MICROBIAL ENZYMES

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DEDICATED TO MY PARENTS

Certified that the work incorporated in thesis: "Microbial Enzymes" submitted by Shri. Ghanashyam D. Ghadge was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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ABBREVIATIONS

AE-globin	-	S-2-Aminoethyl-globin
APME	-	N-Acetyl-DL-phenylalanine methyl ester
ATEE	-	N-Acetyl-L-tyrosine ethyl ester
BAEE	-	N-Benzoyl-L-arginine ethyl ester
BAPNA	-	N-Benzoyl-DL-arginine-p-nitroanilide
Bis	-	N,N'-methylene-bis-acrylamide
BSA	-	Bovine serum albumin
BTEE	-	N-Benzoyl-L-tyrosine ethyl ester
BTPNA	-	N-Benzoyl-L-tyrosine-p-nitroanilide
CHO	-	Chinese hamster ovary
CMC	-	1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate
DFP	-	Diisopropyl phospho-fluoridate
DMSO	-	Dimethyl sulfoxide
EDTA	-	Ethylenediaminetetraacetate
ES_{tet}	-	Enzyme-substrate tetrahedral intermediate
HeLa	-	Human cervix carcinoma
LPNA	-	L-Leucine-p-nitroanilide
MW	-	Molecular weight
PAGE	-	Polyacrylamide gel electrophoresis

PCMB	-	p-Chloromercuribenzoate
pI	-	Isoelectric point
PMSF	-	Phenyl methanesulfonyl fluoride
PU	-	Proteinase unit(s)
SDS	-	Sodium dodecyl sulfate
TAME	-	p-Toluenesulfonyl-L-arginine methyl ester
TCA	-	Trichloroacetic acid
TEMED	-	N,N,N',N'-Tetramethylethylenediamine
TLCK	-	N-p-Toluenesulfonyl-L-lysine chloromethyl ketone
TPCK	-	N-p-Toluenesulfonyl-L-phenylalanine chloromethyl ketone
Tris	-	Tris(hydroxymethyl) aminomethane
Vero	-	African green monkey kidney

C O N T E N T S

CHAPTER I

INTRODUCTION

SECTION I

INTRODUCTION

Proteinases are hydrolytic enzymes capable of degrading proteins into small peptides and amino acids. Their occurrence is ubiquitous and they occur both in eukaryotes as well as in prokaryotes. Among them, the mammalian digestive enzymes such as trypsin, chymotrypsin and pepsin were the first to be discovered. Literature survey shows that, in microorganisms, the species representing over 150 genera of fungi, protozoa and molds possess proteolytic activity. The proteolytic enzymes from different sources have been extensively studied. A detail study of various proteinases has indicated their involvement in many cellular functions such as proteolysis including intracellular protein turnover, digestion, protein translocation, sporulation and germination. Proteinases are primarily involved in the digestion of the nutrient proteins into small peptides and amino acids which then enter the intermediary metabolism and in cellular protein turnover. In addition to the above mentioned functions proteinases have the ability to carry out selective post-translational modification of certain proteins by limited proteolysis such as activation of hormones which indicates their

role in regulatory processes. Proteolysis plays a role in pathogenesis: in the penetration of host organisms, in encountering host defence mechanism and in nutrition during infection. An understanding of cellular functions of proteolytic enzymes and their mechanism of action may provide valuable information, on the basis of which proteolysis might be controlled.

Proteinases are classified in a number of ways on the basis of their in vitro properties such as the pH range over which they are active (acid, neutral and alkaline), their ability to hydrolyze specific proteins or their similarity to well-characterized proteinases such as pepsin, trypsin, chymotrypsin or mammalian cathepsins. The commonly adopted and the most satisfactory classification of proteinases is that proposed by Hartley (1). This is based on their catalytic mechanism. This classification scheme by Hartley forms the basis for Enzyme Commission classification. Many proteolytic enzymes from different sources show similarities in mechanism of action on various substrates. On this basis, Hartley (1) proposed the following classification scheme for proteolytic enzymes. Accordingly, proteolytic enzymes can be classified into four main groups as follows:

(a) Serine Proteinases: The serine proteinases comprise of a large group of enzymes which is distinguished by the presence of a reactive serine residue at the active site. Their hydrolytic activity is inhibited by the reaction of this serine residue with DFP and PMSF. This group includes the proteinases trypsin, chymotrypsin, subtilisins, thrombin, plasmin and elastase.

(b) Thiol Proteinases: In thiol proteinases, a sulphydryl group is present at the active site. This group includes enzymes such as papain, bromelain, ficin and cathepsin B_1.

(c) Metallo Proteinases : This group requires metal ions for their activity and includes carboxypeptidases, aminopeptidases and dipeptidases which are generally exopeptidases. Carboxypeptidases contain Zn^{2+} and aminopeptidases contain Mg^{2+}, Mn^{2+} or Zn^{2+}.

(d) Acid (aspartic) Proteinases: Acid proteinases are characterized by their activity and stability at low pH values and insensitivity to metal chelating agents, phosphorylating agents and thiol reagents. Aspartic acid residue is involved in the hydrolysis by this group of enzymes such as pepsin, gastricin, rennin, cathepsin D and proteinases from species of

Aspergillus, Penicillium, Paecilomyces and Rhizopus.

Proteolytic enzymes find extensive applications in industries such as laundry , leather industry, cheese making etc. In the field of clinical medicine they are used as digestive aids, for wound debridement, burns, ulcers, chest disease, acne and skin cleansing, fibrinolysis, anti-inflammatory agents and drug potentiators. Proteinases, therefore,are one of the most important enzymes from an economical point of view. Proteinases account for almost 60% of the total enzyme sales. Their applications are discussed in detail in Section VI.

Proteinases for commercial use have been traditionally obtained from mammalian system, plants and microorganisms. The animal and plant proteinases such as calf rennet, pancreatic proteinases, malt proteinases and papain are widely used commercially in cheese manufacture, bating of hides, brewing, protein hydrolysis and meat tenderization. Due to the commercial importance and limited availability of these enzymes from the above sources it became imperative to look for microbial sources. This was mainly because of the ease of handling microorganisms and large scale production of the enzymes from them.

The work described in this thesis pertains to extracellular proteinases from a fungal source viz. Conidiobolus sp. This enzyme is of the alkaline serine proteinase type. Hence the literature survey is restricted mainly to the extracellular alkaline proteinases from fungal sources. Only a passing reference is made on proteinases from bacterial sources except subtilisins. Details on subtilisins are also given because of their significant similarity with the Conidiobolus enzyme.

Microbial alkaline proteinases are reported to possess some general properties such as a serine residue at active site, alkaline pH optimum, sensitivity to organophosphorus reagents (e.g. DFP) and esterolytic activity. Generally they are not inhibited by metal chelating and thiol reagents.

SECTION II

DISTRIBUTION AND ISOLATION OF ALKALINE PROTEINASES

A. Bacterial Alkaline Proteinases

Most of the bacterial alkaline proteinases are extracellular and are obtained from the culture medium. Isolation and characterization of these enzymes have been reported from different genera of bacteria such as *Bacillus* (2 - 6), *Arthrobacter* (7 - 12), *Achromobacter* (13, 14), *Bacteroides* (15, 16), *Streptomyces* (17 - 33), *Actinomycetes* (34, 35), *Thermoactinomyces* (36 - 40) and *Serratia* (41 - 46).

Among the bacterial alkaline proteinases, subtilisins produced by different species of *Bacillus* have been studied in detail. Many Laboratories have reported the isolation, crystallization and characterization of various subtilisins named subtilisin Carlsberg (47 - 50), subtilisin BPN' (51) and subtilisin Novo (52). The latter is now available commercially under the trade name 'Alcalase'. Other than these enzymes, several alkaline proteinases from *Bacilli* have also been reported. Among these, the alkaline proteinase from *B. subtilis* var. *amylosacchariticus* (53), *B. subtilis* var. DY (54 - 58) belong to the subtilisin group. Keay and Moser (59, 60) have purified the alkaline

proteinases from B. subtilis (NRRL B 3411), B. licheniformis and B. pumilis. A subtilisin-like proteinase has been isolated by Rappaport and his colleagues (61, 62) from a transformable strain of B. subtilis. This proteinase has an unusually high molecular weight of 166 000 and is probably composed of several subunits.

B. Fungal Alkaline Proteinases

Alkaline proteinases are widely distributed among molds and fungi. Most of these enzymes are extracellular with few exceptions. Molds and fungi are used in the production of proteinases because of ease in isolation, high productivities and good yields. Among the fungal proteinases, those from Aspergilli have been extensively studied. Alkaline proteinases have been isolated and characterized from many fungi and molds such as Aspergillus oryzae (63 - 70), Aspergillus flavus (71, 72), Aspergillus fumigatus (73, 74), Aspergillus sydowi (75), Aspergillus sojae (76, 77), Aspergillus candidus (78 - 80), Aspergillus sulphureus (81), Aspergillus ochraceus (82), Penicillium notatum (83), Penicillium cyaneofulvum (84 - 88), Penicillium lilacinum (89), Penicillium roqueforti (90), Penicillium caseicolum (91), Alternaria tenuissima (92 - 94) Alternaria alternata (Fr.) Kiessl (95, 96), Gliocladium

roseum (97), Scopulariopsis brevicaulis (98), Fusarium semitectum (99), Fusarium vasinfectum (100), Cephalosporium sp. (101 - 105), Torula thermophilia (106), Acremonium kiliense (107, 108), Acremonium chrysogenum (109), Malbranchea pulchella var. sulfurea (110 - 113), Sorangium sp. (114 - 117) and Phymatotrichum omnivorum (118 - 120).

Alkaline proteinase production by fungi belonging to phycomycetes has been little investigated. Few published reports are available on enzyme production as well as characterization of purified alkaline proteinases from these groups. A patent granted to American Cynamid Company (121) describes alkaline protease production by Entomophthora, Basidiobolus and Conidiobolus species. A.G. Jonsson (122) has reported proteolytic activity by Entomophthora species in submerged cultures, most of which are insect pathogens. Alkaline proteinase is also reported from Entomophthora coronata by Hurion and his coworkers (123). Tokuyama and Asano (124, 125) have shown proteinase production by a Conidiobolus sp. related to Conidiobolus adiaeretus which was purified and characterized. An extracellular proteinase accompanied by chitin binding hemagglutinin of Conidiobolus lamprauges (126) was partially purified and characterized to be alkaline proteinase.

