC and suggested involvement of hydrophobic interactions in the formation of the complex (Fig.3.1.13). A steady increase in ANS fluorescence was observed upon gradual cooling of this complex to room temperature which shows that the accessible hydrophobic surfaces of the α-crystallin-APC complex were significantly enhanced upon cooling.

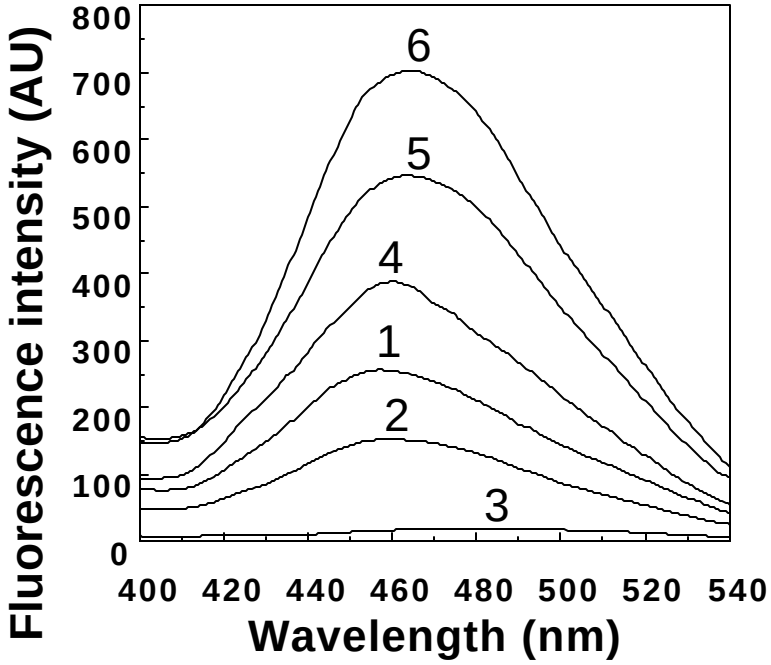

Fig. 3.1.13 ANS fluorescence spectra of APC

α-crystallin at 58°C (1), I_A state of APC at 58°C (2), I_A state of APC added to α-crystallin at 58°C (3), complex of α-crystallin and I_A state cooled to 42°C (4), cooled to 26°C (5), and mixture of α-crystallin and I_A state at 26°C (6).

The emission intensity of the ANS fluorescence of the mixture of α-crystallin and I_A state of APC was comparatively more than that of the complex revealing that α-crystallin forms a complex with the molten globule state of the APC only under thermal stress and that hydrophobic interactions are involved in the formation of the complex.

Refolding of the alkaline protease in presence of a -crystallin:

Dialysis of the unfolded protein against buffer of pH 3.5 or against buffer of pH 7.5 resulted in aggregation of the protein with lack of secondary or tertiary structure as detected by CD and fluorescence spectra (data not shown) respectively. The degree of aggregation was comparatively less when the pH was adjusted to 7.5 by dilution in the presence of salt as revealed by the absorbance of the protein at 340 nm. When the protease was unfolded in the presence of α-crystallin (in a molar ratio of 1:2) and refolded by dilution in presence of salt, the absorbance at 340 nm was negligible suggesting absence of aggregating protein species. The λ_{max} of such refolded enzyme shifted to 342 nm (closer to that of the N state) indicating regain of substantial tertiary structure (Fig.3.1.14). Structural characterization showed that the refolded enzyme has a secondary structure comparable to that of the native state (Fig.3.1.15). The λ_{max} of ANS fluorescence also shifted to 490 nm (Fig.3.1.16) indicating internalization of hydrophobic surfaces. On the contrary, when α-crystallin was added to the already denatured protein and then dialyzed or diluted, there was no decrease in aggregate formation.

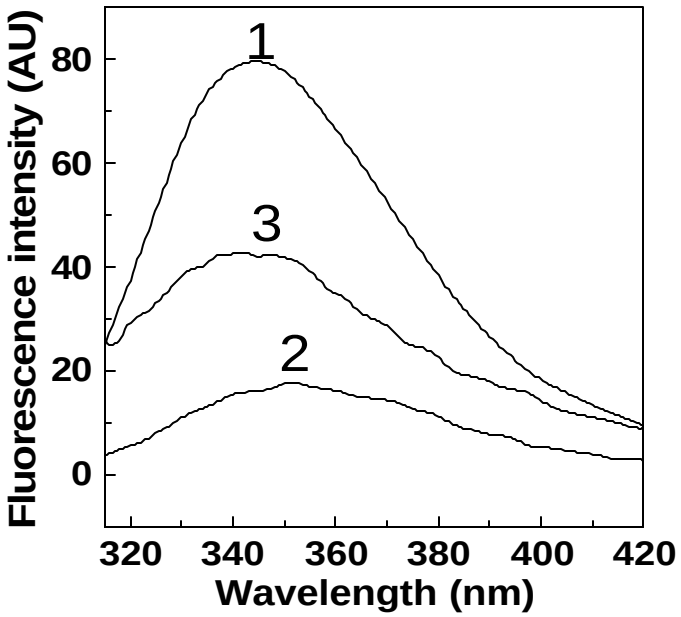

Fig. 3.1.14 Refolding of APC in presence of a -crystallin

Trp fluorescence of APC (1) N state, (2) U state, (3) APC unfolded in presence of α-crystallin and refolded by dilution in presence of 0.5M Na2SO4.

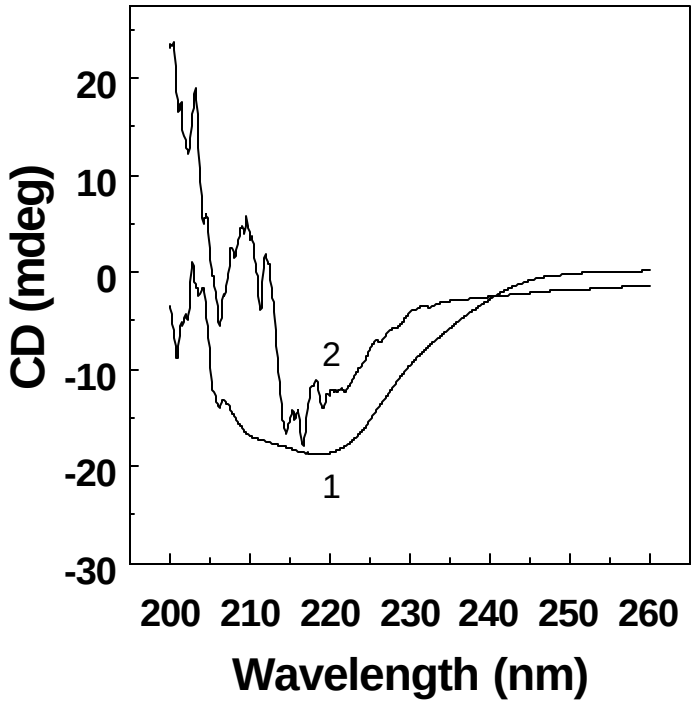

Fig. 3.1.15 Far UV-CD spectra of the refolded APC

(1) Native APC and (2) APC unfolded in presence of α-crystallin and refolded by dilution to pH 7.5 in presence of salt.

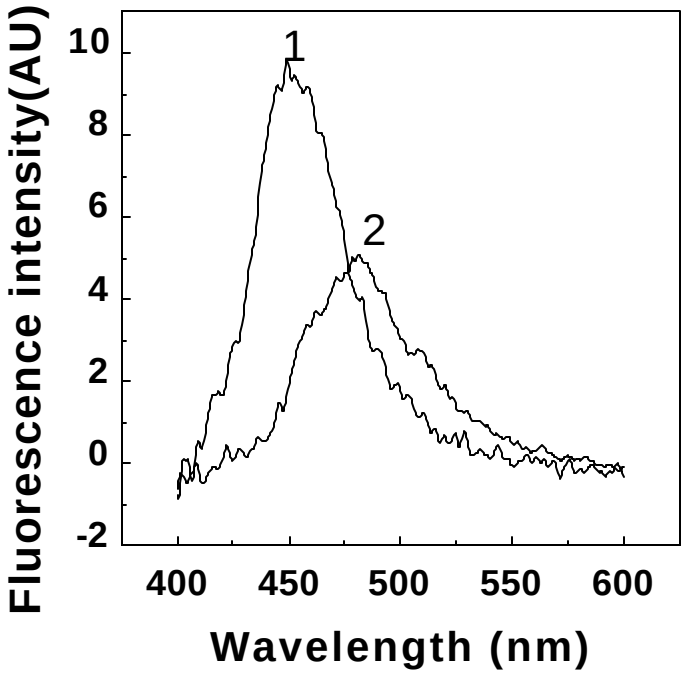

Fig. 3.1.16 ANS fluorescence spectra of APC

APC unfolded and refolded in absence (1) and presence (2) of α-crystallin.

DISCUSSION

A combination of various spectroscopic techniques have contributed to the following picture of the intermediates in the acid-induced unfolding pathway of the *Conidiobolus* alkaline protease. At lower ionic strength, the process of unfolding can be explained by the three-state model represented as $N \rightarrow I \rightarrow U$, where N, I and U are the native (pH7.5), intermediate (pH 3.5) and unfolded (pH 2.0) states of the protease respectively. The unfolding transition is complete at pH 2.0, as indicated by the complete loss of tertiary as well as secondary structure. Under conditions of extreme pHs, the main forces responsible for unfolding of the protein are the repulsions between the charged groups on the protein molecule [34]. APC is a basic protein with a pI of 9.8 and below this pH, it unfolds due to repulsion between the positively charged groups. The I state is characterized by the loss of activity, no rigid tertiary structure as revealed by near UV-CD signal and by the decrease in fluorescence intensity as well as a red shift in the emission maximum. It retains considerable native-like secondary structure, however, it does not show either increased ANS binding or the lack of thermal cooperativity and therefore, cannot be considered as a molten-globule intermediate on the unfolding pathway of APC. An increase in pH or addition of anions, either in the form of salt or acid decrease the repulsive forces, which arise from the protein net charge and consequently, increases the folding forces. In case of APC, the increase in pH from pH 2.0 to pH 7.5 had no effect on fluorescence intensity as well as on the emission maximum. Proteins exhibit differential behaviour upon acid-denaturation [35]. Some proteins do not unfold at low pH values, some undergo transition to a compact molten-globule state A and the third type unfolds first to an extended conformation and then undergoes transition to a compact state A on addition of anions. Addition of salt such as sodium sulfate to APC below pH 4.0 induced a new intermediate state, I_A, which is similar to the " A " state observed in case of many type III proteins such as β-lactamase, α-amylase, ribonuclease A, cytochrome C, carbonic anhydrase, and lysozyme [33]. The strong affinity of ANS to the I_A state (as compared to the N and U states) is due to the absence of rigid packing of hydrophobic clusters in this state resulting in a greater accessibility of the protein hydrophobic core for a solvent. In presence of salt, ANS binds

more tightly to the protease at pH 2.0 than at pH 3.5, which can be attributed to the increased negative charge of ANS due to its sulfonyl groups and increased positive charges on the protein at pH 2.0 [36]. Enhanced binding of ANS is considered as the main criteria of molten globule state of the enzyme. Another criterion to confirm the molten globule state is to examine the behaviour of the intermediate state towards thermal stress. The $I_{330/350}$ ratio for the IA state remained almost constant over a wide temperature range of 30°C to 70°C, revealing its least thermal cooperativity and confirming the molten globule nature of this state.

The molten globule state of proteins is known to contain unstable secondary structure and is thought to expose hydrophobic patches, resulting in a tendency to aggregate. Indeed, the aggregation was detected for the I_A state of APC at higher temperatures indicating its lability. α-Crystallin has been reported to function as a molecular chaperone by suppressing aggregation of proteins undergoing denaturation [14]. However, there are no reports of binding of α-crystallin with proteases. Most of the folding/unfolding studies regarding proteases have been carried out using the propeptide which functions as an intramolecular chaperone [6-8]. Molten globule-like intermediate states have been observed for subtilisin [37] and α-lytic protease [7]. Ours is the first report on the interaction of the intermolecular chaperone α-crystallin with the acid-induced molten globule state of the protease. The salt induced I_A state of APC heated at 58°C does not have any secondary structure as seen in the far UV-CD spectrum, indicating complete denaturation of the intermediate state of the protein at higher temperatures. However, the far UV-CD spectrum of the α-crystallin bound-enzyme exhibited considerable amount of native-like secondary structure showing that α-crystallin forms a complex with the molten globule state of the APC at higher temperatures and protects the molten globule state of the enzyme from thermally induced irreversible denaturation due to aggregation. This is also supported by fluorescence studies. The emission maximum of α-crystallin-bound enzyme shifted from 336 nm to 344 nm, which is intermediate between that of the N and the U states. The interaction of α-crystallin and protease was also confirmed by fluorescent chemoaffinity labeling of the protease. The emission maximum of the labeled protease (410nm) enabled to detect the conformational changes in the enzyme alone in

presence and absence of α-crystallin, eliminating the contribution of the latter. When the I state of APC at pH 3.5 was heated at 58°C, it unfolded without aggregation in contrast to the I_A state. Incubation of the I state of APC with α-crystallin before heat shock didn't prevent its unfolding as detected by fluorescence and far UV-CD, thereby revealing that 'a conformational state which is prone to aggregation' seems to be a prerequisite for binding of α-crystallin indicating its highly specific nature of recognition of the target protein.

The chaperoning activity of α-crystallin is enhanced at higher temperatures due to structural perturbations [38]. At pH 3.5, in presence of salt and at higher temperatures, however, α-crystallin was not unfolded as evident by its λ_{max} (337nm) and its near UV-CD spectrum. The far UV-CD spectrum showed a single negative minimum at 219nm, characteristic of α-crystallin. It has been earlier hypothesized that α-crystallin prevents the aggregation of non-native structures by providing appropriately placed hydrophobic surfaces. Our results on decrease in ANS fluorescence intensity of α-crystallin upon addition of APC support this hypothesis. The observation that the hydrophobic surfaces of mixture are comparatively greater than that of the complex cooled to room temperature which in turn are more than that of the α-crystallin alone further supports that hydrophobic interactions are involved in the formation of the complex.

Proteases play a critical role in a multitude of physiological and pathological processes and under stress conditions like heat shock, UV irradiation, photooxidation or oxidative stress, they may get inactivated. Since the cell has a highly complex heterogeneous environment with high concentrations of proteins in various stages of folding and with potentially interactive surfaces, the protease are likely to get irreversibly inactivated in absence of the prosequence. Under such conditions there will always be a possibility of interaction of molecular chaperones with the enzyme which will bind transiently to the latter preventing its irreversible denaturation and holding it in a folding-competent state.

Attempts to refold the protease by dilution or dialysis resulted in aggregation of the protein, the degree of aggregation being more in case of dialysis. Unfolding of the protease in presence of α-crystallin and its further refolding by dilution to pH 7.5 in presence of 0.5M sodium sulfate resulted in an intermediate state having considerable

tertiary and secondary structure and reduced ANS binding. Such an enzyme when diluted in assay buffer, and incubated at 4°C for 7h, exhibited activity, although very little (2.5%) compared to the native enzyme. Thus, although α-crystallin prevents the thermally induced irreversible aggregation of the alkaline protease and keeps it in a more ordered compact state; it is not able to restore the catalytic activity of the enzyme after refolding. This is also true for certain other proteins like carbonic anhydrase [39]. It could, however, restore the activity (upto 58%) in case of xylose reductase [40]. In case of protease, the addition of the propeptide is essential for folding of the enzyme both structurally and functionally. α-Crystallin failed to assist the refolding of the already denatured protease pointing to a clear difference in the mechanism of chaperoning action of propeptide and α-crystallin. It also reveals the mechanism of self-chaperoning of α-crystallin wherein the α-crystallin denatured along with protease is able to refold itself as well as assist the refolding of the protease. Our results on the refolding of the protease indicate the major role of α-crystallin as a chaperone in the structural reconstitution of the enzyme when added before denaturation of the protein. However, it differs from the propeptide in its chaperoning action. Propeptide can refold even the denatured protease and restore its activity by folding the protein to its native state. α-Crystallin, however, refolds the protease only to its near-native state. Both propeptide and α-crystallin thus 'catalyze' the protein folding in biological systems, propeptide does it by lowering the activation energy of the productive folding pathway whereas α-crystallin does it exclusively by blocking pathways leading to misfolding and aggregation.

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CHAPTER THREE

SECTION II

IN VITRO REFOLDING OF THE MATURE FORM OF APC IN THE ABSENCE OF PROPEPTIDE

SUMMARY

The unfolding of APC in presence of guanidine-hydrochloride (GH) and its refolding were studied using spectroscopic techniques such as CD, fluorescence, second-derivative absorption spectroscopy and size-exclusion chromatography. The unfolding was complete at 2.75 M GH and followed a two-state transition mechanism. The conformational stability for the native state indicated by the ΔG_{H2O} value (2.87 Kcal/mol) was found much higher than that for the unfolded state (0.03Kcal/mol). The reversibility of the unfolding reaction was found to be dependent on the denaturant concentration. The APC unfolded by GH concentration of upto 3.5 M was able to refold merely by dilution, whereas, only 5% refolding was observed for the APC denatured with 4 M GH. Addition of magnesium sulfate to the refolding buffer, however, accelerated the folding rate for this slow-refolding species with the maximum of 44% activity recovered in presence of 0.1 M $MgSO_4$. Addition of sodium sulfate instead of magnesium sulfate could not assist refolding, thereby revealing the role of Mg^{2+} rather than that of the salts. This is the first report of *in vitro* refolding of an alkaline protease in the absence of a propeptide.

INTRODUCTION

Protein folding is currently one of the most intensely investigated areas of structural biology. Although the importance of conformational integrity for enzyme activity is generally recognized, and the unfolding of many enzymes by heat, acid and denaturants is thoroughly studied, there is a need for more experimental studies on folding of different, preferentially small, monomeric proteins to get better insight into the mechanism of protein folding [1,2]. By fast kinetic studies, it has been shown that the secondary structure formation [3] and the collapse to a more compact state [4] are rapid events, followed by slower rearrangements of the backbone structure and side-chains to the final, biologically active conformations.

Apart from molecular chaperones and some other accessory proteins like protein isomerases catalyzing cis-trans isomerization of peptide bonds or disulfide exchange [5,6], there is one more mechanism of assisted protein folding, which was initially demonstrated in serine proteases. The serine proteases when denatured are unable to spontaneously refold to their bioactive conformations even when placed in conditions that favor folding. They are synthesized as precursors containing an amino-terminal propeptide usually preceded by a presequence or a signal peptide [7]. While the signal peptide is essential for its secretion across the membrane, the propeptide seems to perform two distinct functions viz., assistance in folding and inhibition of their protease domains [8]. The propeptide continues its chaperoning function subsequent to its cleavage from the mature protease and structural imprinting that results in conformational diversity originates during this reorganization stage [9].

In the earlier section, we have shown the interaction of α-crystallin with the molten-globule state of the protease. In this section, we have demonstrated another interesting feature of APC while elucidating the mechanism of guanidine hydrochloride (GH)-induced unfolding and refolding of the protease. The unfolding of the APC followed a two-state transition. However, unlike other proteases, which require propeptide for their correct folding into active conformation, the GH-denatured APC was able to refold in the absence of a propeptide. To the best of our knowledge, this is the first report on *in vitro* refolding of a mature protease without the assistance of a propeptide.

MATERIALS AND METHODS

Enzyme production and purification:

The APC was purified and assayed as described in Chapter 2.

Equilibrium unfolding and refolding of the APC:

Samples of the enzyme (0.3 μM) were incubated with various concentrations of GH, ranging from 0 to 6 M, for 2 h at 25°C in 0.05 M potassium phosphate buffer, pH 7.5 and the residual activity was monitored by using sAAPF-pNA as a substrate. For refolding experiments, APC (3 μM) was denatured as above and refolded by dilution with 0.1 M potassium phosphate buffer (pH 7.5) to a final GH concentration of 0.3 M. Magnesium sulfate or sodium sulfate (0-1 M) were incorporated in the dilution buffer when the APC was denatured by 4-6 M GH. The activity recovered was estimated by usual assay procedures.

Fluorescence spectroscopy: Emission spectra were recorded using a Perkin-Elmer LS50B spectrofluorimeter of the APC samples incubated with different concentrations of GH at 25°C for 2 h and refolded by diluting the denaturant in presence or absence of magnesium sulfate or sodium sulfate (0-1 M). An excitation wavelength of 295 nm and a bandwidth of 7.5 nm were used. For fluorescence decay kinetics, samples of APC added to different concentrations of GH were excited at 295 nm and the fluorescence intensity at 340 nm was monitored as a function of time.

Assay for aggregation: Rayleigh light scattering experiments were carried out using the fluorimeter to assess protein aggregation. The excitation and emission wavelengths were set to 295 nm and 340 nm respectively and the change in scattering intensity was monitored. The protein concentration used was 0.3 μM.

Circular dichroism measurements: The CD measurements were carried out on a Jasco J715 spectropolarimeter fitted with xenon lamp. Changes in secondary structure of APC (0.3 μM) induced by GH were monitored in the far-UV (190-250 nm) region using a 10 mm path length cell. The spectra were averaged over five accumulations. The denaturation curves were analyzed by assuming a two-state model [10] in which

$$f_F + f_U = 1 \quad \text{.....(1)}$$

where f_F and f_U represent the fraction of total protein in the folded and unfolded conformations respectively. The value of f_U and f_F at any point of the transition curve can be calculated as follows:

$$f_U = (y_F - y)/ (y_F - y_U) \text{ and}\ldots\ldots\ldots\ldots\ldots(2)$$

$$f_F = (y - y_U)/ (y_F - y_U)\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

where y is any observable parameter chosen to follow unfolding, and y_F and y_U represent the values of y characteristic of the folded and unfolded protein. The equilibrium constant K_U and the free energy change, ΔG_U, for the unfolding reaction can be calculated using the following formula,

$$K_U = f_U / (1- f_U) = f_U / f_F \text{ and}\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

$$\Delta G_U = -RT\ln K_U \ldots\ldots\ldots\ldots\ldots..\ldots..(5)$$

Where R is the gas constant and T is the absolute temperature (K). The value of $\Delta G(H_2O)$ can then be calculated according to the linear extrapolation model as,

$$\Delta G_U = \Delta G(H2O) - m\ [denaturant]..\ldots\ldots\ldots..\ldots(6)$$

The value of m is a measure of dependence of free energy on the denaturant concentration.

Size-exclusion chromatography: Analytical gel-filtration experiments were performed as described in Chapter 2 by using TSKG2000SW column connected to Pharmacia-LKB HPLC system. The APC (0.5 μM) was incubated with different concentrations of GH from 0 to 6 M for 2 h at room temperature. The sample (0.025 μM) was loaded onto the column equilibrated with the same buffer as that of the sample. Elution was carried out at a flow rate of 0.5 ml per min.

RESULTS

Equilibrium unfolding of APC by guanidine hydrochloride is a two-state process

The CD spectrum of APC in the far UV region (200-260 nm) showed typical double negative peaks at 219.6 and 210 nm, which are characteristic of proteins having an α-helical structure. The amplitude of the 219 nm trough decreased rapidly with the increase in GH concentration (Fig.3.2.1). Both negative peaks disappeared at about 3 M GH, indicating total disruption of the secondary structure.

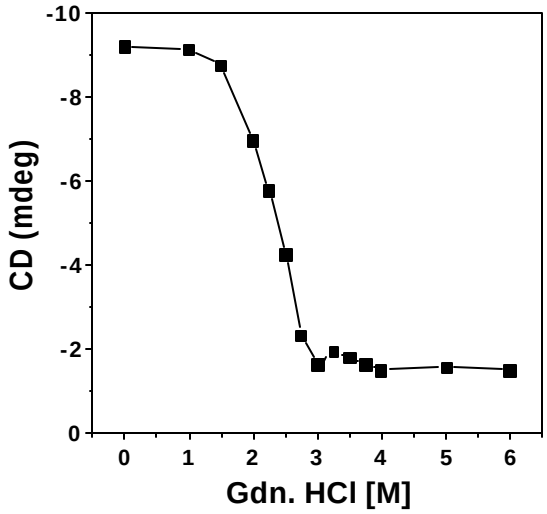

Fig. 3.2.1 Effect of GH on the ellipticity of the APC.

GH (0-6M) was added to APC (0.5μM) in 0.05M sodium phosphate, pH 7.5 and incubated for 6h at 25°C. Ellipticity of APC was monitored at 220nm.

The denaturation curve was analyzed to investigate the nature of the unfolding process. The values of f_F, f_U, and the equilibrium constant are listed in Table 3.2.1. The sum of the f_F and f_U values at various GH concentrations is unity, indicating that the unfolding of the APC followed a two-state mechanism. This is also corroborated by the overall pattern of the denaturation curve (Fig. 3.2.1 and 3.2.2b). Linear extrapolation of the f_F and f_U values indicated that 50% of the unfolding occurred at a GH concentration of 2.35 M. For a 45% unfolded protein, the equilibrium constant, K_U was calculated to be 0.812 and the free energy change ΔG_U was 53.5 cal/mol. The ΔG (H2O) value was further calculated

according to the linear extrapolation model (Fig. 3.2.2a) and was found to be 2.87 Kcal/mol.

Table 3.2.1 Analysis of guanidine hydrochloride-denaturation curve of APC

GH [M]	y[a]	f_F	f_U	K_U	ΔG_U, cal/mol
1	-9.14	0.992	.0078	.00786	1245.9
1.5	-8.74	0.940	.0597	.0635	708.8
2	-6.96	0.701	0.291	0.410	229.2
2.25	-5.75	0.552	0.448	0.812	53.5
2.5	-4.24	0.356	0.644	1.81	-152.7
2.75	-2.3	0.103	0.896	8.62	-553.6
3.25	-1.9	0.052	0.948	18.23	-746.4
3.5	-1.78	0.036	0.964	26.5	-842.4

[a]These data points have been taken from Fig. 3.2.1. For each point, y_F=-9.2 and y_U= -1.5. The values for K_U and ΔG_U have been calculated according to equations (4) and (5) from materials and methods.

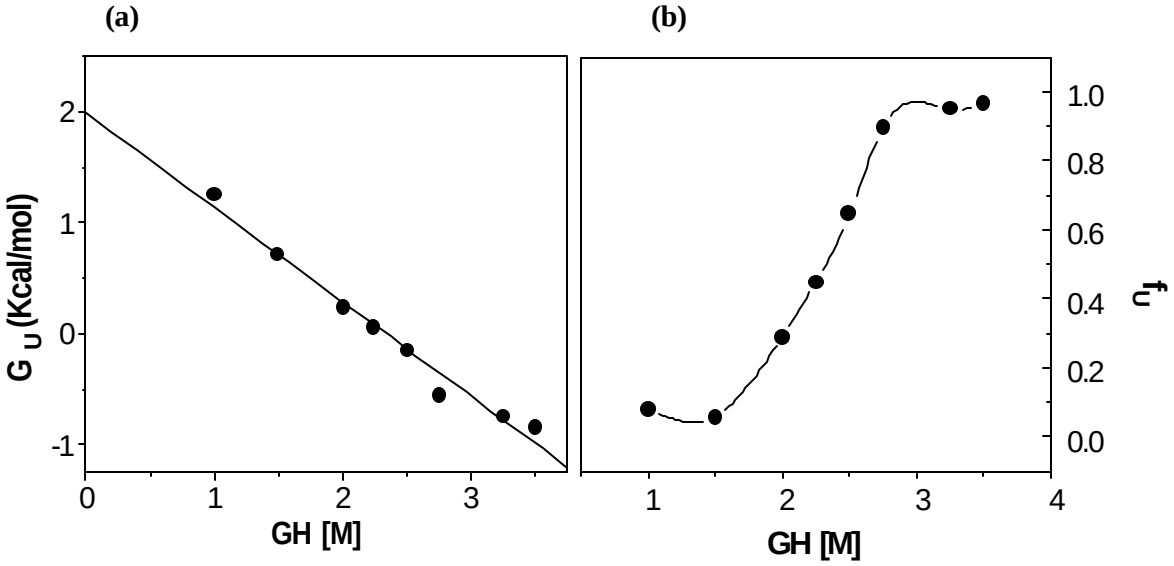

Fig. 3.2.2 The variation of ΔG_U and f_U with GH concentration for APC.