Purification

Most of the proteinases have been purified by conventional chromatographic procedures. An alkaline proteinase from a crude preparation of A. flavus was isolated by adsorption on CM-Sephadex at pH 4.5 followed by desorption at pH 7.0. Further, chromatography on DEAE-Sephadex in phosphate buffer at pH 6.0 gave a 120-fold purified enzyme preparation (71). The purified proteinase was homogeneous on disc gel electrophoresis, immunoelectrophoresis and sedimentation analysis in the ultracentrifuge (71). Impoolsup and his coworkers purified an alkaline and a neutral proteinase from wheat bran koji media in solid state fermentation of A. flavus var. columnaris by using ammonium sulfate precipitation and chromatography on DEAE-Sephadex A-50, CM-cellulose and Sephadex G-100 (72). An alkaline proteinase of A. sojae was obtained in purified form by (i) batchwise treatment with ion-exchange resin Duolite CS 101; (ii) fractional precipitation with ammonium sulfate; (iii) precipitation with acetone; (iv) column chromatography on DEAE-cellulose and (v) gel filtration with Sephadex G-100. The enzyme was homogeneous when analyzed by paper and moving boundary electrophoresis, and by ultracentrifugation analysis (76).

Five different alkaline proteinases have been isolated from A. oryzae by different workers :

Subramanian and Kalnitsky (65) obtained an alkaline proteinase named aspergillopeptidase B from the culture filtrate of A. oryzae by a five step procedure. These steps were (i) dialysis; (ii) batchwise treatment with ECTEOLA-cellulose; (iii) fractional precipitation with ammonium sulfate (75 - 85% saturation); (iv) chromatography on Amberlite CG 50 at pH 6.5 and then (v) chromatography on Amberlite CG 50 at pH 7.5. Subramanian and Kalnitsky (65) obtained 55-fold purification with 14% recovery. This proteinase was homogeneous on paper and moving boundary electrophoresis and by ultracentrifugation analysis. An alkaline proteinase of A. oryzae, called aspergillopeptidase C was purified from culture filtrate by dialysis, precipitation with acetone, separation by carrier free electrophoresis and fractionation with acetone to get 40-fold purification (66). The purity of the proteinase was examined by ultracentrifugation, disc gel electrophoresis and immunoelectrophoresis. These methods showed the proteinase preparation to be homogeneous (66, 127).

Bergkvist (63) isolated three alkaline proteinases from A. oryzae culture filtrate in following steps:

(i) CM-cellulose treatment at pH 5.5 to the tannin-precipitated enzyme; (ii) desorption of adsorbed Proteinase I at pH 7.0; (iii) adjustment of filtrate obtained in Step (i) to pH 4.5; (iv) repetition of Step (i) at pH 4.5 to get Proteinase II; (v) repetition of Step (i) at pH 3.0 to get Proteinase III, and (vi) finally each enzyme fraction was purified on DEAE-cellulose column separately. Proteinase I was eluted from the column with 0.01 M sodium phosphate buffer, pH 6.0; Proteinase II with 0.1 M sodium phosphate buffer, pH 6.0 and Proteinase III with 0.5 M sodium phosphate buffer, pH 6.0. All the three proteinases were found homogeneous by ultracentrifugation and electrophoretic methods (63). An alkaline proteinase of A. sydowi (75) was purified 4.5 fold from the culture filtrate by fractionation with ammonium sulfate, treatment with acrynol and Alumina gel C_r and then DEAE-Sephadex column chromatography. The enzyme was finally crystallized in the form of needles. This preparation of alkaline proteinase was homogeneous by ultra-centrifugation and polyacrylamide gel electrophoresis.

The isolation procedures and evidences for homogeneity for the alkaline proteinases from A. candidus (78), A. sulphureus (81), A. fumigatus (74), A. tenuissima (94), P. cyaneofulvum (85 - 87),

P. notatum (83), G. roseum (97) and S. brevicaulis (98) are described in literature.

Tokuyama and Asano (125) purified and crystallized the alkaline proteinase from Conidiobolus sp. The culture filtrate was dialyzed against running water for one day. The dialyzed crude enzyme solution was saturated to 75% with ammonium sulfate at pH 8.0. The precipitate obtained was collected by centrifugation at 3000 x g for 30 min. and dissolved in distilled water followed by dialysis for 24 h against water. To this enzyme solution, an equal volume of cold acetone was added to precipitate the enzyme which was then collected by centrifugation at 5800 x g for 30 min. It was dried in a vacuum desiccator. The dry crude enzyme was dissolved in 0.02 M potassium phosphate buffer, pH 8.0. The enzyme was then adsorbed on DEAE-Sephadex A-50 column equilibrated with 0.02 M potassium phosphate buffer, pH 8.0 and subsequently eluted with a linear gradient of 0.0 to 0.3 M NaCl in 0.02 M potassium phosphate buffer, pH 8.0. The fractions containing protease activity were pooled and dialyzed. The dialyzed enzyme solution was lyophilized and a crystalline alkaline proteinase was obtained.

The alkaline proteinase obtained was homogeneous as shown by gel filtration, polyacrylamide gel electrophoresis and ultracentrifugation analysis (125).

SECTION III

PROPERTIES OF ALKALINE PROTEINASES

A. Bacterial Alkaline Proteinases

Properties of subtilisin Carlsberg, BPN' and Novo are described here.

(a) Physical properties of subtilisins

1. Molecular weight and physical constants. Molecular weights of subtilisin Carlsberg, BPN' and Novo are in the range of 26 000 - 28 000 (Table I). The isoelectric points of subtilisins were found to be in the alkaline region. The molecular weights, physical constants and isoelectric points of subtilisins are summarised in Table I.

2. Stability. Subtilisin Carlsberg is more stable at acidic pH (pH 5.3 to 6.5) than at alkaline pH values (pH 8.0 to 9.5), since autolysis of the enzyme occurs at higher pH values. The enzyme loses activity below pH 5.0. It is stable for several months at room temperature in glycerol solutions and at -10°C in lyophilized state. Subtilisin BPN' exhibits the same stability characteristics as subtilisin Carlsberg and is stabilized by calcium, ammonium, sodium or cobalt salts, even at a concentration of 0.001 M (135). The stability properties of subtilisin Novo are similar

TABLE I : PHYSICOCHEMICAL PROPERTIES OF MICROBIAL ALKALINE PROTEINASES

Property	Subtilisin Carlsberg (50,128-130)	Subtilisin BPN' (131-133)	Subtilisin Novo (52,134)	Aspergillopeptidase B (159)	Aspergillopeptidase C (127)	Alkaline proteinase of A. tenuissima (94)	A. flavus (71,160)	A. sojae (76,161)	A. oryzae (68)	A. candidus (78,79)
pI	9.4	7.8	9.1	-	-	>10.5	-	5.1	-	4.9
$E^{1\%}_{280\ nm}$	9.6	11.7	11.7	9.0	-	13.3	9.04	8.98	7.81	-
$S_{20,w}$	2.85	2.77	-	-	-	2.35	3.05	2.82	-	3.0
Partial specific volume[a] ($\bar{v}$)	0.725	0.731	0.731	0.682 (0.72)	0.70	0.706	-	0.726	-	0.70
Molecular weight										
Ultracentrifugation	27 400	26 000	-	18 000	20 500	23 000	23 620 to 27 180	22 600	-	-
Amino acid analysis	27 277	27 532	27 532	17 700	19 650	24 750	18 000	25 750	-	22 000
Gel filtration	-	-	-	-	-	-	19 800	25 000	23 000	23 000

[a]Calculated from amino acid analysis.

(-) indicates that the data is not cited in the reference.

to other subtilisins. Gaunaris and Ottesen (136) have reported that all subtilisins are resistant to denaturing agents such as 6M urea and 50% ethanol. These enzymes are stable to a variety of detergents such as SDS and sodium tripolyphosphate (137).

(b) Chemical properties of subtilisins

1. Amino acid composition and terminal amino acid residues. Amino acid compositions of subtilisins are described in Table II. These enzymes exhibit high similarity in amino acid composition. The amino acid composition of subtilisins shows the absence of disulfide bond, presence of methionine and high contents of glycine, alanine, serine and valine. The amino terminal residue of subtilisin Carlsberg, BPN' and Novo is identical, viz. alanine.

2. Active site studies. Both subtilisin Carlsberg (50) and BPN' (138) are inactivated by DFP indicating the presence of a serine residue at the active site. One mole of DFP binds with one mole of subtilisin (128, 139). Studies by Sanger and Shaw (140), and Noller and Bernhard (141) showed the active site sequence to be Asn-Gly-Thr-Ser*-Met-Ala. The active site is located in a long sequence of constant residues from 218 to 240 (142). Oosterbaan

TABLE II : AMINO ACID COMPOSITION OF SUBTILISINS AND FUNGAL ALKALINE PROTEINASES

Amino acid	Subtilisin		A. oryzae[a]	A. oryzae[b]	A. oryzae	A. flavus	A. sojae	A. sulphureus	A. candidus	A. tenuissima
	Carlsberg (129)	BPN'/Novo (132)/(134)	(159)	(127)	(68)	(71)	(76)	(81)	(78)	(94)
Lysine	9	11	11-12	12	17	11	14	12	14	1
Histidine	5	6	4	4	5	3 - 4	5	5	4	1
Arginine	4	2	2	3	3	2	3	4	3	10
Aspartic acid[c]	28	28	21	21-22	26	21	31	27	25	18
Threonine	19	13	11	13	14	11	18	19	15	33
Serine	32	37	19	23	24	20	28	20	23	35
Glutamic acid[c]	12	15	12	13	14	12 - 13	19	12	15	12
Proline	9	14	4	5-6	7	4 - 5	6	5	7	4
Glycine	35	33	19	21	25	20	27	31	22	55
Alanine	41	37	23	23	29	23	32	31	24	22
Cysteine	0	0	0	0	-	0	2	0	0	6
Valine	31	30	15	16	16	15	18	21	16	19
Methionine	5	5	0	1	1	1	2	0	2	0
Isoleucine	10	13	9-10	10-11	12	9 - 10	14	10	11	7
Leucine	16	15	9	10	12	9	14	14	10	7
Tyrosine	13	10	5	4-5	7	5	8	7	6	13
Phenylalanine	4	3	5	6	6	5	7	5	7	6
Tryptophan	1	3	2	2	2	2	2	2	2	4
Total	274	275	171-173	187-191	220	173-177	250	225	206	253
Molecular weight	27 287	27 532	17 200	19 650	18 000	18 000	25 750	-	22 000	24 750

[a]Aspergillopeptidase B

[b]Aspergillopeptidase C

[c]Values of aspartic acid and glutamic acid include aspargine and glutamine, respectively.