(a)Least square analysis of the data from Table 3.2.1 was carried out. The black solid line represents the regression line. The slope (m) of the line is 0.873. (b) The plot of f_U values against the denaturant concentration.

Inactivation of APC parallels changes in conformation

Fluorescence emission spectra of Trp residues were used to monitor qualitative differences in the polarity of their microenvironment upon unfolding by GH. One of the three Trp residues in APC is located at or near the active site (26). There was a slight increase (8%) in fluorescence intensity (excitation 295nm) in presence of 1M GH (Fig. 3.2.3a) accompanied by an increase in protease activity by 20% (Fig.3.2.3b).

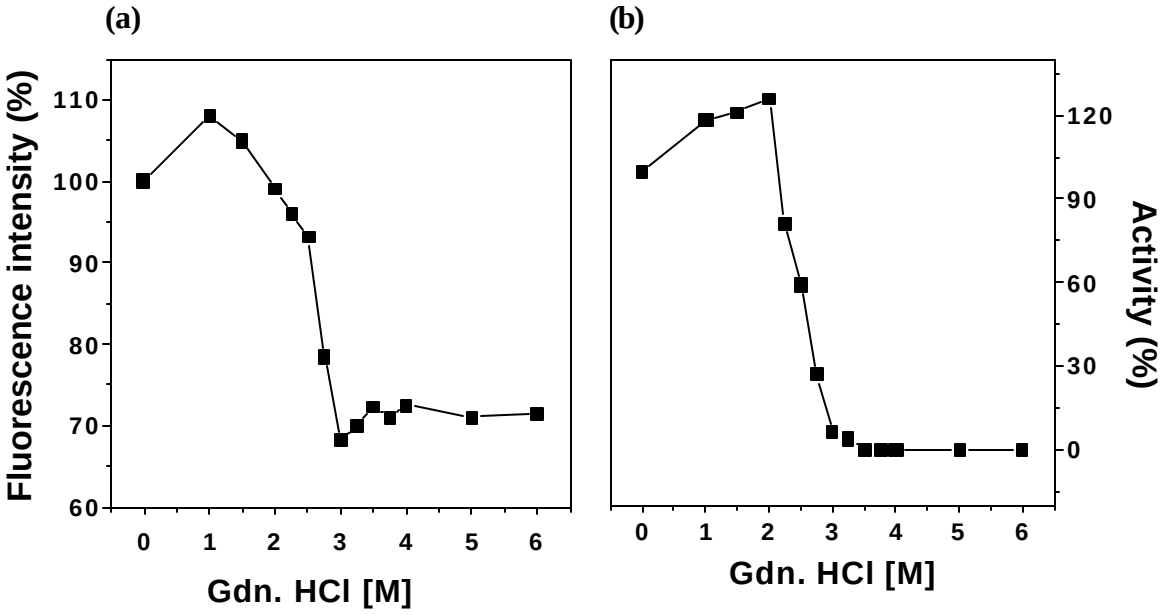

Fig. 3.2.3 Effect of GH on the fluorescence intensity and activity of the APC.

APC (0.3 μM) in 0.05M sodium phosphate buffer, pH 7.5 was incubated with increasing concentrations of GH at 25°C for 6h. (a) The fluorescence intensity (λ_{ex} 295nm) and (b) the residual activity were determined.

The initial increase in fluorescence and activity of APC in presence of GH can be attributed to the conformational changes around the active site leading to a slightly increased accessibility of the active site residues to the substrate. This is also indicated by the red shift in the λ_{max} of 2.5nm and 6.5 nm for 1 M and 2 M GH-treated enzyme, spectively. The denaturation of APC in increasing concentrations of GH was accompanied by a decrease in the emission intensity of fluorescence, a further red shift of the emission maximum by 10-14 nm and a gradual loss of activity. Total loss of activity and a minimum of fluorescence intensity were observed at 3 M GH. Increase in GH

concentration upto 6 M caused a marginal increase in fluorescence intensity indicating complete exposure of the Trp residues at and above 3 M GH due to the total denaturation of the protein. The unfolding of APC was also monitored by fluorescence decay kinetics (Fig.3.2.4).

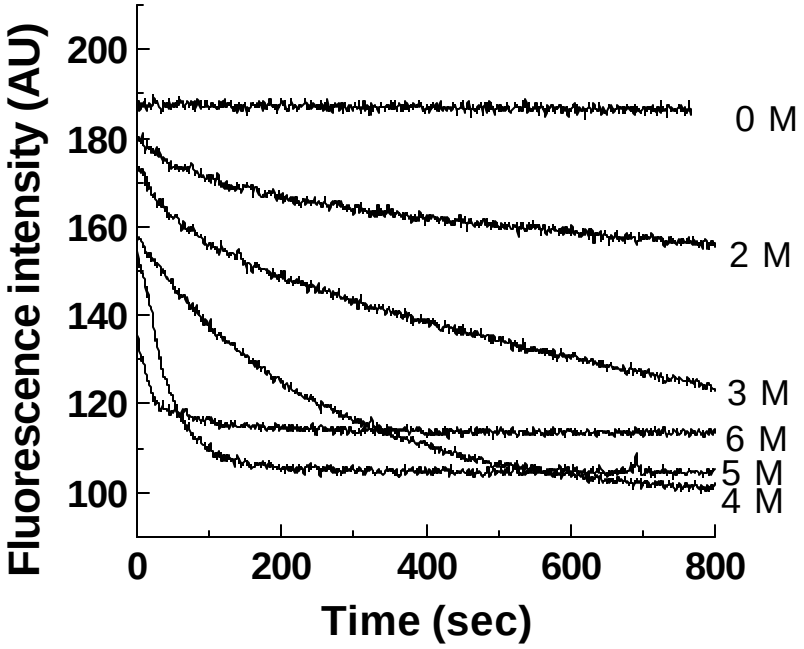

Fig. 3.2.4 Fluorescence decay kinetics of the APC.

The effect of increasing concentrations of the GH on the fluorescence intensity of APC (0.3μM) at 340nm was monitored with respect to time.

The fluorescence of the native protein at 340 nm remained constant with respect to time whereas there is a gradual decrease in intensity with increasing concentrations of GH. The time required for reaching equilibrium decreased as the denaturant concentration was increased. This was revealed by the fast decrease in the fluorescence intensity, which remained constant beyond 100 seconds in presence of 5 and 6 M GH, indicating rapid unfolding of the protein.

In order to further assess the effect of GH on the conformation of APC, the relative hydrodynamic volumes were determined by gel-filtration chromatography (Fig.3.2.5). Native APC eluted from the column at a volume of 40 ml. The protein denatured with 6 M GH, however, eluted at 32 ml.

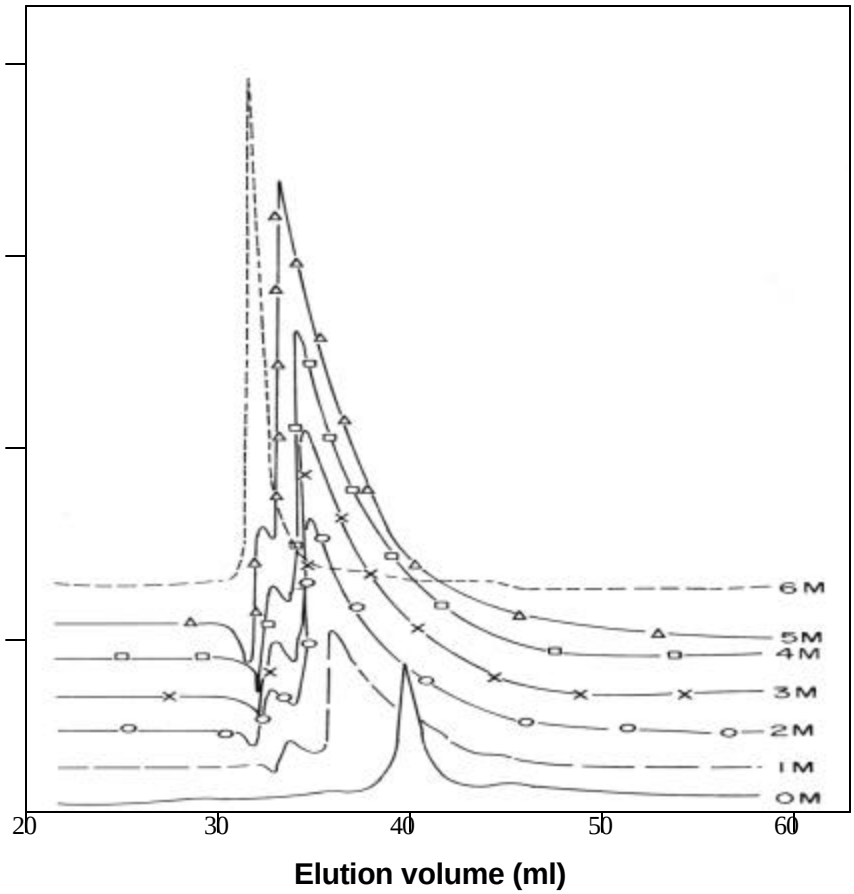

Fig. 3.2.5 Gel-filtration profile of the APC incubated with increasing concentrations of GH.

The flow rate was maintained at 0.5ml/min.

The protein denatured with increasing concentrations of GH between 0 to 6 M eluted at volumes intermediate between that of native and unfolded protein, suggesting a gradual increase in the hydrodynamic volume of the polypeptide chain upon unfolding. The peak corresponding to the native protease was symmetric, indicating a defined structure. The intermediates, however, had a short hump, the height of which increases with increasing concentrations of GH and the peaks tailed considerably, suggesting that these intermediates represent an ensemble of different conformations rather than the presence of a single defined structure. The peak for the APC denatured in presence of 6M GH was again symmetric, indicating a single population of completely unfolded polypeptide. Results of structural analysis together with the activity measurements clearly indicate that the inactivation of APC was accompanied by a change in the protein conformation.

Refolding of the GH-denatured APC:

The APC denatured by 3 M GH for 6 h was able to recover its full activity immediately after it was refolded by dilution to adjust the final concentration of GH to 0.3 M (which has no effect on enzyme activity) whereas, APC denatured with 3.5 M GH for 6h required 30 min to recover its activity indicating decreased folding-competence of the protein species. Further increase in the concentration of the GH led to the increase in time required to recover the activity completely, thus for APC denatured in presence of 3.75 M GH, 60% of the activity was recovered in 6 h. However, only 5% activity was recovered after 6 h for the APC denatured with 4 M GH suggesting formation of an intermediate with a larger disruption of tertiary and secondary structure. No activity was recovered after diluting the APC denatured with 5 and 6 M GH indicating irreversible denaturation of the protein. The CD spectra of APC after dilution of the denaturant were identical to those before dilution and indicated mostly random structures. The pathway towards intermediate or native state for this form of the protein therefore appears to be irreversibly blocked. At the relatively low protein concentrations of APC used in the spectroscopic experiments (0.3-1μM), there was no evidence of protein association or aggregation as monitored by the absence of an increase in light scattering. Increase in temperature to 58°C did not induce aggregation. Addition of the molecular chaperone, α-crystallin is known to assist refolding of proteins [11]. Incorporation of increasing amounts of α-crystallin alone (0-25μg) or coupled with ATP (10 mM) in the dilution buffer for APC denatured with 3-6 M GH, did not result in the recovery of activity or any regain of fluorescence spectra. These results support the previous observations [12] that presence of an aggregation-prone state of a protein is a prerequisite for the α–crystallin-assisted protein folding.

Mg^{2+}assists the refolding of APC

Our observation that magnesium stabilizes the protein against thermal inactivation (results described in Section I of chapter 3) and that sodium sulfate assisted the refolding of acid denatured APC (results descried in the section I of this chapter) prompted us to study the effect of sodium sulfate and magnesium sulfate on the refolding reaction. Interesting results were obtained in the refolding of the APC denatured with 4 M GH in

presence of salt. There was an increase in the amount of activity recovered with the increasing concentrations of magnesium sulfate with a maximum of 44% recovery of activity at the MgSO4 concentration of 0.1 M. The recovery of activity was accompanied by partial recovery of secondary structure (Fig.3.2.6a), of fluorescence intensity, as well as by a shift in the emission maximum from 355 nm to 345 nm indicating partial recovery

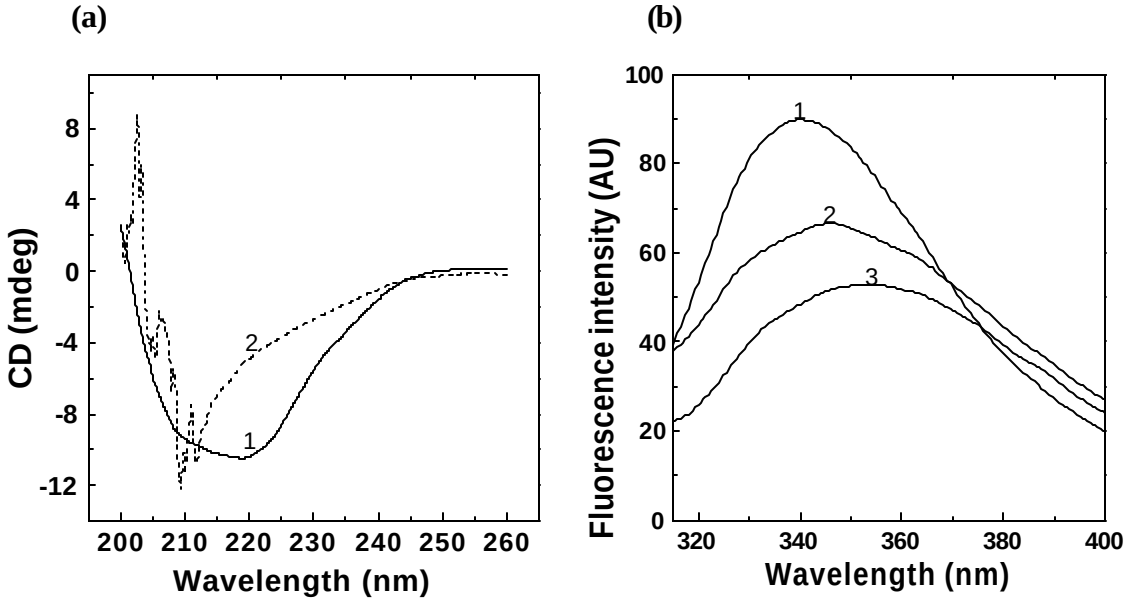

Fig. 3.2.6 Refolding of APC in presence of magnesium sulfate.

(a) Far UV-CD spectra of (1) native state and (2) APC unfolded in presence of 4M GH and refolded by dilution in presence of 0.1M $MgSO_4$. (b) Trp fluorescence of APC (1) native state, (2) refolded as above, and (3) unfolded state (treated with 6M GH).

of the tertiary structure (Fig.3.2.6b). Concentrations of MgSO4 higher than 0.1M, however, reduced the recovery of activity indicating reduced accessibility of the enzyme to the substrate at high salt concentrations. The 4 M GH-denatured APC, however, could not recover its activity when diluted in presence of sodium sulfate. The ability of the salts to accelerate folding, therefore, was not correlated with its effectiveness as a salting out agent, but related to the nature of the specific cation i.e. magnesium.

DISCUSSION

Folding is a cooperative process and many small globular proteins are often found to follow a two-state unfolding transition, wherein the native and the unfolded polypeptide chains are the most stable conformations [13]. On the other hand, an unusual folding mechanism of several small serine proteases provides a means to circumvent the high cooperativity of the folding process. These proteases require a propeptide for their correct folding. The propeptide is a part of the folding protein and therefore has been termed as an intramolecular chaperone to distinguish from the molecular chaperones. Our results provide evidence for the first time that the APC, inspite of being a serine protease, is able to refold in the absence of a propeptide. The only precedence of *in vitro* folding of a mature protease in the absence of a propeptide is that of subtilisin wherein the calcium-binding site was mutated [14].

A combination of various spectroscopic techniques such as CD, fluorescence, second derivative absorption spectroscopy and size-exclusion chromatography revealed the existence of a two-state model for the process of unfolding of the APC by GH. The free energy change, ΔG (H_2O), for the equilibrium reaction

Native state (N) « Denatured state (D)

is referred to as the conformational stability of a protein and is determined by various factors such as amino acid sequence of the protein, variable conditions of pH and temperature and the concentrations of salts and ligands [15,16]. The native state of APC with a ΔG_{H2O} value of 2.87 Kcal/mol is more stable than its unfolded conformation. In comparison with other proteins of similar size, proteases are more tightly packed since they have smaller than average surface areas, smaller radii of gyration, higher C_α densities, fewer helices, and more loops. All these properties are speculated to be coevolved in proteases to avoid autolysis [17]. This observation can be correlated to the high concentration of the denaturant (3-4 M GH) required to unfold the APC and is supported by the studies on another protease, papain, which also unfolds at similar GH concentrations [18]. The calorimetric data on the unfolding of subtilisin has also been reported to conform to a two-state model, indicating that the unfolding reaction is fundamentally reversible [19]. The APC unfolded progressively at increasing

concentrations of GH (0-3 M) as indicated by gradual loss of tertiary and secondary structure. The intermediate at 3 M GH is characterized by the total loss of activity, complete exposure of Trp residues to the solvent as indicated by the wavelength of emission maximum (352 nm), a minimum of fluorescence intensity and a loss of secondary structure. This intermediate is highly folding-competent since it recovers full activity immediately after the dilution of the denaturant. Increase in denaturant concentration upto 3.5 M had no effect on fluorescence or CD signal but its folding competence is slightly decreased, as it required a longer time (1h) to recover full activity. However, the APC denatured in presence of 4 M GH could recover only 5% of the activity indicating that at this denaturant concentration, APC exists as a slowly refolding intermediate with a larger disruption of tertiary compared with secondary structure. Thus, the intermediates observed at 3 and 4 M GH can be termed as fast and slow refolding intermediates respectively. The smooth changes observed in CD and fluorescence signals on the unfolding pathway can be explained by the continuous replacement of folding competent species by the more disordered states. In marked contrast, APC treated with 5 and 6 M GH doesn't recover their activity as well as fluorescence, indicating irreversible denaturation of the protein.

It is generally believed that the various intermediates along the folding pathway of a particular protein are similar if not identical to those along its unfolding pathway [20]. If it is assumed, therefore, that unfolding of a protein occurs via the reverse of its folding pathway, then a hierarchy of intermediates involved on the unfolding pathway of a protein can be outlined as:

folded/native protein ⇔ highly ordered intermediates ⇔ disordered intermediates ⇔ unfolded protein

↓

aggregation and precipitation

Our results on the denaturation and renaturation of APC also suggest that several intermediates are in equilibrium before the protein is completely unfolded by 6 M GH, and the following model can explain the denaturation and refolding of APC by GH:

$$N \Leftrightarrow I_F \leftrightarrow I_S \rightarrow D$$

Where N, D, I_F, and I_S are the native, denatured, the fast and slow refolding intermediate states observed at 0, 6, 3 and 4 M GH, respectively.

Many proteins are difficult to fold *in vitro*. One common obstacle to proper folding is aggregation during the folding process. Unfolded mature subtilisin does aggregate at neutral pH at concentrations of 1mg/mL. At lower protein concentrations, the primary impediment to folding seems to be the high free energy of activation for the process. One of the ways by which this barrier can be reduced is by the addition of salt to the folding reaction [21]. Addition of salts increases the protein stability because of their preferential destabilization of the unfolded state, thereby facilitating the refolding process. Another possible mechanism by which salts promote folding is by 'salting out' effect. At high concentration, salt competes with water such that the unfolded form of the protein is salted out of solution, resulting in the protein being driven to the native form [22]. This occurs because the native form has less area exposed to solvent than the unfolded form. To check the effect of the salts, therefore, we chose the APC denatured with 4 M GH, which was not able to recover its activity upon mere dilution of the denaturant. While addition of Na_2SO_4 had no effect on refolding, incorporation of $MgSO_4$ in the dilution buffer led to a partial recovery of the activity. The folding process was highly dependent on the concentration of the salt and maximum recovery of activity (44%) was observed for 0.1M $MgSO_4$. The results indicate the role of magnesium ions rather than the sulfate ions in the process of refolding of APC. Subtilisin is an unusual example of a monomeric protein with a substantial kinetic barrier to folding and unfolding [23]. The high thermodynamic and kinetic stability of subtillisin is in large part mediated by the high-affinity calcium site A. Calcium is an integral part of the structure, and its association with or dissociation from the protein probably requires significant but transient disruption in surrounding protein-protein interactions. Ribonuclease T1 is also known to be stabilized by the preferential binding of monovalent and divalent cations to the native state [24]. The proteolytic activity of SAM-P45, a novel member of subtilisin-like protease family from *Streptomyces albogriseus* was stimulated by the divalent cations Ca^{2+} and Mg^{2+} [25]. The activity of the native state of APC is also enhanced by Mg^{2+} Mg^{2+} ions. Of the metal ions tested, only magnesium (100 mM) was able to offer up to 25% protection to APC against thermal inactivation (results described in Chapter 3). In

contrast to the many microbial serine proteases such as subtilisin, thermitase, and thermomycolase (26), calcium ions did not enhance the thermal stability of the alkaline protease. In this respect, it differs from subtilisin although its biochemical properties such as alkaline pH optimum, Mr of 28,000 and inhibition by *Streptomyces* subtilisin inhibitor (SSI) are typical of a subtilisin-type protease.

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CHAPTER FOUR

THERMAL STABILITY OF THE APC

SECTION ONE

UNFOLDING PRECEDES AUTOPROTEOLYSIS DURING THERMAL INACTIVATION OF APC

SUMMARY

Thermal inactivation of APC was studied both at its stability pH (7.5) and optimum pH (10) in the presence and absence of additives. The enzyme was stable at 40°C at pH 7.5 for 1 h. There was a progressive loss in activity with increasing temperature and complete loss of activity occurred at 60°C. The kinetics of inactivation of the APC at various temperatures at pH 7.5 does not follow the first-order kinetics. Thermal inactivation was accompanied by the conformational changes in the protein structure as revealed by the diminished α-helical content and red shifted fluorescence spectrum of the enzyme. The effect of various additives to enhance the thermal stability of APC by means of modifying its microenvironment was studied. Glycerol (50 %) and sorbitol (25 %) offered 43 and 49 % protection respectively against inactivation of the enzyme at 55°C, at pH 7.5. Glycine (1 M) or magnesium sulphate (100 mM) offered 25 - 30% protection. Stabilization of the enzyme by sugar alcohols or by glycine was due to the prevention of the unfolding of the protein tertiary structure, as revealed by the decrease in the extent of red shift of fluorescence spectra of the enzyme. At 55°C at pH 10.0, the loss in enzyme activity was too rapid to be measured. The APC undergoes autoproteolysis under these conditions as evident by the decrease in the intensity of the protease band upon SDS-PAGE. No thermal stabilization was offered by the additives at this pH indicating that they prevent the unfolding but not the autoproteolysis of the APC.

INTRODUCTION

Proteases are one of the three highest tonnage commercial enzymes, amyloglucosidase and xylose isomerase being the other two. Microbial proteases account for approximately 40 % of the total worldwide enzyme sales, of which alkaline proteases account for about 25 % [1]. Recent awareness of environmental pollution caused by chemical-based industries has necessitated the development of enzyme-based processes as alternatives for current chemical processes. In this regard, alkaline proteases have potential application in leather and other industries, for partial or total replacement of currently employed toxic chemicals. Bacterial alkaline proteases have a long history of application in the detergent industries. Their application in the leather industry for dehairing and bating of hides is a relatively new development and has conferred added biotechnological importance to these enzymes [2]. Fungi elaborate a wider variety of proteases as compared to that by bacteria. Fungal alkaline proteases offer a distinct advantage over the currently used bacterial proteases in terms of the ease of preparation of microbe-free enzyme as against the cost-intensive filtration technology required for the isolation of a bacterial enzyme. A highly active alkaline protease from the fungus *Conidiobolus macrosporus* has properties such as high pH optimum, broad substrate specificity, and pI close to the pH of the detergent solution, which are some of the useful features for its application in detergent industry. However, its tolerance to heat is inferior to that of the commercial bacterial enzyme, subtilisin [20]. Our research on the stability of the purified *Conidiobolus* alkaline protease is directed towards understanding the mechanism of thermal inactivation of the enzyme and increasing its stability to enhance its potential for industrial application. Such studies are very helpful from the point of view of the protein engineering of the enzyme and for reducing enzyme replacement cost. Enzymes can be tailored for optimal performance in industrial applications by evolutionary molecular engineering, which is also called as *in vitro* evolution [3]. In the present studies, thermal inactivation of *Conidiobolus* alkaline protease has been investigated as a function of pH, and the effect of various additives on the thermal stability has been determined. Our results on the structure-function relationship of the APC show that the thermal inactivation of the enzyme is primarily due to the unfolding of the protein followed by its subsequent autoproteolysis. Glycerol, sorbitol, and glycine conferred stability by protecting the enzyme against thermal unfolding.

MATERIALS AND METHODS

Enzyme production and purification

The APC was purified and assayed as described in Chapter 2.

Thermal stability measurements

Thermal stabilization of the enzyme was studied by incubating APC (0.15 U) in potassium phosphate buffer (0.05 M), pH 7.5, in the presence or absence of an additive at a desired temperature for a stipulated period. At the end of the incubation period, the enzyme was cooled on ice for 15 min and the residual activity was determined under the standard assay conditions. The APC incubated at 4°C in the absence of an additive was considered to be a control and was assumed to have 100% activity. *Streptomyces* alkaline protease inhibitor (API) was isolated and purified according to Vernekar *et. al.* [4]. Modified API was prepared by treatment of the API with dithiothreitol (10 mM) for 15 min and stopping the reaction with two fold molar excess of iodoacetamide. For studies on pH-dependent stabilization, the APC was incubated in 0.05 M acetate buffer, pH 5.0; citrate phosphate buffer, pH 6.0; potassium phosphate buffer, pH 7.5; Tris.HCl buffer, pH 8.5; carbonate- bicarbonate buffer, pH 10.0 and KCl- NaOH buffer, pH 12.0, for 1 h at a desired temperature and estimated as above.

Fluorescence measurements

Fluorescence measurements were performed with a Perkin-Elmer LS-50B spectro-fluorimeter equipped with a Julabo F- 20 water-bath. APC with absorption of 0.1 at 280 nm was excited in a 1cm path length quartz cuvette at 280 nm, with an excitation and emission bandwidth of 7.5 nm.

Circular dichroism measurements

CD spectra were recorded on a Jasco J- 715 spectropolarimeter fitted with a xenon lamp. The Far-UV CD spectra of the APC (7.5 μg) were recorded at 25°C between 200 to 250 nm using a 1cm path length quartz cuvette.