(-) indicates that the data is not cited in the reference

and Cohen (143) demonstrated by photooxidation studies, the involvement of a histidine moiety at the active site of subtilisins. Subsequently, Noller and Bernhard (141) suggested on the basis of their studies on the sequence around the reactive site of subtilisins, that a histidine residue may be getting acylated prior to acylation of reactive serine group. They concluded that the catalytically active histidine residue is in proximity to the serine group. Further, Glazer (144) provided kinetic evidence for involvement of a histidine moiety which showed that protonation of a group with pK' of 6.58 resulted in inactivation of subtilisin Carlsberg. Similar results were obtained with subtilisin BPN' and Novo (145). Shaw and Rascica (146) synthesized a highly reactive phenylalanine derivative, benzyloxycarbonyl-L-phenylalanine-bromomethyl ketone (ZPBK) and demonstrated that this reagent reacts stoichiometrically with subtilisin BPN' with loss of enzyme activity. The reaction was specific and resulted in the loss of a single histidine residue. Subsequent to this work Markland et al. (147) isolated and identified a covalently labelled peptide from ZPBK-reacted-subtilisin Carlsberg. These studies lead to the conclusion that His_{64} is the active histidine. Similarly, a peptide was isolated from

subtilisin BPN' containing the labelled histidine residue and was found to be His_{64}. Further Wright et al. (148) on the basis of X-ray analysis of subtilisin BPN' demonstrated that His_{64} and the active serine 221 are in close proximity in the three dimentional structure of subtilisin.

(c) Primary structure of subtilisins

The amino acid sequences of subtilisin BPN' and Carlsberg have been determined by Smith and his coworkers (142, 149). The enzymes consisted of a single polypeptide chain and were devoid of cysteine and cystine. From the peptide mapping (150) and chromatographic separation and analysis (134) of tryptic digest of subtilisin Novo, it was found that subtilisin Novo appeared to be identical to subtilisin BPN' in all respects but, they were different from subtilisin Carlsberg. Smith and his coworkers (142, 149) showed that subtilisin BPN' consists of a single polypeptide chain of 275 amino acid residues devoid of any disulfide bridges. The serine residue at active site is at position 221 and the sequence around active serine is Thr-Ser*-Met-Ala. Several repeat sequences were found in the polypeptide chain. There were many di-, tri- and in one case tetra-peptide repetitions of the same residues.

Subtilisin Carlsberg was found to consist of a single polypeptide chain containing 274 amino acid residues. It differs from subtilisin BPN' in total 85 positions including one deletion at position 56 in subtilisin Carlsberg (142). The tryptic peptides of the Carlsberg and BPN' enzymes (142) are different, although the total base composition of the two enzymes (11 lysine and 2 arginine residues in subtilisin BPN' and 9 lysine and 4 arginine residues in subtilisin Carlsberg) are identical. This is because of the difference in positions of these basic amino acids in the sequence. The four methionine residues are in the same positions (viz. residues 119, 124, 199, and 222) in all the three subtilisins.

Amino acid compositions, active site sequences and other properties mentioned above indicate that the three dimensional structure of these enzymes is very similar, if not identical. The differences between subtilisin Carlsberg and BPN' occurs mainly at the exterior chain segment. The amino acid residues determining the interior conformation of subtilisin BPN' are found in identical positions in subtilisin Carlsberg (148). On this basis Wright et al. (148) have suggested that the two subtilisins may have very similar three dimensional structure.

(d) Substrate specificity of subtilisins

From the hydrolytic studies on various proteins and synthetic substrates it is evident that subtilisins exhibit a broad specificity. Subtilisins are endopeptidases and are capable of hydrolyzing peptide bonds as well as ester bonds. Subtilisins are shown to catalyze the transesterification reactions (151) and not the transpeptidation reactions (145). Like most of the other proteolytic enzymes, subtilisins also catalyze aminolysis reactions (152) such as:

L-Leucine benzyl ester + glycylglycine n-amyl ester $\rightleftharpoons$

L-Leucine glycylglycine n-amyl ester + benzyl alcohol.

1. Protein substrates. Tuppy (153), Haugaard and Haugaard (154) and Meedom (155) have studied, in detail, the hydrolysis of insulin chains by subtilisin Carlsberg. Their work indicates that subtilisin Carlsberg has a broad specificity in the cleavage of peptide bonds. Tuppy (153) used the B-chain of oxidized beef insulin for digestion studies by subtilisin and could separate five free amino acids (Ala, Tyr, Phe, Gln, Leu), nine dipeptides, five tripeptides, three tetrapeptides and other peptides. Haugaard and Haugaard (154) digested

native beef insulin by subtilisin Carlsberg and Meedom (155) used native pork insulin for these studies. Meedom found that 20 out of 49 peptide bonds in porcine insulin were cleaved. From their studies, subtilisin Carlsberg appeared to attack peptide bonds at the carboxyl side of leucine, cysteine, glutamic acid and glutamine little more specifically than other linkages. However many other bonds were cleaved on extensive hydrolysis. Tuppy (153) also suggested that the enzyme can hydrolyze not only internal bonds but also terminal bonds in peptides, yielding free amino acids. Tuppy (156) in 1953, has reported that the digestion of oxytocin by subtilisin Carlsberg resulted in cleavage at the peptide bond in which carboxyl group is donated by glutamine and leucine.

Subtilisin BPN' also shows a very wide specificity. It can attack about one third of the peptide bonds in casein and one fifth of those in gelatin. Many free amino acids were detected in the casein digestion mixture.

Hydrolysis of insulin with subtilisin BPN' under restricted conditions gave more definite information regarding its specificity. Morihara and Tsuzuki (157) carried out the digestion of oxidized B-chain of insulin for 2 - 3 hours with 0.1% by weight of subtilisin BPN' at room temperature and pH 9.0. They found that

cleavage occurred at the peptide bonds between Gln_4-His_5, Ser_9-His_{10}, Leu_{15}-Tyr_{16}, Tyr_{16}-Leu_{17} and Phe_{25}-Tyr_{26}. This is consistant with the finding that hydrolysis of small ester and amide substrates, such as Acetyl-X-ethyl ester and carbobenzoxy (Cbz)-Gly-X-NH_2, occurs at the carboxyterminal side of residue X,where X represents aromatic or apolar residues such as L-tyrosine, L-phenylalanine and L-leucine. When initial stages of degradation of insulin were investigated (158), it was found that the peptide bond Leu_{15}-Tyr_{16} was cleaved much faster than any other bond, by both subtilisin Carlsberg and Novo (enzyme to substrate ratio, 1:3960). When the enzyme concentration was increased, the extent of hydrolysis was also increased which resulted in small fragments. Analysis of these fragments demonstrated that hydrolysis by subtilisin Carlsberg takes place at peptide bond between Gln_4-His_5, Ser_9-His_{10}, Leu_{11}-Val_{12} Leu_{15}-Try_{16}, Try_{16}-Leu_{17}, Leu_{17}-Val_{18} and Tyr_{26}-Thr_{27}. Similar results were obtained with subtilisin Novo but the rate of hydrolysis was different. The peptide bonds Ser_9 - His_{10} and Tyr_{26}-Thr_{27} were hydrolyzed faster by subtilisin Novo than by subtilisin Carlsberg, while Gln_4-His_5 and Leu_{11}-Val_{12} bonds were cleaved faster by subtilisin Carlsberg than by Novo enzyme.

2. Synthetic substrates. Subtilisins are capable of hydrolyzing a variety of esters of amino acids. They show wide specificity towards synthetic substrates. Ottesen and Spector (52), and Hunt and Ottesen (150) observed that N-benzoyl-L-tyrosine ethyl ester (BTEE) was a better substrate than N-benzoyl-L-leucine ethyl ester for subtilisin BPN' and Novo but the rate of hydrolysis of both the substrates was same for subtilisin Carlsberg. They also found that subtilisin Carlsberg was more active on both substrates than subtilisin BPN' and Novo. Glazer (144), Barel and Glazer (152), and Myers and Glazer (145) have studied the kinetics of hydrolysis of various N-acetyl amino acid esters by subtilisin Novo, Carlsberg and BPN'. All the subtilisins show markedly higher activity on esters of aromatic amino acids than those of aliphatic amino acids. It was observed that the V_{max} values for subtilisin Carlsberg were higher than those for other subtilisins with N-acetyl amino acid esters. The highest V_{max} values for subtilisin Carlsberg suggest that its deacylation rate is faster than the other subtilisins. This was further supported by Barel and Glazer (152) who observed that the deacylation rate of N-trans-cinnamoyl subtilisin Carlsberg was higher than that of subtilisin Novo. Although subtilisin Carlsberg has higher V_{max}

for N-acetyl amino acid esters than subtilisin Novo, the K_m values for the substrates and K_i values for inhibitors were found similar for all subtilisins. This indicated that all subtilisins possess similar substrate binding site, even though their amino acid sequences are different. Glazer (144) observed that even if subtilisin Carlsberg has higher V_{max} values with N-acetyl amino acid esters than subtilisin Novo, both enzymes have similar V_{max} values with free amino acid esters (152). This suggests that the binding site of subtilisin Carlsberg is less polar than that of the Novo enzyme and hence more sensitive to the polar α-amino group in the free amino acid esters.

Some aromatic compounds such as phenol, indole, hydrocinnamate and indole propionate were competitive inhibitors of the hydrolysis of N-acetyl-L-tyrosine ethyl ester (ATEE) but were non-competitive inhibitors with N-benzoyl-L-arginine ethyl ester (BAEE) (144). This suggests that these two ester substrates are bound in different manner at the active sites. Morihara and Tsuzuki (157) have reported that subtilisin BPN' hydrolyzes the synthetic substrates of the type Cbz-Gly-X-NH_2, where X can be Ala, Leu, Phe, Tyr, Ser, or Trp. Benzoyl-Tyr-NH_2 is not hydrolyzed by subtilisin BPN'.

B. Fungal Alkaline Proteinases

(a) Physical properties of fungal alkaline proteinases

1. Molecular weight, physical constants and isoelectric point. The molecular weights of the various alkaline proteinases from fungi are within the range of 20 000 - 25 000 (Table I). A few enzymes, however, were reported to have higher molecular weights. *A. oryzae* EI 212 (69), *A. oryzae* OUT 5038 (64), *P. cyaneofulvum* (85), *Phymatotrichum omnivorum* (120) alkaline proteinases have molecular weights of 35 000, 52 000, 45 000 and 33 000, respectively. An alkaline proteinase of *T. thermophila* (106) is reported to be present both in monomeric (MW 16 200) and dimeric (MW 33 700) form, the latter being more stable.