RESULTS

Stability of alkaline protease as a function of temperature and pH:

The optimum temperature and pH for the APC activity are 37°C and pH 10 respectively. Thermal stability of APC was determined over the temperature range of 40 to 60°C, at pH 7.5. Fig.4.1.1 shows the temperature stability profile of purified APC when incubated for 1 h at different temperatures.

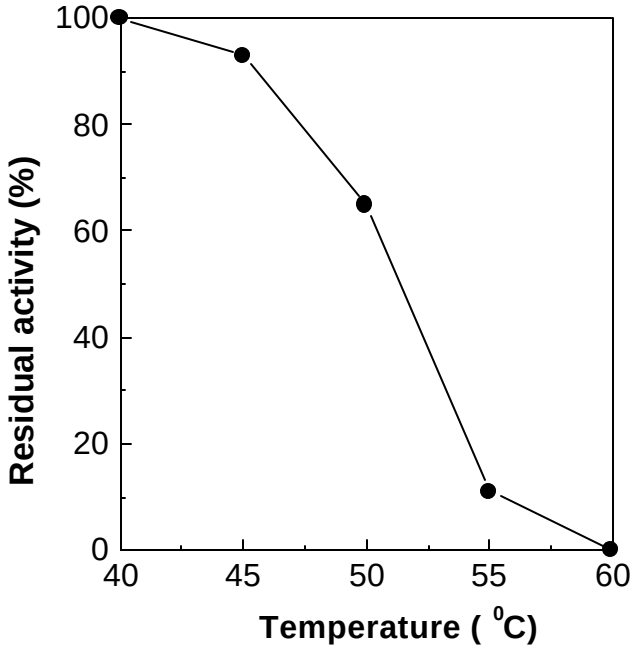


Fig. 4.1.1 Temperature stability of the APC

The APC (0.15 U) was incubated at the desired temperature at pH 7.5 for 1 h and assayed for residual activity.

The enzyme was stable at 40°C for 1 h. However, there was a progressive loss in activity with increasing temperature and complete loss of activity occurred at 60°C. The kinetics of thermal inactivation of the APC at various temperatures (Fig.4.1.2) at pH 7.5 revealed that inactivation does not follow the first-order kinetics, suggesting that besides thermal denaturation other factors such as autoproteolysis etc. may also be responsible for the observed loss in activity. The thermal stability of APC is highly pH dependent (Fig. 4.1.3). The enzyme was quite stable between pH 5.0 to 8.5 and at pH 12 at both 40°C and 45°C. However, there was 25 % loss in activity at 40°C when incubated for 1 h at pH 10, which is the optimum pH of the enzyme. The loss in activity further increased to 95 % at 45°C.

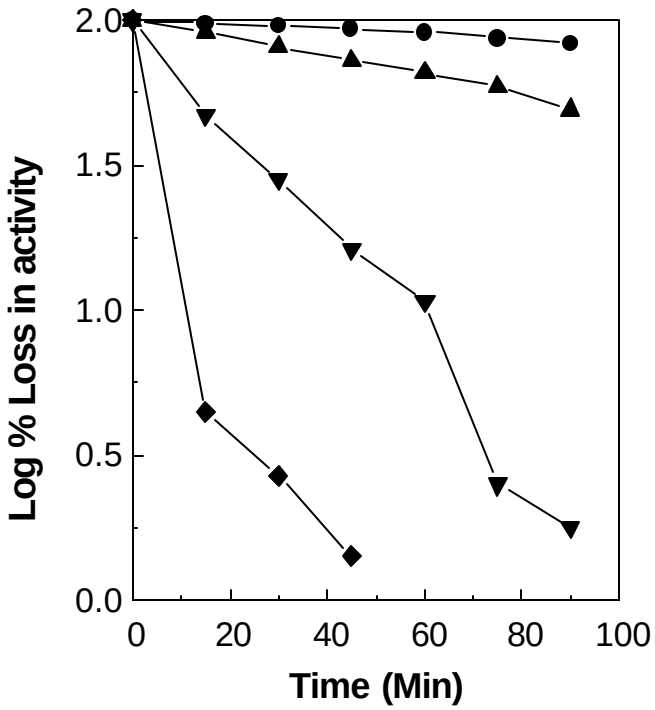


Fig. 4.1.2. Kinetics of thermal inactivation of APC

The APC (1 U) at pH 7.5 was incubated at 45°C (λ), 50°C (σ), 55°C (τ) and 60°C (υ). Aliquots were removed after every 15 min and estimated for residual activity.

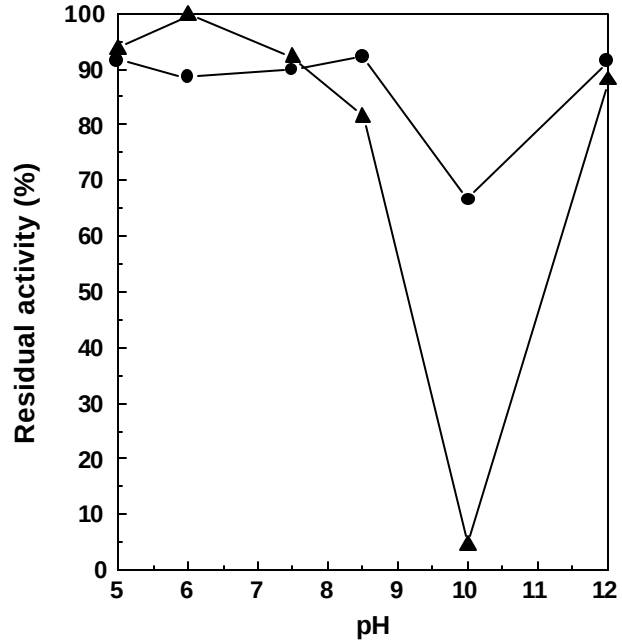


Fig. 4.1.3 Effect of pH on thermal stability of APC

The APC (0.15 U) was incubated at various pHs at 40°C (λ) and 45°C (σ) for 1 h and assayed for activity.

Effect of additives on thermal stability of APC:

The stability studies on APC were carried out at 55°C, as the enzyme is inactivated at a suitable rate at this temperature. The enzyme is inactivated irreversibly at 55°C with a $t_{1/2}$ of 14 min. The influence of various additives such as sugar and sugar alcohols, amino acids, metal ions, denaturants, reducing agents, substrate and inhibitor on the thermostability of the enzyme at 55°C was studied in an aqueous medium (Table 4.1.1).

Table 4.1.1 Effect of various additives on the thermal stability of APC

Additive	Concentration	Residual Activity (%)
Trehalose	0.5M	0
Glycerol	50%	43
Sorbitol	25%	49
Xylitol	50%	0
Alanine	0.5 M	0
Glycine	1M	30
$CaCl_2$	50mM	0
$MgSO_4$	100mM	25
Sorbitol + $MgSO_4$	25% + 100mM	62
GH	2M	0
Dithiothreitol	10mM	0
Casein	10mg	0
Modified API*	1U	0

*API was modified as described in Materials and Methods. Under these conditions, inhibitor binds to the enzyme but shows no inhibition.

Among the various sugar alcohols tested, sorbitol (25%) offered maximum (49%) protection against denaturation. Stabilization was found to be concentration-dependent and in the presence of sorbitol (25%) the $t_{1/2}$ increased from 14 min to 75 min (Fig. 4.1.4).

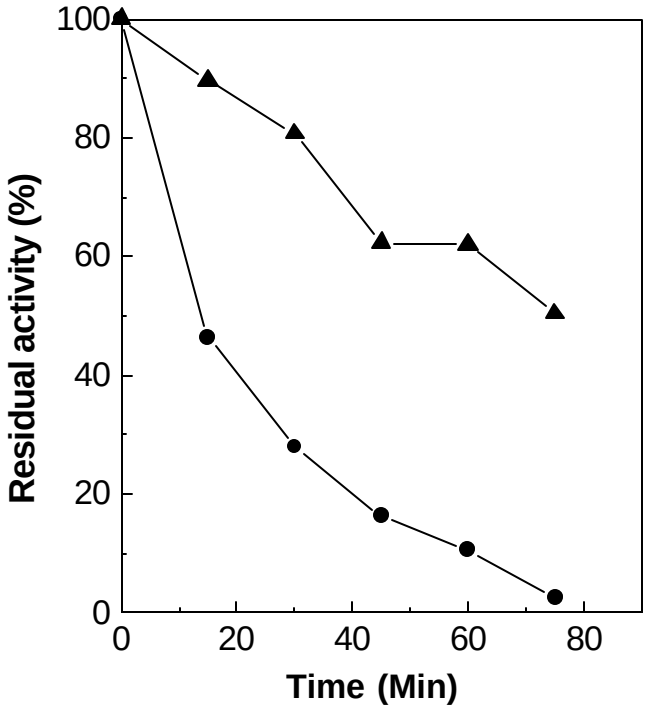


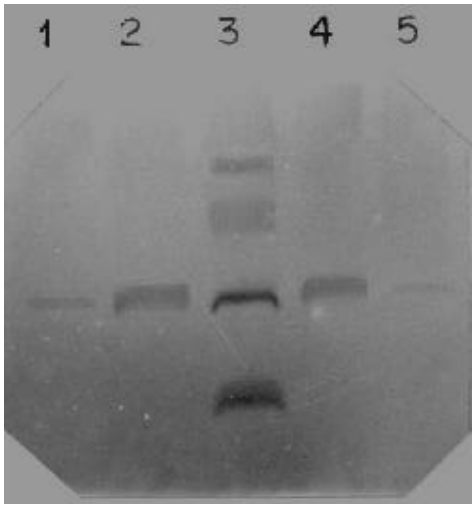
Fig.4.1.4 Effect of sorbitol on thermal stability of APC

The enzyme (0.9U) was incubated at 55°C, at pH 7.5 in the absence (▲) and presence (●) of sorbitol (25%). Aliquots were removed after every 15 min, and estimated for activity.

No direct relationship was observed between the protective effect of polyhydric alcohols on APC and their molecular sizes which is similar to the results obtained for rulactine, a protease from *Micrococcus caseolyticus* [5]. Of the various metal ions tested, only magnesium (100 mM) was able to offer up to 25% protection against inactivation. In contrast to the observations with many microbial serine proteases such as subtilisin, thermitase and thermomycolase (27), calcium ions did not enhance the thermal stability of the alkaline protease. Sorbitol and magnesium sulphate have an additive effect, which results in 62% stabilization of the APC, revealing their differential mode of stabilization. Among the various amino acids tested, only glycine (1 M) offered 30% thermal protection. The binding of the enzyme to the substrate or inhibitor is likely to confer a stable conformation by protecting the active site of the enzyme. However, neither substrate (casein) nor inhibitor (API) offered any protection against thermal inactivation of the enzyme, implying that the loss of activity does not result from the change in conformation at the active site, but is due to the overall change in conformation. The absorption spectrum of the alkaline protease heated at 55°C at pH 7.5 showed no increase in the light scattering at 360 nm, suggesting that no aggregation of the

protein occurred upon the heat treatment. The extent of thermal stabilization by small molecular weight additives was found to depend both upon the temperature of inactivation and on the concentration of the stabilizer. No protection against thermal denaturation was offered by these additives at 60°C. The loss in enzyme activity at 55°C at pH 10.0 was too rapid to be measured. No thermal stabilization was offered by the additives at this pH. The APC undergoes autoproteolysis upon heating at 55°C at pH 7.5 and 10 as revealed by the decrease in the intensity of the protease band on SDS-PAGE (Fig. 4.1.5).

Fig.4.1.5 Analysis of autoproteolysis of APC during thermal inactivation:



Lane 1, enzyme (10 μg) heated at 55°C, at pH 7.5 for 1h; lane 2, enzyme at pH 7.5; lane 3, molecular weight markers; lane 4, enzyme at pH 10.0; lane 5, enzyme heated at 55°C, at pH 10.0 for 1 h.

However, the extent of autoproteolysis is more at pH 10 than at pH 7.5 and no protection is offered by the additives against autoproteolysis of the enzyme. The rate of self-digestion of extracellular proteases is reported to be maximum between 50-60°C [7]. This is associated with the fact that autolysis is most effective when both the native and the denatured forms of the enzyme coexist and the former degrades the latter. Electrophoresis analysis indicated that the polypeptide chain suffered no fragmentation when exposed to inactivating conditions.

Conformational changes during thermal inactivation and stabilization

Intrinsic fluorescence- Changes in the tertiary structure of the APC during thermal inactivation were followed by monitoring its fluorescence spectra upon excitation at 280 nm. The native enzyme exhibits an emission maximum at 340 nm. There is a red shift in fluorescence emission spectrum upon heat treatment of the enzyme (Fig.4.1.6a). The shift in the emission maximum is more at 65°C than at 55°C, indicating temperature- dependent denaturation and greater exposure of aromatic amino acids to the solvent. Simultaneously, there is a decrease in the quantum yield of the fluorescence. In the presence of glycine (Fig.4.1.6b), glycerol (Fig.4.1.6c) or sorbitol (Fig.4.1.6d), the shift in the emission maximum of the enzyme heated at 55°C is less than in the absence of the additive and correlated well with the extent of protection. Thus, thermal stabilization is provided by preventing the change in conformation of the protein. Quantum yield for the heated enzyme was more in the presence of the additive except for the sample heated in presence of sorbitol. Thermal denaturation of the enzyme is more at pH 10 than at pH 7.5 as indicated by the extent of red shift of the enzyme heated at pH 10.

Far- UV CD spectra The CD in the far- UV region reports on the backbone of a protein and is used to characterize the secondary structure and the changes therein. The APC displays a strong and characteristic CD spectrum in the far- UV region. Two distinct negative minima, at 219 nm and 209 nm were displayed (Fig. 4.1.7). The 219 nm band is of larger magnitude and more prominent than that at 208 nm band. The enzyme appears to be an α/β type of protein with intermixed segments of α- helix and β- sheet [8]. The α–helical content diminished upon heating the enzyme at both pH 7.5 and pH 10.0 with simultaneous increase in the random coil. The shape of the CD spectrum of thermally inactivated APC is similar to those of other thermally unfolded proteases [9,10].

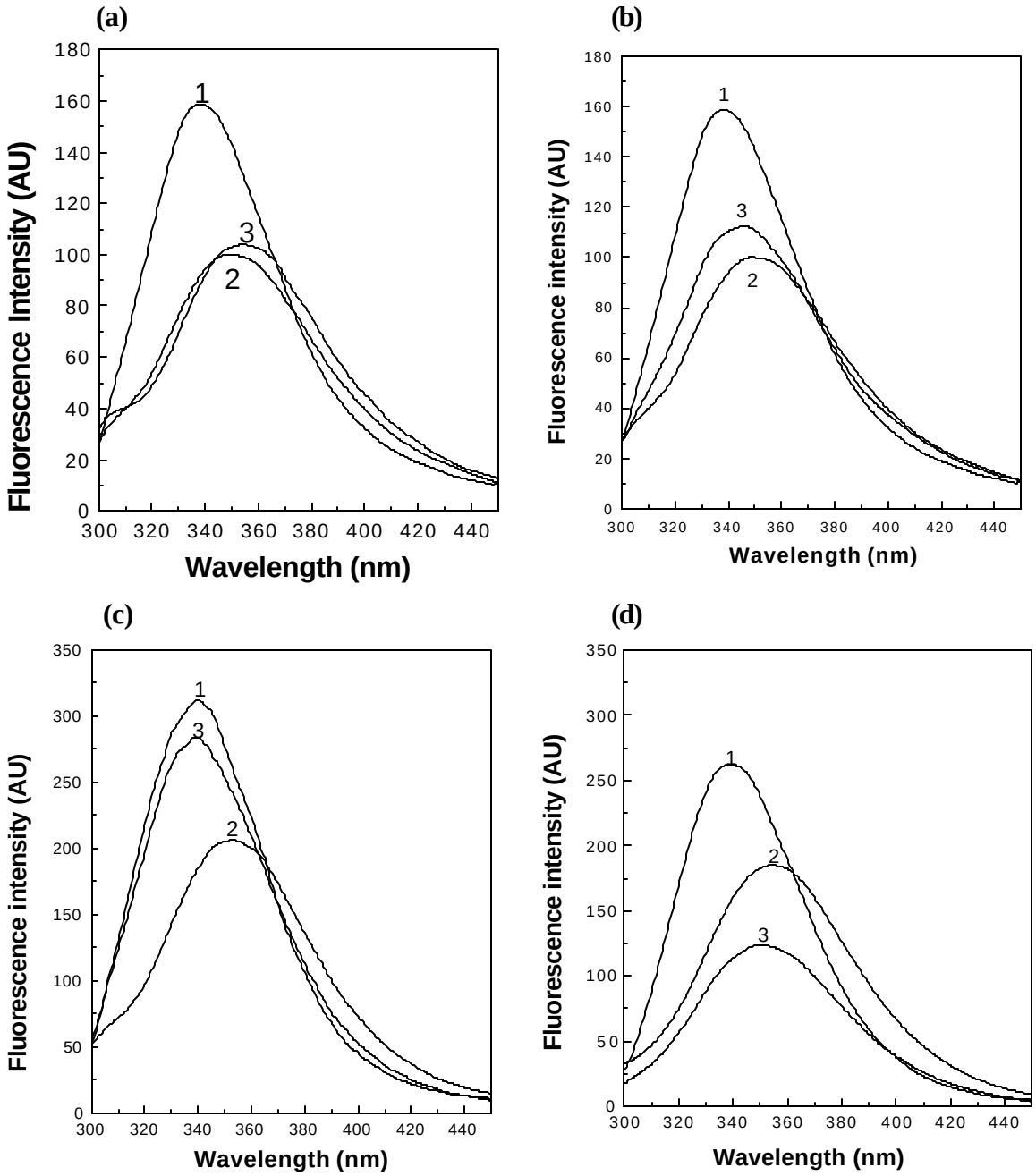


Fig. 4.1.6. Effect of thermal inactivation on the intrinsic fluorescence of APC

Fluorescence spectra were recorded at an excitation wavelength of 280 nm. The enzyme (1), heated at pH 7.5 for 1 h, **(a)** at 55°C (2) and 65°C (3); **(b)** at 55°C in the absence (2) and presence (3) of glycine (1M); **(c)** at 55°C in the absence (2) and presence (3) of glycerol (50%) and **(d)** at 55°C in the absence (2) and presence (3) of sorbitol (25%).

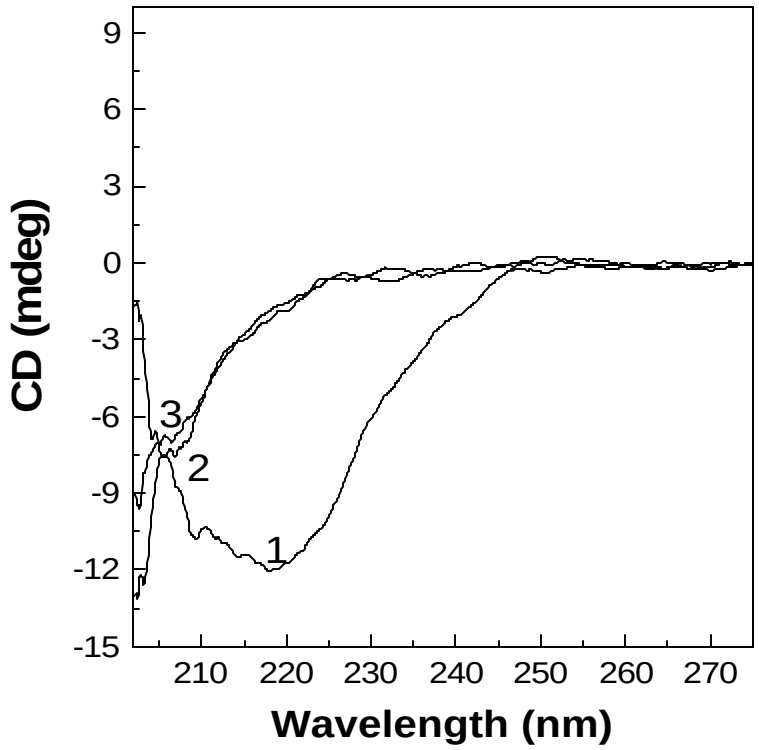


Fig.4.1.7 Effect of thermal inactivation on far UV-CD spectra of APC

Enzyme (1) at pH 7.5, heated at 55°C (2) and 65°C (3) for 1h.

DISCUSSION

The systematic study and modeling of thermal inactivation phenomenon has greatly aided in the formulation of principles of enzyme stabilization. We report herein the molecular mechanisms of thermal inactivation and stabilization of APC at 55°C, at two pHs viz. pH of the maximum stability (pH 7.5) and pH of maximum activity (pH 10). When enzymes readily inactivate at temperatures below 70°C at a pH close to neutral, the inactivation process involves no changes in the primary structure [7]. The major causes of inactivation of proteins are: aggregation, alterations in primary structure, cleavage of S-S bonds, thiol- disulphide exchange, dissociation of prosthetic group from the active center of the enzyme, dissociation of oligomeric proteins into subunits and conformational changes in the macromolecule [12]. In practice, all these inactivation processes are interrelated. However, the primary role is mostly played by conformational changes, which often trigger other mechanisms of inactivation that cannot occur prior to a change in the conformation of the protein. Thermal inactivation of the *Conidiobolus* alkaline protease at 55°C at pH 7.5 was accompanied by the loss of secondary and tertiary structure. Unfolding of the enzyme molecule was indicated by the diminished α- helical content and the red shift in the emission maximum. Therefore, prevention of unfolding of the protein chain is of overriding importance in improving the stability of designed enzymes. It is not surprising that almost all known approaches to enzyme stabilization deal, explicitly or implicitly, with trying to eliminate (or minimize) the process of unfolding. This can be achieved by addition of specific compounds to the enzyme containing solution, by immobilization or by the chemical modification of the protein.

Addition of small compounds to protein solution and changing its microenvironment provides a simple and practical means to increase the stability of the enzyme. Several additives widely used in stabilizing the enzymes include solutes involved in osmoregulation, cryoprotectants, carbohydrates, polyols, thickners, polyelectrolytes, metal ions, amino acids, thiol-compounds, non- ionic detergents and also substrates, coenzymes or inhibitors of the respective enzyme. In most of these cases, either increased viscosity or lowered water activity of the enzyme preparation is the underlying phenomenon in the stabilization of the enzyme [12]. Glycerol and sorbitol have a stabilizing effect on APC. Sorbitol protected the enzyme against thermal denaturation by preventing the changes in tertiary structure. Stabilization at higher temperatures under lyophilized state by sugars and sugar alcohols is reported for an

aspartyl protease from *Rhizomucor pusillus* [13]. Sugars or polyhydric alcohols modify the structure of water and/or strengthen hydrophobic interactions among non-polar amino acids, rigidify protein molecules thereby making them resistant to unfolding and thermal denaturation.

Autolysis or self- digestion is greatly accelerated at elevated temperatures and therefore, thermal inactivation of proteolytic enzymes is, in fact, most often due to autolysis. Our results on the pH dependent thermal inactivation of the APC also reveal that it is the unfolding and subsequent autolysis of the enzyme molecule that leads to thermal inactivation. The extent of autolysis was more at pH 10 than at pH 7.5. The phenomenon of thermal inactivation was found to be highly pH dependent for serine proteases from other microbial sources as well [14,10]. Thermitase is degraded by autolysis and thereby inactivated, especially at elevated temperatures, at alkaline pH values as shown by complete disappearance of the protease band on polyacrylamide gel electrophoresis [15]. The decrease in protein amount at pH 9.1 position in the isoelectric focussing experiment was correlated to the absence of active/ inactive intermediates during the course of autolysis of the thermitase [16]. In the autolytic process, the first cleavage step of the intact polypeptide chain is slow, followed by a rapid proteolytic degradation of the arising unfolded polypeptide fragments. The rate-limiting step for autolysis is not the proteolysis itself but a preceeding unfolding of the enzyme molecule, which facilitates the proteolysis.

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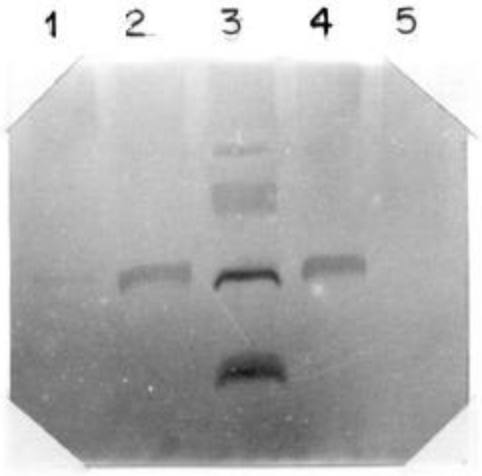
Fig. 5



CHAPTER FOUR

SECTION TWO

IMMOBILIZATION OF THE ALKALINE PROTEASE FOR REUSE AND INCREASED THERMAL STABILITY

SUMMARY

Alkaline protease from a fungus *Conidiobolus macrosporus* (NCIM 1298) was immobilized on an inexpensive support, polyamide, using glutaraldehyde as a bifunctional agent. The immobilized enzyme showed a higher optimum temperature of 50°C as compared to that of the free enzyme (40°C). The polyamide-bound enzyme showed maximum activity in the pH range of 8-9, whereas the optimum pH of the soluble enzyme was pH 10. The efficiency of immobilization was 58% under the optimal conditions of pH and temperature. The immobilized enzyme was stable over a wide range of pH (5.0 - 12.0) and exhibited an increased thermostability (50% activity at 60°C). There was a fourteen-fold decrease in the K_m of immobilized enzyme compared to the free enzyme indicating increased affinity for the substrate. The immobilized enzyme was fully active even after twenty-two cycles of repeated use displaying excellent durability. It catalyzed the hydrolysis of ovalbumin and hemoglobin in addition to casein revealing its broad substrate specificity. The polyamide-bound enzyme retained 80% activity at 50°C in presence of 8 M urea exhibiting its stability to the denaturant at elevated temperatures. It was compatible with the detergents such as Rin Shakti, Ariel, and Surf-Excel as deduced by the retention of significant amount of its activity (>57%) in their presence. The improved properties of the immobilized enzyme such as (i) increased stability at higher temperatures, (ii) retention of activity in presence of denaturants and detergents, (iii) broad substrate specificity and (iv) an excellent durability for repeated use make the enzyme a suitable candidate for its application in industries.

INTRODUCTION

Higher thermostability is an important factor for the suitability of an alkaline protease for commercial application. The strategies used for improving the thermostability include use of additives, introduction of disulfide bonds [1], site-specific mutagenesis [2] and chemical modification or crosslinking [3]. In all these strategies, however, the recovery yield and reusability of free enzymes as industrial catalysts are quite limited. Therefore, an increased attention has been paid to enzyme immobilization which offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture and adaptability to various engineering designs [4]. The commercial immobilization processes are of two types viz. whole-cell immobilization and cell-free enzyme immobilization. In case of alkaline protease, the whole-cell immobilization is mainly carried out to increase the production of the enzyme. The cells or mycelia are entrapped or encapsulated in different gel matrices such as calcium alginate or κ-carrageenan [5,6] or sheep wool [7]. The mycelium of *Conidiobolus* (NCL 82-1-1) was entrapped in κ-carrageenan or in cubes of polyurethane sponge [8]. The cell-free immobilization is generally carried out to increase the stability of the immobilized enzyme towards pH and/or temperature and to facilitate its storage and reuse.