The isoelectric points for proteinases from *A. sojae* (76), *A. candidus* (79) and *A. tenuissima* (94) were 5.1, 4.9 and above 10.5, respectively.

Molecular weights of other proteinases and physical constants of some of these enzymes are given in Table I.

2. Stability. Most of the alkaline proteinases from fungal sources were found to be stable in the pH range of 5.0 to 10.0 at low temperatures and unstable at temperatures above 55° - 60°C. Alkaline proteinases from *A. sydowi* (75), *A. candidus* (79), *A. fumigatus* (74)

and A. sojae (162) were stabilized against thermal inactivation by Ca^{2+}.

Tokuyama and Asano (125) have reported that the alkaline proteinase of Conidiobolus sp. is stable at pH 7.0 - 8.0 and loses its activity completely below pH 5.0 and above pH 12.0 when incubated at 5°C for 24 h. The enzyme was stable upto 40°C (pH 8.0) but there was a sharp fall in its activity above 40°C. Table III shows pH and temperature stability characteristics of various alkaline proteinases.

(b) Chemical properties of fungal alkaline proteinases

1. Amino acid composition, terminal amino acid residues and non-protein components. The amino acid compositions of various alkaline proteinases are summarized in Table II. Aspergillopeptidase B from A. oryzae (159), aspergillopeptidase C from A. oryzae (127), alkaline proteinases from A. flavus (71) and A. candidus (78) have similar amino acid compositions. These enzymes, except from A. sojae (76) and A. tenuissima (94) do not contain cysteine residues. Presence of methionine is reported in some of these alkaline proteinases (68, 71, 76, 78, 127). The alkaline proteinase of A. tenuissima (94) showed difference in

TABLE III : ENZYMATIC PROPERTIES OF FUNGAL ALKALINE PROTEINASES

Enzyme	Optimum pH[a]	Optimum Temp. (°C)	Stability pH	Stability Temp.	Reference
A. flavus	7.0 - 9.0	-	-	-	71
A. flavus var. columnaris	8.0 - 11.0	50 - 55	-	-	72
A. sojae	11.0	-	4.5 - 10.0 at low temp. and 6.0 at 50°C	Unstable at 60°C for 10 min, stabilized by Ca^{2+} (2×10^{-3}M)	162
A. oryzae					63
Proteinase I	-	-	5.0 - 8.5	All three inactivated completely at 60°C in 2 min, slowly at 40°C	
Proteinase II	-	-	4.5 - 10.5		
Proteinase III	-	-	3.0 - 6.0		
A. oryzae (aspergillo-peptidase B)	10.3 - 10.4	-	-	Inactivation starts at 45°C, irreversible, pH dependent, completely inactivated at 55°C at any pH	163
A. oryzae (aspergillo-peptidase C)	9.0 - 10.0	-	-	-	127
A. oryzae	9.3 - 9.5	40 - 42	5.0 - 8.0 at 40°C, 30 min	Stable at 45°C (pH 7.0, 15 min)	67
A. oryzae 460	10.5 11.0(polyLys) 4.0(polyGlu)	-	5.0 - 8.5 at 37°C, 3 h	Stable upto 50°C, unstable above 60°C	68
A. oryzae EI 212	10.0 10.0 (Hb)	-	6.0 - 8.0 at 30°C, 60 min	More stable at 4°C	69
A. sulphureus	7.0 - 10.0	50 at pH 7.0	6.0 -11.0 at 5°C	Stable below 37°C	81
			6.0 - 10.0 at 37°C	Unstable above 50°C (10 min)	
A. sydowi	8.0	40	6.0 - 9.0 at 5°C 7.0 at 37°C	5% activity lost at 45°C in 10 min, 95% activity lost at 50°C, Ca^{2+} protects	75
A. candidus	11.0 - 11.5	47 at pH 7.0	5.0 - 9.0 at 30°C, 6.0 at 50°C	Most stable at 40°C lost completely at 60°C in 10 min, Ca^{2+} protects to some extent	79
A. fumigatus	7.5, 10.0 4.0, 9.0(Hb)	40	-	Half inactivation at 50°C completely inactivated at 60°C, Ca^{2+} protects	74
A. alternata(Fr.) Kiessl	8.0 - 9.5	-	Wide range	-	95
A. tenuissima	9.5	-	-	-	92
P. cyaneofulvum	9.5 - 11.0 6.5 - 8.5 (Hb)	-	4.0 -11.0	Inactivated at 60°C in 15 - 20 min	85
S. brevicaulis	10.5 - 11.0 7.0 - 9.5 (Hb) 6.0 - 8.0(gelatin)	-	-	-	98
G. roseum					97
Proteinase I	11.0	45	-	-	
Proteinase II	10.0	45	-	-	
Conidiobolus sp.	9.0	40 at pH 9.0 50 at pH 8.0	7.0 - 8.0 at 5°C, 24 h	Stable upto 40°C at pH 8.0 and 9.0	125

[a] Optimum pH mentioned is generally for casein as a substrate unless otherwise stated in parenthesis.

(-) indicates that the data is not cited in the reference.

the amino acid composition when compared with other enzymes.

Aspergillopeptidase B from A. oryzae (159) and the alkaline proteinase from A. flavus (71) and A. sojae (76) have identical N and C terminal amino acid residues viz. glycine and alanine, respectively.

Small amount of carbohydrates was detected in aspergillopeptidase B (163) and alkaline proteinase of A. flavus (71). The A. sojae alkaline proteinase was reported to be devoid of any sugar or phosphate compounds (76).

2. Active site studies. Alkaline proteinases from fungi are inhibited by organophosphorus compounds such as DFP and PMSF indicating the role of an active serine in the mechanism of action of these enzymes. This reaction of organophosphorus compounds with enzyme molecule is stoichiometric. The active site sequence of alkaline proteinase from A. flavus was found to be Gly-Thr-Ser*-Met-Ala (164) and that of A. oryzae was Thr-Ser*-Met-Ala (165). On the basis of kinetic studies Turkova (166) has determined the role of active histidine in the mechanism of action of the alkaline proteinase from A. flavus.

(c) Enzymatic properties of fungal

1. Optimum pH and temperature. Alkaline proteinases show maximum activity at higher pH values. Hydrolysis of proteins, peptides and synthetic substrates occurs over a wide pH range (pH 7.0 - 11.0). However, some enzymes are reported to have a fairly sharp pH optima (64, 65, 67 - 69, 75, 79, 92, 97, 98, 127, 162, 163). The optimum pH values for various alkaline proteinases are given in Table III. For some alkaline proteinases the pH optima are substrate dependent. The alkaline proteinase of A. fumigatus showed two pH optima, pH 7.5 and 10.0 for casein and pH 4.0 and 9.0 for hemoglobin hydrolysis (74). The pH optimum for P. omnivorum proteinase was reported to be around pH 5.0 for hemoglobin and pH 7.0 - 8.0 for ATEE (120). The alkaline proteinase of P. cyaneofulvum exhibited an optimum activity at pH 9.5 - 11.0 for casein (85), pH 4.3 for poly-L-glutamic acid and pH 10.7 for poly-L-lysine (87).

The alkaline proteinase of Conidiobolus sp. (125) showed maximum activity at pH 9.0. Optimum temperature for proteolytic activity of the enzyme was pH dependent, being 50°C at pH 8.0 and 40°C at pH 9.0 (125). The optimum temperatures for some of the alkaline proteinases are given in Table III.

2. Substrate specificity. Alkaline proteinases, in general, show wide specificity, however, careful study has shown preference of some substrates or amino acid residues over others. Martin and Jonsson (74) have reported that the alkaline proteinase of A. fumigatus hydrolyzed poly-L-glutamic acid and poly-L-lysine optimally at pH 4.0 and 10.0, respectively; hydrolysis of poly-L-glutamic acid being ten times that of poly-L-lysine. Products of hydrolysis were found to be peptides, without any free amino acid formation. The enzyme also showed milk clotting activity, however, on prolonged incubation the clot was dissolved. Aspergillopeptidase C of A. oryzae (127) could digest denatured serum albumin and β-lactoglobulin. It had no action on the native proteins. Studies on oxidized A and B chains of insulin suggested that the enzyme attacked preferentially those bonds, in which an acidic amino acid or its amide participates. Serine bonds also appeared to be broken relatively quicker. Aspergillopeptidase C could not split the peptide bonds of imino acids i.e. of proline and hydroxyproline. The enzyme, however, could digest native collagen.

Turkova and Mikes (167) showed, by digestion studies of synthetic peptides, that the alkaline proteinase of

A. flavus cleaved the peptide bonds at the carboxyl side of phenylalanine, leucine, tyrosine, aspartic acid and methionine. They also found that the proteinase cleaved the bond at the carboxyl side of glycine, if the carboxyl group of the adjacent phenylalanine residues was bound by a peptidic or atleast pseudopeptidic residue but could not cleave when the phenylalanine residue was esterified. Turkova and Mikes (167) also studied the digestion of B-chain of oxidized insulin and found that the specificity of A. flavus proteinase resembles with that of A. oryzae proteinase reported by Sanger et al. (168) but it differs from the specificity of aspergillopeptidase of A. oryzae reported by Nordwig and Jahn (127). The specificity of A. flavus (167) corresponds to that of A. oryzae proteinase reported by Morihara and Tsuzuki (64).

A comparative study was made on specificities of five alkaline proteinases from B. subtilis (Nagarase), S. fradiae, and A. oryzae by Morihara and Tsuzuki (64) using synthetic substrates and oxidized insulin B-chain. These proteinases preferentially hydrolyzed linkages involving the carboxyl group of tyrosine, phenylalanine, leucine etc. They also showed esterase activity on BAEE, ATEE, Ac-Lys-OMe, Ac-Phe-OEt and Ac-Try -OEt.

The digests of oxidized B-chain of insulin by the alkaline proteinases from A. oryzae and A. flavus showed identical peptide maps indicating the similarity in their specificity (169). The alkaline proteinase of A. oryzae 460 isolated by Nakadi et al. (68) did not show either carboxypeptidase or aminopeptidase activity but could hydrolyze poly-L-glutamic acid and poly-L-lysine optimally at pH 4.0 and 11.0, respectively. The enzyme did not show activity on N-benzoyl-DL-arginine p-nitroanilide (BAPNA). It is reported to be an endopeptidase and not an exopeptidase.