Increasing use of immobilized enzymes in recent years has led to studies concerned with their applications in industrial processes. The alkaline proteases have now been used for a multitude of purposes. Some of the important applications of immobilized alkaline proteases in peptide synthesis [9], in deproteinization [10], as biosensors [11] and in food industry [12]. The alkaline proteases of microbial origin occupy an important position as biobuilders in the detergent industry. Immobilization of bacterial alkaline proteases on various matrices has been reported [13-18]. However, there are very few reports on the immobilization of fungal alkaline proteases. High cost of production of the bacterial enzyme is a major barrier for its application in developing environmentally friendly technologies. The fungus *Conidiobolus macrosporus* (NCIM 1298) produces high yields (30U/ml) of alkaline protease [19] in short fermentation cycles (48h) comparable to the bacterial enzyme. Fungal origin of the enzyme offers a distinct advantage in terms of ease

of downstream processing as against cost-intensive high-speed centrifugation technologies required for the bacterial enzyme. The objective of our present work has been to immobilize the enzyme for its repeated use and to improve the thermostability with a view of increasing its efficiency for application in leather and detergent industries. The enzyme was immobilized on an inexpensive support, polyamide, using glutaraldehyde as the spacer. The kinetic parameters, reuse potential, and stability of the immobilized enzyme are described.

MATERIALS AND METHODS

Glutaraldehyde was obtained from Loba chemicals, India. Polyamide was a gift from Polymer Division, National Chemical Laboratory, India. All other chemicals used were of analytical grade.

Organism and culture conditions:

Growth, maintenance of *Conidiobolus macrosporus* (NCIM 1298), and production of alkaline protease were carried out as described in the Section A of Chapter 2.

Enzyme activity and protein estimation:

The protease activity was determined in 0.1 M carbonate-bicarbonate buffer, pH 10.0 or in 0.1 M Tris-Cl buffer, pH 8.0 at 40°C using casein (10mg) [20]. One unit of enzyme activity is defined as the amount of the enzyme required to cause an increase of 1 absorbance unit at 280 nm per ml of reaction mixture per minute when casein was used as a substrate. Protein was estimated according to the method of Bradford [21] using BSA as a standard.

Immobilization of the enzyme:

All the steps were carried out between 4 to 10°C unless otherwise mentioned. Polyamide (0.5 g) was equilibrated with 10 ml of 0.1 M potassium phosphate buffer, pH 7.5 by stirring it for 1 h. Glutaraldehyde was added to a final concentration of 2% and the stirring was continued for 3 h. Polyamide was allowed to settle for 30 min and the clear supernatant containing excess of glutaraldehyde was removed. The volume was made up to 12 ml by the addition of 0.1M phosphate buffer. The concentrated enzyme (0.5 ml, 4 mg/ml) was added drop-wise to the polyamide suspension under constant stirring. After 6 h, the mixture was centrifuged at 10,000 rpm for 30 min. The unbound enzyme was removed by repeated washing of the polyamide using 0.1M phosphate buffer until the supernatant showed no activity. The polyamide with the immobilized enzyme was further resuspended in 0.1 M phosphate buffer. The activity of the immobilized enzyme was determined by vigorous shaking in presence of substrate at 40°C for 20 min.

Properties of the immobilized enzyme:

Optimum pH of the enzyme was determined by estimating the enzyme activity in the pH range of 7 to 12. The buffers used were 0.1M sodium phosphate pH 7.5, Tris - HCl (pH 8.0-9.5), sodium carbonate - bicarbonate (pH 10.0) and glycine - NaOH (pH 11.0 & 12.0). Activity of the immobilized enzyme was determined at different temperatures ranging from 40°C to 80°C at pH 10.0. To check the effect of pH on the stability of the immobilized enzyme, it was incubated with 100 µl of 0.1 M buffer of desired pH for 1 h at 25°C. Residual activity was determined by caseinolytic assay at pH 8.0 and 50°C and compared with that of the control sample kept at 4°C at pH 7.5. The enzyme samples were incubated at various temperatures ranging from 40°C to 80°C at pH 7.5. After 1 h, residual activity was determined at pH 8.0 at 50°C and compared with the control sample kept at 4°C at pH 7.5. The free and immobilized proteases were incubated with increasing amounts of casein (0.025-10 mg). The kinetic constant (K_m) was determined from the Lineweaver-Burk plots. The immobilized enzyme was kept with the substrate at 50°C for 20 min and immediately transferred onto ice. The immobilized enzyme was recovered by centrifugation, washed with buffer and the hydrolysis of substrate was monitored by usual assay procedure. The caseinolytic assay was applied to determine the activity with other protein substrates such as hemoglobin, ovalbumin, gelatin, and bovine serum albumin.

Effect of denaturants and detergents:

The detergents were diluted in double distilled water to give a final concentration of 560 mg/ml. The free or immobilized protease (1 U each) was incubated at 30°C in various detergents (final concentration 7 mg/ml, which is the concentration, required to simulate washing conditions) for zero to 60 min. The residual activity in each sample was determined at 40°C by caseinolytic assay and compared with the control sample incubated at 40°C without any detergent. Similarly, the effect of denaturants such as urea and SDS was studied by incubating the free and immobilized enzyme with urea (0-8 M) or SDS (0-0.3 %) for 30 min and the residual activity was determined under their optimal conditions of pH and temperature.

RESULTS AND DISCUSSION

Optimization of the immobilization conditions:

Conidiobolus macrosporus produces high yield of protease (30 U/ml) and the crude extracellular broth consists of about five different alkaline proteases as visualized by the gel-X-ray film contact print technique [19]. To utilize the activity of all these proteases in commercial application, the crude culture filtrate was used for immobilization on polyamide.

Glutaraldehyde acts as a spacer arm for the immobilization of alkaline protease on polyamide. The effect of varying concentrations of glutaraldehyde on the immobilization showed that there was an initial increase in the extent of immobilization with the increase in glutaraldehyde concentration and maximum immobilization was obtained at a glutaraldehyde concentration of 1.76 %. As the glutaraldehyde concentration was further increased, there was a decrease in the efficiency of immobilization. This may be because of denaturation of protein in the presence of excess amount of glutaraldehyde. The alkaline protease lost 45 % and 72 % of its activity in presence of 0.5 % and 2.5 % glutaraldehyde respectively. This provides an explanation for the decrease in the extent of immobilization in presence of higher concentrations of glutaraldehyde. Therefore, in all further experiments 1.76 %, glutaraldehyde was added to polyamide and the excess of glutaraldehyde was removed before the addition of the protease. At such a low concentration, the polyamide-bound glutaraldehyde did not affect the enzyme activity and acted only as a spacer arm for the enzyme.

The efficiency of immobilization was also dependent on the time for which the enzyme was incubated with the polyamide. Maximum immobilization was obtained by stirring the enzyme with polyamide for 6h after which there was no increase even up to 18 h. Therefore, 6 h was chosen as the optimum time for immobilization in further experiments. The efficiency of immobilization was 58 % when the activity was determined under the optimum conditions of pH (8.0) and temperature (50°C).

Characterization of the immobilized enzyme:

The thermal stability of the immobilized enzyme is one of the most important criteria for its application. The native *Conidiobolus* protease shows maximum activity at 40°C whereas temperature optimum for the immobilized enzyme increased to 50°C (Fig.4.2.1a).

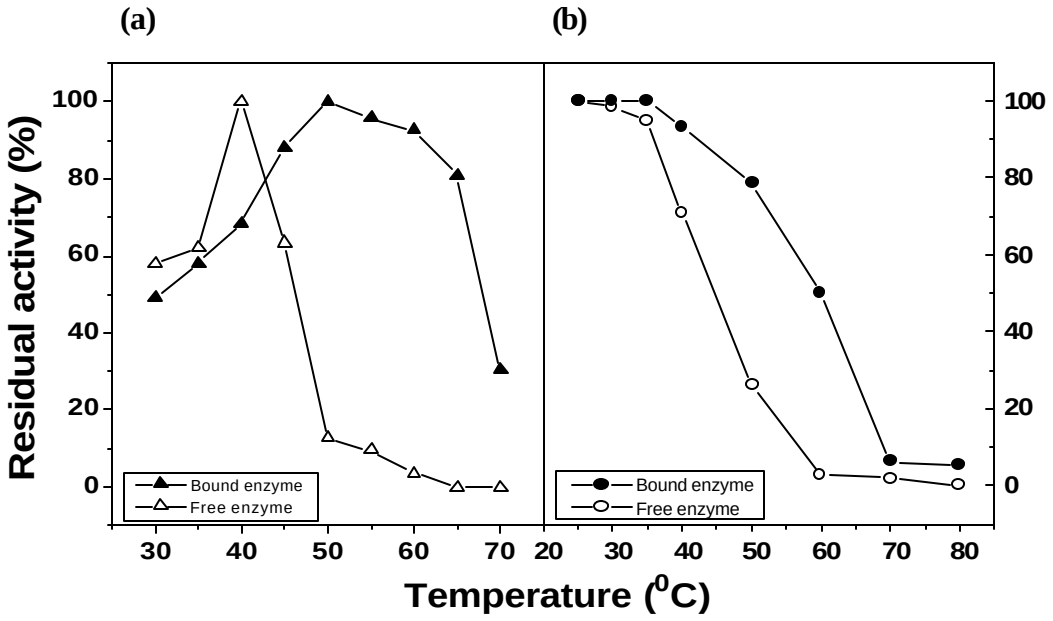


Fig.4.2.1 Optimum temperature (a) and temperature stability (b) of the free and the immobilized alkaline protease

(a)The free and the bound enzyme (0.3 U and 1 U respectively) were incubated at different temperatures (30°C - 70°C) at pH 10.0 for 20min and the residual activity was determined (Δ, free protease; ▲, bound protease). (b) Immobilized enzyme (1U) was incubated in 0.1M phosphate buffer, pH 7.5 at different temperatures (25°C - 80°C) for 1 h and assayed for activity at pH 8.0 and 50°C. Soluble crude alkaline protease (0.3 U) was also treated under the same conditions and assayed at 40°C at pH 10.0. (○, free protease, ●, bound protease).

The immobilized enzyme exhibited 10 times higher activity at 60°C compared to the native enzyme (Fig.4.2.1b) thereby displaying increased thermostability. In case of some proteases, the optimum temperature remained constant, however there was an increase in the temperature stability [13,22], whereas some immobilized proteases exhibited both

higher optimum temperature and increased thermostability [14,16]. Covalently bound immobilized enzyme system is more resistant to heat and denaturing agents than its soluble form probably due to the limitation of free diffusion of the substrate. This confers a good operational stability to an immobilized preparation i.e. the ability to maintain a constant level of activity over a significant period during actual use, resulting in an easier process control. This higher stability can also be attributed to the prevention of autodigestion and/or thermal denaturation because of the fixation of enzyme molecules on the surface of polyamide and can be exploited for its application in detergents.

The effect of pH on the activity of the free and immobilized alkaline protease for casein hydrolysis was studied in buffers of various pHs at 40°C (Fig.4.2.2a).

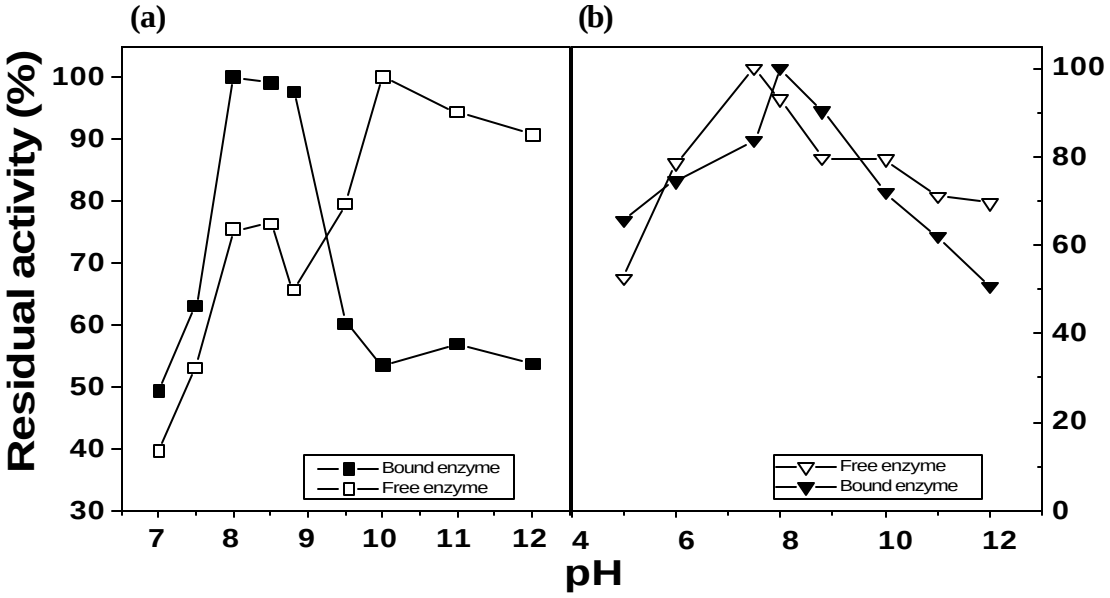


Fig.4.2.2 Optimum pH (a) and pH stability (b) of free and bound alkaline protease

(a) The activity of the immobilized enzyme (1U) and the free enzyme (0.3 U) was determined by incubating the enzyme with casein (1%) in 0.1 M buffers of different pH (7.0-12.0) at 40°C for 20 min. (, free protease; ■, bound protease). (b) The immobilized enzyme (1 U) and soluble enzyme (0.3 U) were incubated at room temperature in 0.1 M buffers of different pH (5.0-12.0) and assayed for activity at their optimal conditions of pH and temperature (pH 8.0, 50°C for immobilized enzyme (▼) and pH 10.0, 40°C for the soluble enzyme (▽)).