Gertler and Hayashi (170) showed that the alkaline proteinase of A. sojae could hydrolyze the ester bond of acetyl-L-alanyl-L-alanyl-L-alanine methyl ester which is a highly specific ester substrate of elastase (171). They observed that this proteinase was inactive on Congo red elastin, an insoluble substrate of elastase. The explanation was that this enzyme was not adsorbed on elastin at pH range 5.5 - 9.0 in low ionic strength, since the alkaline proteinase of A. sojae is an acidic protein with pI of 5.1. The specificity of the alkaline proteinase from A. sojae was further investigated by using synthetic substrates. The enzyme hydrolyzed peptide bonds involving carboxyl group of leucine, tyrosine, phenylalanine, arginine and lysine.

The enzyme liberated relatively large peptides from natural proteins with traces of free amino acids suggesting that the enzyme is of endo type. It showed low esterase activity on BAEE, ATEE and p-toluenesulfonyl-L-arginine methyl ester (TAME). Hemoglobin, fibrinogen, fibrin and collagen were found to be good substrates for the enzyme, however, elastin was a poor substrate. Recently, Ichishima et al. (172) have determined the specificity of alkaline proteinase from A. sojae by using oxidized insulin A and B chains and angiotensin I and II peptides. They found that hydrolysis of insulin A chain was observed at three peptide bonds viz. oxidized Cys $(CySO_3H)_{11}$-Ser_{12}, Tyr_{14}-Gln_{15}, and Glu_{17}-Asn_{18}. Hydrolysis of B-chain was mainly observed at Leu_{15}-Tyr_{16} bond and additional four bonds (Glu_{13}-Ala_{14}, Ala_{14}-Leu_{15}, Tyr_{16}-Leu_{17} and Val_{18}-$CySO_3H_{19}$). Hydrolysis of angiotensin I was observed at Tyr_4-Ile_5 and Phe_8-His_9 bonds and that of angiotensin II at Tyr_4-Ile_5.

The alkaline proteinase of P. cyaneofulvum could hydrolyze casein, denatured hemoglobin, gelatin, native bovine serum albumin (BSA) and insulin but not denatured or native ovalbumin. It could coagulate milk (85). Martin and his coworkers studied the digestion of A and B chains of insulin by P. cyaneofulvum protease (86). They observed 10, 14 and 15 sites of cleavage in

B-chain in 42, 210 and 1000 minutes, respectively. Four primary sites of attack were between amino acids 3 and 4, 9 and 10, 15 and 16 and 25 and 26 (or possibly 24 and 25); and many secondary ones were observed. This enzyme was shown not to hydrolyze di- and tri-peptides including leucyl-glycine, leucyl-glycyl-glycine and alanyl-glycyl-glycine indicating the absence of peptidase activity. This alkaline proteinase of P. cyaneofulvum was examined for its mode of action on poly-L-glutamic acid, poly-L-lysine and poly-L-aspartic acid (87). It cleaved poly-L-lysine, poly-L-glutamic acid and poly-L-aspartic acid optimally at pH 10.7, 4.5 and 4.3, respectively. Action on poly-L-lysine and poly-L-glutamic acid was random in nature except near the end of peptide chain. Final products were di-, tri- and tetra-peptides. No monomer products were detected. Poly-L-proline was not hydrolyzed. Poly-L-lysine was more susceptible than poly-L-glutamic acid and poly-L-aspartic acid was least susceptible. S. brevicaulis alkaline proteinase hydrolyzed casein, urea denatured hemoglobin, gelatin, fibrinogen, fibrin, insulin A and B chains but not human serum albumin and ovalbumin (98). It could coagulate milk.

3. Inhibitors. Members of the alkaline proteinase group are inhibited by diisopropyl phospho-fluoridate (DFP) (68, 69, 74, 75, 79, 81, 85, 98, 127, 162, 163) indicating a presence of serine residue at the active site. DFP reacts with the active serine stoichiometrically. These enzymes are also inhibited by other serine specific reagents such as phenyl methanesulfonyl fluoride (PMSF) (72, 98, 120, 127), N-bromosuccinamide (69, 75, 81) and diphenyl carbamyl chloride (127). The microbial alkaline proteinases, in general, are not inhibited by N-p-toluenesulfonyl-L-lycine chloromethyl ketone (TLCK) or N-p-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK) (81, 120, 162). However, these reagents inhibit trypsin (173) and chymotrypsin, specifically. The alkaline proteinases from A. oryzae (127), A. sydowi (75), A. flavus (71), A. sojae (162), A. fumigatus (74), A. sulphureus (81), P. cyaneofulvum (85) and S. brevicaulis (98) were not inhibited by the sulfhydryl reagents, p-chloromercuribenzoate (PCMB), cysteine or KCN.

Among the naturally occurring inhibitors, the potato inhibitor is reported to inhibit the alkaline proteinases of A. oryzae (68), A. sojae (162), A. sydowi (75), A. candidus (79) and G. roseum (97). Ethylene-diaminetetraacetate (EDTA) was not inhibitory to the alkaline proteinases of A. oryzae (68, 127),

A. sulphureus (81), A. fumigatus (74) and P. cyaneofulvum (85) with the exception of the enzyme from P. omnivorum (119, 120). Soybean trypsin inhibitor did not inhibit the enzymes from A. sojae (162), A. oryzae (163) and P. cyaneofulvum (85). A.sojae proteinase is reported to be inhibited by animal serum (162). The alkaline proteinase from A. sydowi (75) and A. sulphureus (81) were reported to have no effect of oxidizing reagents except iodine.

Some of the alkaline proteinases are inhibited by metal ions. Zn^{2+} and Hg^{+} ions were reported to inhibit the proteinase from P. cyaneofulvum, this inhibition was reversed with EDTA (85). A.sojae alkaline proteinase was remarkably labile in the presence of Fe^{2+}, Fe^{3+}, Cu^{2+}, Cu^{+} and Hg^{2+} and less labile in the presence of Ni^{2+}, Ag^{2+}, Hg^{+} and Sn^{2+} (162). Aspergillopeptidase C was inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} (127) but aspergillopeptidase B was not inhibited by Cu^{2+} and Zn^{2+} (163). Zn^{2+}, Ni^{2+} and Hg^{2+} were inhibitory to A. sydowi (75) proteinase but Ca^{2+} and Cu^{2+} were not inhibitory. The alkaline proteinase from A. sulphureus (81) was slightly inhibited by Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Hg^{2+}, and significantly by Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. Three alkaline proteinases of A. oryzae isolated by Bergkrist (63), are different from other alkaline proteinases. Proteinase I was

not inhibited by Ca^{2+} but was considerably inhibited by Ni^{2+}, Cu^{2+} and Zn^{2+}. Proteinase II was completely inhibited by Cu^{2+} and Zn^{2+} and Proteinase III was slightly inhibited by Ca^{2+} and significantly by Ni^{2+}, Cu^{2+} and Zn^{2+}. Proteinase I and III were inhibited by ascorbic acid and sodium dodecyl sulfate (SDS) whereas Proteinase II was not. All the three enzymes were inhibited by laurylamine.

The alkaline proteinase of Conidiobolus sp. (125) was inhibited to 50% of its activity by Al^{3+} and Cu^{2+} whereas it was almost completely inhibited by Ba^{2+}, Ag^{+} and Hg^{2+}. This enzyme was markedly inhibited by $KMnO_4$ and 8-hydroxyquinoline whereas potato inhibitor did not show any inhibitory effect. EDTA, at concentration of 10^{-3}M showed 37% inhibition of the enzyme activity (125).

SECTION IV

MECHANISM OF ACTION OF SERINE PROTEINASES

Various hypotheses have been put forth on the mechanism of action of serine proteinases. On the basis of studies by Wang (174), Blow et al. (175), Caplow (176), Polgar and Bender (177), and Fersht (178, 179) a rigid reactive site model has been proposed for the hydrolysis by serine proteinases in which the histidine residue at active site operates in a fixed steric position both as a general base and as a general acid. Role of imidazole group of histidine as general base - general acid in the reaction by serine proteinases is widely accepted (180,181). Involvement of histidine accounts for the high nucleophilicity of the serine residue at the active site. The involvement of serine and histidine in the catalytic activity of subtilisin (182) and mammalian serine proteinases (175) is already demonstrated and a role of aspartic acid has been postulated (175, 182).

The high reactivity of the serine hydroxyl group at the active site of serine proteinases is mainly because of hydrogen bonding between this serine residue and a nearby histidine residue (174). This histidine residue at the active site is bonded to an aspartic acid residue (175,182) However, Polgar and Bender (177) contradicted this view. By kinetic studies, they showed that subtilisin Novo and Carlsberg and their thiol derivatives in the acyl-enzyme

form possess an imidazole group with a pK$'_a$ of about 7.0 in the active site. This group i.e. the active histidine residue is present in the unsubstituted enzymes. But it was not found in the free thiol enzyme. In the free thiol subtilisin Carlsberg, the pK$'_a$ was shifted to 6.15 and in the thiol subtilisin Novo to 5.5. To explain this difference between subtilisins and their thiol derivatives, they postulated that a hydrogen bond is present between thiol group and the nearby histidine residue in free thiol enzyme which caused shift in pK$'_a$ value. But in the serine enzyme, since there was no change in the pK$'_a$ value between free and acyl-enzyme it was concluded that there was no hydrogen bonding. Kinetic studies with D_2O supported this conclusion (177).

Hydrolysis by serine proteinases occurs via acylation (K_2) and deacylation (K_3) steps according to the equation:

$$EH + RCOX \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} EH.RCOX \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} ECOR + HX$$

$$ECOR + H_2O \underset{K_{-3}}{\overset{K_3}{\rightleftharpoons}} RCO_2H + EH$$

On the basis of pH dependent experiments (183-185), chemical modification (176) and X-ray crystallographic

studies (182) the mechanism of action of serine proteinases is proposed as given in Figure 1. It demonstrates the role of amino acid side chains at the active site in acylation and deacylation of the enzyme. Figure 1 shows the mechanism of formation and hydrolysis of acyl-enzyme as described by Polgar and Bender (177) which also includes the features of mechanism proposed by Wang (174) and Blow et al. (175).

It is generally assumed that formation of a tetrahedral intermediate takes place between hydroxyl group of serine and amide or ester substrate. The substrate binds at the active site of the enzyme in such a position that carbonyl carbon of substrate comes in contact with the hydroxyl oxygen of serine at active site. At the same time, the proton acceptor atom of the leaving group i.e.'X'of the substrate comes in close proximity to the hydroxyl group of the serine residue. This positioning of atoms allows the $N^{\epsilon 2}$ nitrogen atom of the imidazole ring of histidine to interact simultaneously with both the proton donar and acceptor atoms. The transfer of proton of serine hydroxyl group to 'X' of substrate is via $N^{\epsilon 2}$ nitrogen atom of histidine at the active site. A favourable collision between the carbonyl carbon atom of the substrate and the oxygen atom of the serine

Fig. 1 A possible mechanism of action of serine proteinases

(a) Formation of acyl-enzyme intermediate

(b) Hydrolysis of acyl-enzyme intermediate

ES_{tet} and X represent the tetrahedral intermediate and the leaving group of the substrate, respectively.

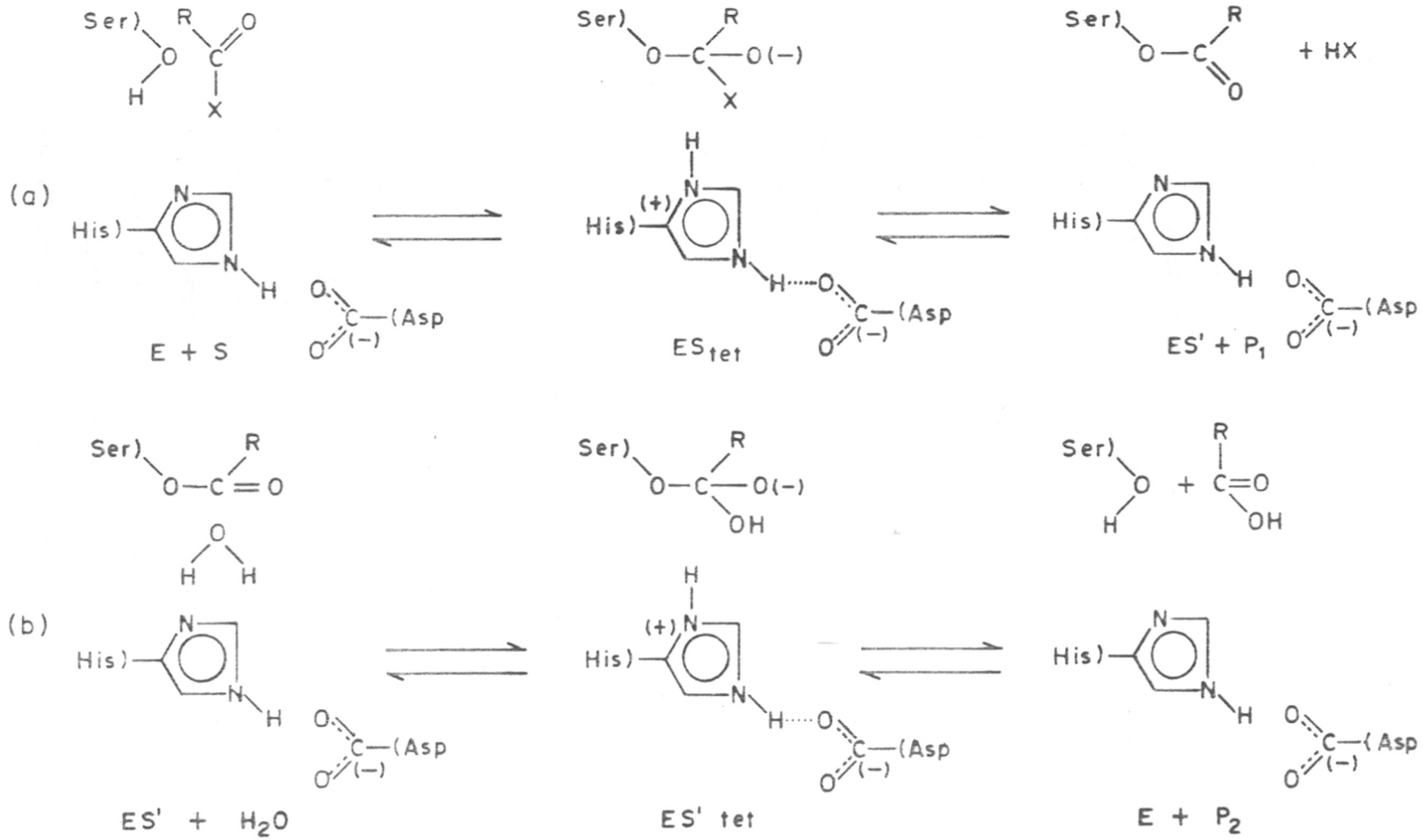


FIG. 1

residue triggers the catalytic reaction. The first step, i.e. bond making is general base catalyzed. In this step, a tetrahedral intermediate 'ES_{tet}' is formed as a result of attack by the serine oxygen on the carbonyl carbon of substrate and this is accompanied by transfer of hydroxyl proton to $N^{\epsilon 2}$ of histidine at the active site.

The second step is general acid-catalyzed by the imidazolium ion formed in the first step. In this step, the bond is broken to release the acyl-enzyme intermediate and a product 'HX'.

The 'charge relay' hypothesis (186) was assumed to account for the catalytic mechanism of serine proteinases. It involved a double proton transfer from 'serine' to histidine and from histidine to aspartate which would relay negative charge from the buried aspartate to the serine side chains during catalysis. Simultaneously, the serine oxygen attacks the substrate and a tetrahedral intermediate is formed. This transforms into an acyl-enzyme which hydrolyzes with the same mechanism. Recently nuclear magnetic resonance studies (187, 188) and neutron diffraction studies (189) contradicted the 'charge relay' hypothesis. Accordingly, the proton is not transferred from histidine to aspartate, rather, an ion pair is formed. Though early quantum chemical calculations supported the His-Asp proton transfer (190 - 192),

recently, it became clear (193, 194) that this was partly due to the inadequacy of methods used to estimate proton affinities of negatively charged species like aspartate. However, it is considered that the electrostatic effect of the proton environment accounts for stabilization of the ion pair (195).

Polgar and Bender (177, 196) have suggested that the possible role of aspartic acid at the active site is to form hydrogen bonding to histidine at the active site which thus possibly stabilizes the imidazolium ion formed along with the 'ES_{tet}' against premature loss of its proton. This was further supported by Naray-Szabo (197).

The evidence for intermediates were obtained only from the acylation step. It is generally accepted that the deacylation mechanism must be the reverse of acylation (198). That is deacylation must proceed through the same sequence of intermediates in the reverse order with water replacing the leaving group of 'HX'.

The rigid active site hypothesis has been assumed as given in Fig. 1 but it is possible that significant conformational changes (induced fit hypothesis) may be taking place (199) which may give rise to additional intermediate enzyme-substrate complexes.

SECTION V

PHYSIOLOGICAL ROLE OF PROTEINASES

A major role of proteinases in nature is nutritional, that is to hydrolyze large polypeptide chain of proteins into smaller peptides or amino acids which are then utilized by the various cells. This is a universal phenomenon and occurs in all living systems. The role of proteinases in regulation of metabolic processes is much more complex and less understood. In all cell systems there is a balance between protein synthesis and protein degradation and intracellular proteinases seem to play an important role in this process. Proteinases are known to be involved in other biological processes such as spore formation and germination, activation of certain viruses to induce pathogenicity (200), protein maturation in viral assembly (201), at several stages of mammalian fertilization process (202), in blood coagulation, fibrinolysis and control of blood pressure (203). Proteolytic enzymes are also intimately involved in many fundamental processes such as hemostasis, inflammation, complement action, cell-cell interactions, gene depression and hormone metabolism.

Physiological role of proteinases is reviewed by Ward (204) which is discussed here in brief.

(a) Protein turnover

Protein turnover is essential for the adaptation of cells to new environmental conditions. In an environment devoid of amino acids, protein turnover provides an amino acid pool for the synthesis of newly required proteins. The rate of proteolysis is generally not proportional to the amount of intracellular proteinase as it may be affected by prior denaturation or modification of proteins. Denaturation by unfolding and chemically induced modifications may also contribute to proteolysis of abnormal proteins (205). The structure of proteins is also important factor in their degradation.

ATP plays an important role in intracellular protein degradation. In bacterial and mammalian cell, for example, inhibition of energy metabolism causes blocking of intracellular degradation of both normal and abnormal proteins (206, 207). It is reported that ATP stimulates the rate of proteolysis in Escherichia coli by two to three fold (204). In bacteria, proteolysis plays an important role in controlling the enzyme levels. The degradation rates of β-galactosidase (208), serine deaminase and alkaline phosphatase (209) from E. coli are measured. All these enzymes were reported to be stable in either growing or starving cells. On the

other hand, glutamine synthetase (210), aspartokinase (211) and certain DNAses appeared to be degraded when E. coli enters a stationary phase and proteolysis is enhanced (212).

In growing bacteria about 1 - 3% of the cell proteins are degraded per hour. Same is true for mammalian and plant cells in culture. The rate of protein turnover in E. coli is high in slowly growing cells and increases during starvation (213). During rapid growth, only abnormal or damaged proteins are degraded whereas most of the cell proteins are resistant to proteolysis (214).

(b) Spore formation

It is reported that starvation induces the formation of bacterial spores (215, 216) and the fruiting body of the slime molds (217). During this process the protein turnover is extensive. The production of extracellular proteinases is frequently associated with sporulation in Bacillus sp. The relationship between proteolysis and sporulation is not restricted to only procaryotes. Under starvation conditions, unicellular slime molds aggregate into multicellular units and differentiate further to a stack and spore containing fruiting body. This process is accompanied by extensive protein degradation (218).

(c) Spore germination

Proteolysis during germination provides amino acids which the spore cannot synthesize at early state of germination. Proteins synthesized newly during germination are also degraded. Proteolysis is involved in eucaryotic germination and hyphal fusion also. It is reported that the alkaline proteinase is involved in macroconidial germination of Microsporium gypseum (219).

(d) Modulation of gene expression

Roberts and his colleagues (220 - 222) demonstrated derepression taking place as a result of repressor degradation by an ATP-dependent proteolytic activity. Maurizi and Switzer (223) have suggested a possible role of proteinases in regulation of translation by modification of ribosomal proteins.

(e) Enzyme secretion

Extracellular enzymes, in general, are considered to be synthesized at cell membrane in a precursor form and then released in active form by proteolysis. Aiyappa et al. (224) reported an extracellular proteinase from B. licheniformis which converted membrane bound penicillinase into the free extracellular form. Some extracellular proteinases are activated

after release. Berg and Pettersson (225) have suggested that cellulases are cell bound and are released into the growth medium through specific release mechanisms. Specific proteases have been suggested to play a key role in regulating the release and activity of cellulase (226). Eriksson and Pettersson (227) have reported that endo-1,4-β-D-glucanases of Sporotrichum pulverulentum, possibly, is present in zymogen form which showed a significant increase in activity on incubation with proteases.