The free and the bound enzyme were stable (>50% activity) over a wide pH range of 5.0-12.0. The maximum activity of the free enzyme was at pH 7.5, whereas that of the immobilized enzyme was at pH 8.0. The free enzyme exhibited maximum activity at pH 10.0, whereas, the bound enzyme exhibited maximum activity between pH 8.0 to 9.0 (Fig. 4.2.2b), which can be explained based on the partitioning of hydrogen ions. Since polyamide is positively charged, it has a tendency to concentrate $[OH]^-$ ions around it, thus increasing the pH around the enzyme. In order to obtain the pH value for optimum activity in the vicinity of the enzyme, the external bulk phase value of pH must be lower than the enzyme's intrinsic pH optimum. Hence, the pH optimum shifted towards more acidic values of pH and as a result, the optimum pH of the enzyme is lowered by 1-2 units. A similar downshift of 1 unit in the optimum pH was observed for an alkaline protease immobilized on ω-dicarboxypolyethylene glycol magnetic nanoparticles [22], whereas a shift of 0.5 units in the optimum pH towards alkaline side was observed in case of an alkaline protease immobilized on epichlorohydrin-activated cellulose beads [13].

The kinetic studies of the soluble and the immobilized alkaline protease showed that there was a 14-fold decrease in the apparent K_m of the immobilized enzyme (101.2 mg/ml for the free enzyme as against 7.3 mg/ml for the bound enzyme) indicating increased affinity for the substrate (casein). The alteration in the kinetic constant can be attributed to the change in the microenvironment around the enzyme as compared to the bulk phase. The covalent binding of the enzyme may reduce the diffusional barrier for the substrate and facilitate the access of the active site of the enzyme to the substrate. K_m value lower than that of the free enzyme was also observed for an alkaline protease from *B. subtilis* [24].

Substrate specificity is another important criterion, which determines the suitability of the enzyme preparation for its biotechnological exploitations. In case of protease, it is important especially for its use in detergent & leather industries. Both native and immobilized protease preparations were able to hydrolyze other substrates such as hemoglobin and ovalbumin besides casein, exhibiting broad substrate specificity. The immobilized enzyme exhibited higher efficiency (9.4 U/ml) of hydrolysis of hemoglobin than casein at 50°C (5.1 U/ml).

Reuse potential of the immobilized enzyme:

The reuse of the immobilized enzyme is very important from the point of view of reducing the cost of the enzyme, which is an important factor while considering its suitability for commercial application. The activity was retained without any significant loss even after the reaction was repeated twenty-two times (Fig.4.2.3) indicating excellent performance of the immobilized enzyme. An alkaline protease from *B. subtilis* could be recycled only 5-6 times [24]. Papain encapsulated in polyacrolein microspheres was recycled 10 times [25].

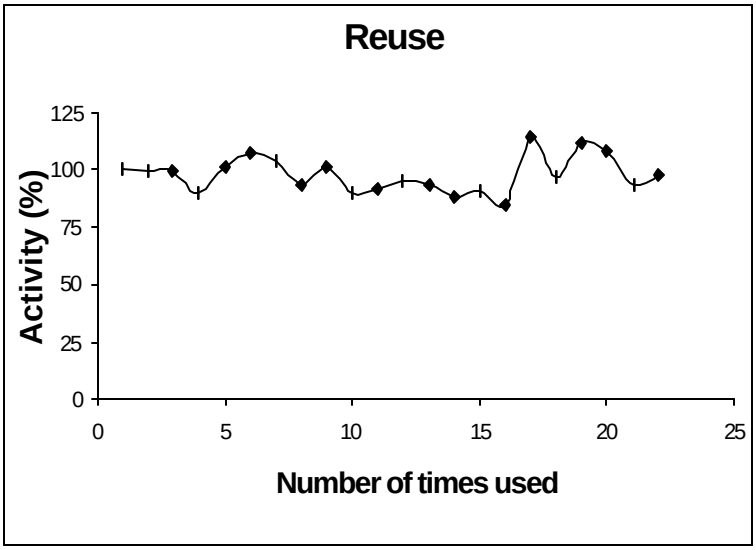


Fig.4.2.3 Reuse of the immobilized enzyme

The polyamide-bound enzyme (1 U) was assayed at pH 8.0, 50°C for 20 min. After every use, the immobilized enzyme was separated from the substrate, washed with buffer, and used for the next assay. The hydrolysis of the substrate was monitored by the usual caseinolytic assay.

Compatibility with the detergents:

Due to their environmentally friendly nature, alkaline proteases have potential application in detergent industries for partial or total replacement of currently employed toxic chemicals. An ideal detergent enzyme should be stable and active in the detergent

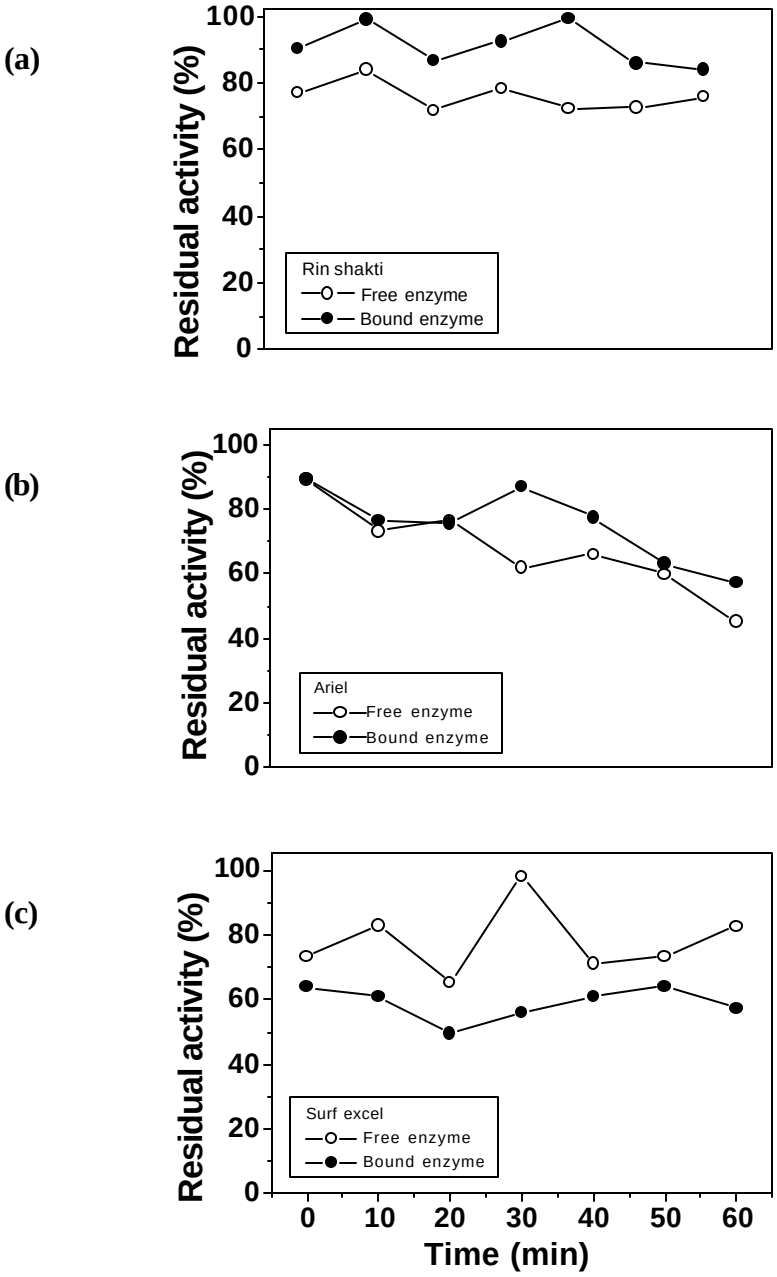


Fig.4.2.4 Compatibility of the alkaline protease with various commercial detergents

Free enzyme (0.3 U) and bound enzyme (1 U) were incubated in presence of various detergents (7mg/ml) at room temperature. The residual protease activity was determined after every 10 min. Activity of control sample devoid of any detergent incubated under similar conditions was assumed as 100%. (a) Rin Shakti, (b) Ariel, and (c) Surf-excel.

solution and should have adequate temperature stability to be effective in a wide range of washing temperatures. Both the free and bound proteases were compatible to detergents such as Ariel, Rin Shakti, and Surf Excel, which are most commonly used in India. The maximum stability of the immobilized enzyme was observed with Rin Shakti, as the enzyme retained 84% of its activity after incubation with the detergent at 28°C for 1 h (Fig. 4.2.4a). The free enzyme retained 76% of its activity under similar conditions. Both the enzymes retained more than 50% of their activity in presence of Ariel (Fig. 4.2.4b) and Surf Excel (Fig. 4.2.4c). The enhanced stability for the immobilized enzyme can be attributed to its covalent binding with the polyamide, which offers greater protection against autodigestion and inactivation. This indicates excellent compatibility of the immobilized enzyme preparation with the detergents. The polyamide bound enzyme is stable over a wide range of pH and exhibits a broad substrate specificity that may be effective in washing a variety of stains. These properties are also shown by the free enzyme. Earlier, the alkaline protease from *Conidiobolus* sp. (NCL 86.8.20) has been shown to be compatible with the detergents like Nirma, Snow-white, Revel, Wheel etc [23]. However, the free enzyme is stable only upto 40°C, whereas, as shown in the present work, the immobilized enzyme from *Conidiobolus macrosporus* is stable upto 60°C, which is an additional advantage for its potential use in washing powders.

Effect of denaturants:

The soluble and the immobilized proteases were treated with increasing concentrations of denaturants such as urea or SDS. There was an increase in the activity of both free and polyamide-bound enzyme with increasing concentration of urea. Maximum 43 % increase in the activity was observed at 3 M urea for the free enzyme. The bound enzyme showed 47 % increase in the presence of 4 M urea at 28°C. Increase in temperature to 40°C led to an inactivation of the free enzyme with increase in urea concentration (Fig. 4.2.5a), whereas, the immobilized enzyme continued to show increased activity (Fig. 4.2.5b). Further, increase in temperature to 50°C led to a complete loss of activity of the free enzyme, whereas the immobilized enzyme was able to retain 80 % of the activity in

presence of 8 M urea. Thus, the immobilized enzyme was stable in the presence of urea at high temperatures.

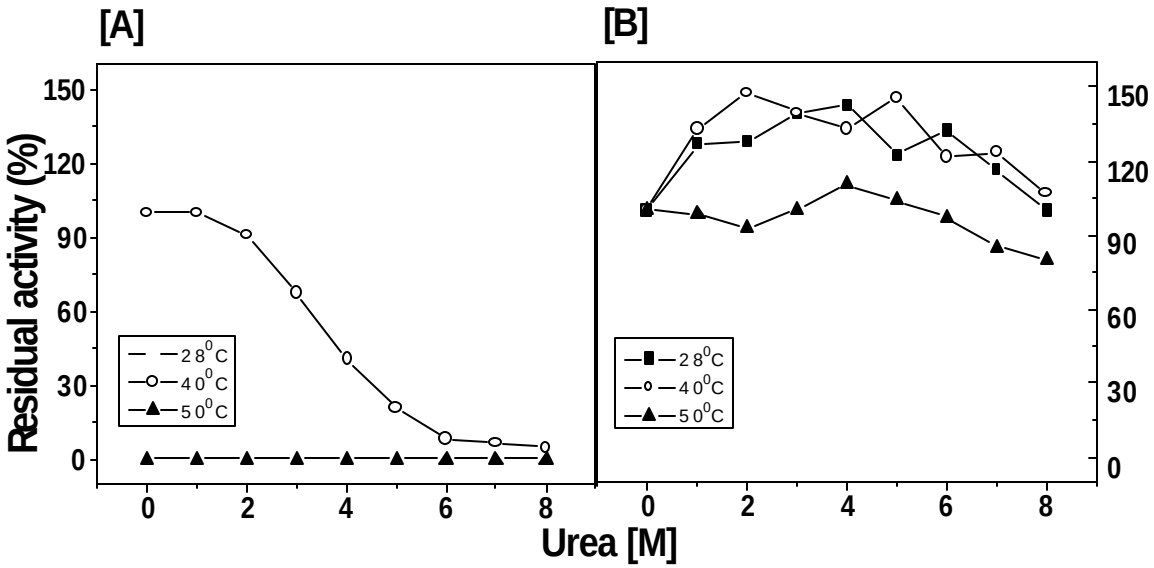


Fig.4.2.5 Effect of urea and on the activity of the free (a) and bound (b) enzyme

The free enzyme (0.3 U) and the bound enzyme (1 U) were incubated with different concentrations of urea (1-8 M) at different temperatures (30°C, 40°C and 50°C) for 60 min and the residual activity was determined at the optimum conditions of pH and temperature for the free and bound enzyme.

Both free and polyamide-bound enzymes exhibited a similar pattern of inactivation due to SDS. Both the enzymes retained about 25 % of the original activity in the presence of 0.3 % SDS at 28°C. The loss of activity may be attributed to the binding of negatively charged detergent to the positively charged alkaline protease. The influence of temperature on the SDS-induced inactivation of the enzyme was studied by incubating the enzyme in presence of SDS at 40°C. The effect of temperature and SDS was seen to be cooperative and the activity decreased rapidly at increased temperature. The immobilized enzyme retained 25 % and 15 % of its activity compared to 13 % and 0 % for the free enzyme in the presence of 0.3 % SDS at 40°C and 50°C respectively indicating higher stability of the bound enzyme to the denaturant at increased temperature.

Table 4.2.1 Comparison of the properties of free and immobilized enzyme:

Enzyme	Free	Immobilized
Optimum temperature	40	50
Optimum pH	10	8.0-9.0
Reuse	0	22
	Activity (%)	
At 60°C	5	50
In presence of		
Urea (8M) at 50°C	17	80
Ariel	47	60
Rin Shakti	76	84
Surf-excel	80	54

In conclusion, immobilization of the alkaline protease on an inexpensive support (polyamide) has several advantages. The improved properties of the immobilized enzyme such as increased thermal stability, increased affinity to the substrate, resistance to denaturants, excellent compatibility to detergents and retention of activity even after twenty-two reuses make the immobilized alkaline protease a potential candidate for use in industry.

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