SECTION VI

APPLICATIONS OF MICROBIAL PROTEINASES

Proteinases are important from an economic point of view. They account for almost 60% of total enzyme sales. Proteolytic enzymes find extensive application in industries such as laundering, leather industry, cheese making etc. In the field of clinical medicine they are used as digestive aids, for wound debridement, burns, ulcers, chest disease, acne and skin cleansing, fibrinolysis, anti-inflammatory agents and drug potentiators (228).

An alkaline proteinase of B. licheniformis is used in detergents. Mucor proteinase finds use in cheese manufacture as a substitute for calf rennet. A. oryzae proteinase has application in modification of dough for bread and craker making. For centuries, fungal proteinases have been used for the production of soy sauce, tamari sauce and miso. The applications of microbial proteinases are discussed in detail by Ward (204) which are briefly mentioned below.

(a) Laundary detergents

Proteinases used in detergents should have the following properties: stability at pH 9.0 - 10.5

and at high temperature, activity in the presence of oxidizing and chelating agents and broad specificity. Proteinases in detergents help to remove the protein stains on clothes. The proteinases from B. licheniformis and alkalophilic Bacillus sp. have been used in detergents.

(b) Tanning industry

The conventional method used for dehairing of hides is by employing lime. However, proteinase from B. licheniformis is now used with sulfites at mild alkaline pH values for dehairing of hides. Proteinases from A. oryzae, B. amyloliquefaciens and B. licheniformis have also been used in the process of bating to make the leather soft and elastic. Enzymes are superior to the conventional dehairing process as they impart better characteristics, such as softness and elasticity to the leather.

(c) Preparation of protein hydrolysates

Proteinases have been found useful in the production of hydrolysates from soy protein and other vegetable proteins. Hydrolysates of fish, meat and microbial proteins are also prepared using various proteinases. Protein hydrolysates find extensive use as food for children and invalids. Proteinases have been used to

separate meat scrap into a high grade fat fraction, a soluble protein fraction, an insoluble fraction and a bone fraction. They have also been found applicable in fish industry to speed up processing of inedible or scrap fish to produce oil, meat and fish solubles. Pronase and proteinases from genus Aspergillus are used for this purpose.

Microbial proteinases in combination with plant proteinases have been used for tenderization of meat.

(d) Dairy industry

Proteinases from M. miehei, M. pusillus and Endothia parasitica are used as calf rennet substitute for the manufacture of cheese and curdling of milk.

(e) Brewing

In the brewing industry, proteinases are used during mashing where they increase the actual yield of the extract and the level of α-amino nitrogen in wort. They are also used in chill proofing of beer. Microbial neutral proteinase from Bacillus and Aspergillus species can be used in addition to papain.

(f) Milling and baking

A proteinase preparation of A. oryzae has been

used in the baking industry. Addition of proteinases to doughs improves their handling properties, elasticity and texture of gluten. It also increases the loaf volume with better crust texture. Fungal and bacterial proteinases have also been found applicable in craker doughs where they improve the extensibility and strength of the dough which allows it to be rolled very thinly without tearing.

(g) Peptide synthesis

Use of proteinases in peptide synthesis reactions (229) is one of their major applications. They are used in ordered condensation of amino acids or peptide subunits to obtain polypeptides of defined sequences. Kullman (230) and Konopinska and Muzalewski (231) have reported the proteinase catalyzed peptide synthesis using trypsin, chymotrypsin, papain and thermolysin (231). According to them the enzymatic peptide synthesis are advantageous for following reasons: (i) short reaction time; (ii) water solution as reaction medium is possible; (iii) no protection of functional groups in side chain is necessary; (iv) because of stereospecific action of enzymes the use of racemic amino acids is possible and racemization is excluded and (v) scaling upto industrial scale is

possible , particularly through use of biphasic mixture of solvent and immobilized enzymes. Specific proteinases have also been used in peptide synthesis to remove α-amino and α-carboxyl blocking groups and groups blocking side chain amino and carboxy functions (204).

(h) Medical applications

Most of the proteinases being used for medicinal purpose are from either plant or animal sources. Aspergillus proteinases have been used as digestive aids in gastro-intestinal disorders such as dyspepsia, The streptokinase has been reported to have proteolytic properties with possible application in the treatment of thrombosis. The streptokinase-plasminogen complex is reported to convert free plasminogen to the active fibrinolytic enzyme plasmin (232).

(i) Optical resolution of amino acids

Preparation of optically pure compounds is often achieved by introducing asymmetry into the molecule by using stoichiometric amount of a chiral reagent. But it is, sometimes, impractical and gives product of less optical purity. For some substrates such as amino acids, resolution by enzymatic methods gives

an attractive alternative to the use of chiral reagents.

Subtilisin Carlsberg is used for resolution of (D) and (L) phenylalanine from a racemic mixture of its esters (233). (D) and (L) phenylalanine find use in the production of chloramphenicol and aspartame, respectively.

(j) Animal cell cultures

In general, trypsin is used in animal cell cultures for dissociation of cells from monolayer cell cultures, dissociation of cells from tissues for primary cell cultures and for treatment of metaphase chromosome preparations for the production of G-band preparations. Microbial proteinases have also been tried as substitute for trypsin in animal cell cultures. Froemmel and Boehmer (234) have described the successful use of thermitase, an alkaline proteinase of Thermoactinomyces vulgaris for obtaining single cells from tissue, for detachment of cultivated cells from substratum and for isolation of cells with successive passage of mouse embryonic fibroblasts.

Other applications of microbial proteinases include their incorporation into animal feeds to

increase nutritional value of the feed, their use in gelatin hydrolysis on photographic film to aid silver recovery and their general application in cleaning delicate biological processing and medical equipments.

PRESENT INVESTIGATION

A saprophytic strain of Conidiobolus was isolated from plant detritus in our laboratory and designated as strain NCL 82.1.1. High amount of alkaline proteinase is secreted when Conidiobolus sp. is grown under submerged conditions on the medium containing peptone, casein, sucrose, salts and metal ions. A preliminary report on production of the enzyme has been published (235). In depty study of isolation, purification, characterization, physico-chemical properties, substrate specificity and applications is carried out in the present work. During these studies, a versatile electrophoresis apparatus is developed and used for large scale purification of the enzyme.

Objective of this work was to understand the specificity of alkaline proteinase from Conidiobolus sp. (NCL 82.1.1) to hydrolyze various proteins and to examine its ability for application in industries and clinical uses. The thesis describes studies on purification, physicochemical properties, substrate specificity of 'alkaline proteinase A' from Conidiobolus sp. and its applications in animal cell

cultures, preparation of protein hydrolysates and in resolution of amino acids.

Chapter I describes review of literature on various microbial alkaline proteinases, purification of some fungal alkaline proteinases, their physicochemical properties, physiological role, mechanism of action and applications.

Chapter II describes different materials and methods used for the present studies.

Isolation and purification of the major alkaline proteinase i.e. 'alkaline proteinase A' from the culture filtrate of Conidiobolus sp. (NCL 82.1.1) is given in Chapter III.

Chapter IV includes two sections. Physicochemical properties and substrate specificity of 'alkaline proteinase A' are discussed in Section I. Applications of crude enzyme preparation are illustrated in Section II.

Summary of the work carried out is described at the end of the thesis.

CHAPTER II

MATERIALS AND METHODS

Materials

The following chemicals and biochemicals were obtained from the sources indicated in parenthesis: DEAE-cellulose (0.92 meq/g, medium mesh), CM-cellulose (0.6 meq/g, medium mesh), Tris (hydroxymethyl) aminomethane, N-benzoyl-L-tyrosine-p-nitroanilide (BTPNA) BAPNA, TAME, BTEE, L-leucine-p-nitroanilide, TLCK, TPCK, PMSF, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (CMC), phenylglyoxal, N-acetylimidazole, azocasein, azoalbumin, oxidized B chain of insulin, lysozyme (egg white), cytochrome c (horse spleen), myoglobin (horse heart), ovalbumin, bovine serum albumin (BSA), fibrinogen (bovine), standard protein mixture for SDS-polyacrylamide gel electrophoresis, subtilisin Carlsberg (subtilopeptidase A), thrombin (human plasma), Coomassie Brilliant Blue R-250 and G-250, standard amino acid mixture (Cat. No. AA-S-18) and elastin-orcein |Sigma Chemical Co., USA|; 2,3-butanedione and PCMB |Fluka AG, Switzerland|; hemoglobin |Serva Laboratories, Germany|; iodoacetamide |Koch Light Laboratory, U.K.|; chymotrypsinogen A, blue dextran 2000, and Sephadex G-50 and G-75 |Pharmacia Fine Chemicals, Uppsala, Sweden|; azocoll |Calbiochem, U.S.A.|;

ATEE |Biochemicals Unit, Delhi|; cysteine hydrochloride |Loba-chemie Ind. Co., Bombay|; proteose peptone, meat extract and bacto yeast extract |Difco Lab., U.S.A.|; ampholine carrier ampholytes (LKB Produktor, Bromma, Sweden|.

The chemicals used for polyacrylamide gel electrophoresis viz. acrylamide, N,N'-methylene-bis acrylamide and N,N,N', N'-tetramethylethylenediamine (TEMED) were purchased from Eastman Kodak Company, U.S.A. Sodium dodecyl sulfate (SDS) |Sisco Research Labs. Pvt. Ltd., Bombay| was crystallized twice from ethanol.

N-acetyl-DL-phenylalanine methyl ester (APME) and N-acetyl-DL-phenylglycine methyl ester were kindly supplied by Dr. Natu from this laboratory.

For enzyme assay, casein (Hammarstein) was obtained from Sisco Research Laboratory Pvt. Ltd., Bombay, India and for enzyme production defatted casein was procured from Amul Products (Khaira, Gujarat, India).

All other chemicals were of analytical grade and obtained from local suppliers.

Organism

The saprophytic strain of Conidiobolus (NCL 82.1.1) isolated by Srinivasan et al. (235) was used in the present work.

The culture was isolated by superimposing on MGYP-agar (3 g malt extract, 3 g yeast extract, 5 g peptone, 10 g glucose, 20 g agar per litre), fine particles of decomposing plant detritus mixed with soft agar and attached to the inner surface of petridish lid, following the procedure of Drechsler (236). Single colonies developing from forcibly discharged conidia were transferred to MGYP-agar slants and incubated at 24° - 28°C for 2 - 3 days followed by conservation at 10° - 15°C. The fungus grew rapidly and developed numerous conidia which were forcibly discharged forming a visible whitish deposit on the glass above the growing culture. Microscopic examination showed that several of the conidia also developed microconidia on radial sterigmata.

On the basis of spore measurements and other salient morphological features, the strain was found to be related to Conidiobolus coronatus (Cost.) Batko,

following the taxonomic key suggested by Srinivasan and Thirumalachar (237).

Preparation of crude enzyme extract

Seed was prepared by inoculating about one square cm piece from 72 h old subculture grown on MGYP-agar slant to 100 ml of seed medium (Table IV) in 500 ml Erlenmeyer flask. It was incubated at 28° - 30°C for 48 h on a rotary shaker at 150 rpm. 12 ml of this seed medium was then transferred to 100 ml of production medium (Table IV) in 500 ml Erlenmeyer flask. After incubation on a rotary shaker (150 rpm) at 28° - 30°C for 40 h, the culture was harvested by filtration through Whatman No. 1 filter paper. The clear filtrate obtained was processed for purification of the enzyme.

The culture has retained its initial enzyme activity for over 4 years with frequent subculturing on MGYP-agar slants. The stock culture is maintained on MGYP-agar slants and stored at 15°C. However, it gets degenerated on lyophilization or on keeping below 10°C.

In this work wherever phosphate buffers were used only potassium salts were employed except for SDS-PAGE where sodium phosphate buffer was used.

TABLE IV : COMPOSITION OF SEED AND PRODUCTION MEDIUM*

Constituents	Seed medium g/L	Production medium g/L
Glucose	10.0	-
Sucrose	-	50.0
Starch	5.0	5.0
Peptone	7.5	7.5
Casein	-	20.0
Meat extract	7.5	-
NaCl	3.0	3.0
$MgSO_4.7H_2O$	1.0	1.0
K_2HPO_4	1.0	1.0
$CuSO_4.5H_2O$	7×10^{-3}	7×10^{-3}
$FeSO_4.7H_2O$	1×10^{-3}	1×10^{-3}
$MnCl_2.4H_2O$	8×10^{-3}	8×10^{-3}
$ZnSO_4.7H_2O$	2×10^{-3}	2×10^{-3}

*pH of the medium is generally 6.8 and adjusted if necessary before autoclaving. The medium was autoclaved at 15 lbs for 20 minutes.

Enzyme assays

(a) Caseinolytic assay. The method of Kunitz (238) was used routinely for assay of proteinase. Reaction mixture contained an aliquot of suitably diluted enzyme solution, 10 mg of Hammerstein casein and 0.1 M sodium carbonate-bicarbonate buffer, pH 10.0 in a final volume of 2.0 ml. The reaction was initiated by addition of 1.0 ml of 1% casein solution. Reaction was carried out at 35°C for 10 minutes and was terminated by adding 3.0 ml of 5% trichloroacetic acid (TCA). After standing for about 30 min it was filtered through Whatman No. 1 filter paper and absorbance of the filtrate was measured at 280 nm.

One unit of proteinase activity (PU) was defined as the amount of enzyme resulting in an increase of 0.001 absorbance per ml of reaction mixture per minute at 35°C. Specific activity was calculated as proteinase units per mg of enzyme protein.

(b) Proteinase activity on various protein substrates. Substrate specificity of the enzyme was tested by using the following protein substrates: casein, bovine serum albumin, ovalbumin, native and denatured hemoglobin, azocoll, azocasein and azoalbumin. The caseinolytic assay was followed for the substrates

except for azocoll, azocasein and azoalbumin. Hemoglobin was denatured with urea under alkaline condition as described by Walter (239).

Activity of the enzyme on azocoll, azocasein and azoalbumin was determined by the method of Ansari and Stevens (240). One ml of the chromogenic substrate azocoll (20 mg/ml), azocasein (2.5 mg/ml) or azoalbumin (2.5 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.0 was incubated with an aliquot (0.1 ml) of suitably diluted enzyme for 30 min at 35°C. The reaction was stopped by adding 0.04 ml of 50% (w/v) TCA. After centrifugation, the absorbance was measured at 520 nm (azocoll) and 440 nm (azocasein or azoalbumin). The enzyme units and specific activity for these substrates was also calculated as described above.

(c) Esterolytic activity. Esters of different amino acids were used for determining esterolytic activity of the enzyme. The esterolytic activity on TAME was determined by potentiometric titration at constant pH 8.0, according to the procedure of Ottesen and Svendsen (241). Reaction was carried out in 0.1 M KCl at 30°C in a pH-stat Radiometer with PHM-62 standard pH meter and TTT-60 titrator. 0.02 N NaOH was used for

the titration of hydrogen ions liberated during the hydrolysis of ester bond. The reaction was initiated by adding 80 µg of enzyme in 50 µl of 0.01 M phosphate buffer, pH 7.5 to 10 ml of substrate solution of various concentrations.

Esterolytic activity of the enzyme with ATEE and BAEE was determined according to the procedure given by Glazer (144) using pH-stat Radiometer as described above with 10 ml of substrate solution of different concentrations.

The esterolytic activity of the enzyme with BTEE was determined spectrophotometrically at 256 nm (10 mm light path). The reaction was initiated by adding 0.1 ml of suitably diluted enzyme to 1.0 ml of substrate solution (various concentrations) in 0.1 M Tris-HCl buffer, pH 8.0 containing 0.1 M $CaCl_2$. Rate of change of absorbancy (ΔE) was linear only upto 0.12 O.D. The value of ΔE (change in molar extinction coefficient) for complete hydrolysis of BTEE was taken to be 964 (242).

(d) Peptidase activity using p-nitroanilide substrates. The method of Ansari and Stevens (240) was followed. 0.16 ml of the substrate solution (1 mM solution of BAPNA, BTPNA or leucine-p-nitroanilide in DMSO) was mixed with 0.5 ml of 0.05 M Tris-HCl buffer, pH 8.0 and 0.1 ml of enzyme (50 µg) and incubated for

1 h at 35°C. Reaction was terminated by adding 0.5 ml 1 M Na_2CO_3 and the p-nitroanilide formed was measured at 420 nm. One unit of activity was defined as the amount of enzyme which released 1 nmol of p-nitroanilide per minute at 35°C.

Determination of protein

(a) Lowry's method. Protein determination of purified enzyme fractions was carried out by the method of Lowry et al. (243). The colour developed was read at 660 nm. Crystalline bovine serum albumin, fraction V was used as standard protein. The concentration of serum albumin was calculated from its extinction coefficient at 280 nm ($E^{1cm}_{1\%}$ = 6.6) according to Cohn et al. (244). Interferring substances such as glucose, phosphate, thioethanol were removed by dialysis prior to estimation of protein.

(b) Spectrophotometric method. Method of Warburg and Christian (245) was used routinely to determine protein concentrations during the purification procedure. It was assumed that a 0.1 percent protein solution shows an absorbance of 1.0 at 280 nm for 1 cm light path. A blank was also taken using a buffer of same composition. Correction for ultraviolet absorbing impurities was made by substracting the absorbance at 340 nm.

(c) Dye binding method. Protein determinations of alkaline proteinase obtained after electroelution was carried out by the method of Bradford (246), since acrylamide impurities do not interfere in this assay. Crystalline bovine serum albumin fraction V was used as standard protein. Concentration of bovine serum albumin was calculated from its extinction coefficient at 280 nm ($E_{1\%}^{1cm}$ = 6.6) (244). The absorbance of Coomassie Brilliant Blue G-250 bound protein was read at 595 nm.

Gel filtration

Sephadex G-50 was suspended in water and was allowed to swell by heating in a boiling water bath for about one hour, cooled and deaerated. Swollen gel was then packed in a column (1.6 x 100 cm) and was equilibrated by passing 500 ml of 0.05 M phosphate buffer, pH 7.0. The enzyme (5.0 mg) in 0.5 ml of 0.05 M phosphate buffer, pH 7.0 was loaded on the column and eluted with the same buffer. 2 ml fractions were collected at a flow rate of 12 ml/h and were assayed for protein and enzyme activity.

Ion-exchange chromatography

CM-cellulose (2.0 g) equilibrated with 0.02 M phosphate buffer, pH 7.0 was packed in a glass column

(1.2 x 15 cm). The enzyme (alkaline proteinase A) was adsorbed on CM-cellulose at pH 7.0 and the column was washed with 0.02 M phosphate buffer, pH 7.0 (120 ml) to remove unadsorbed impurities. The adsorbed 'alkaline proteinase A' was then eluted with 120 ml of 0.06 M phosphate buffer, pH 7.0. Fractions of 6 ml were collected at a flow rate of 12 ml/h and were assayed for protein and enzyme activity.

Analytical polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at pH 4.3 according to the method of Reisfeld et al. (247) and at pH 7.6 using the cationic system C as described by Zuidweg et al. (248) with some modifications.

(a) Electrophoresis at pH 4.3. The separation gel composition was 0.36 M acetic acid and 0.06 M KOH, pH 4.3; 7% acrylamide; 0.1% Bis; 0.1% TEMED and 0.14% ammonium persulfate. Electrode buffer contained 0.035 M β-alanine, 0.014 M acetic acid pH 4.5. During electrophoresis anode was at the top and cathode at the bottom.

(b) Electrophoresis at pH 7.6. The separation gel contained 0.1 M KOH and 0.5 M boric acid, pH 7.6; 7.5% acrylamide; 0.2% Bis; 0.125% TEMED and 0.07% ammonium persulfate. Electrode buffer composition was

0.13 M Tris and 0.2 M boric acid, pH 7.8. Anode was at the top and cathode at the bottom.

Basic fuchsin was used as a tracking dye in both the above procedures. Electrophoresis was run with 4 mA current per tube gel for a period of 150 - 180 min. Gels were stained with Xylene Cyanine Brilliant G (also referred to as Coomassie Brilliant Blue G-250) prepared according to Blakesley and Boezi (249).

Preparative polyacrylamide gel electrophoresis

(a) Polyacrylamide gel electrophoresis run. Preparative PAGE method was used for purification of the enzymes. The cationic system C described by Zuidweg et al. (248) was followed with the modifications described earlier. Electrophoresis was carried out in the apparatus developed during the present work wherein gel making and subsequent electrophoresis is carried out in the same apparatus (250).

The apparatus was fabricated from perspex sheets. Figure 2 and 3 show the photographs of the empty and assembled unit. Figures 4 and 5 give the dimensions of the unit.

Fig. 2 Empty electrophoresis unit

Fig. 3 Assembled electrophoresis unit

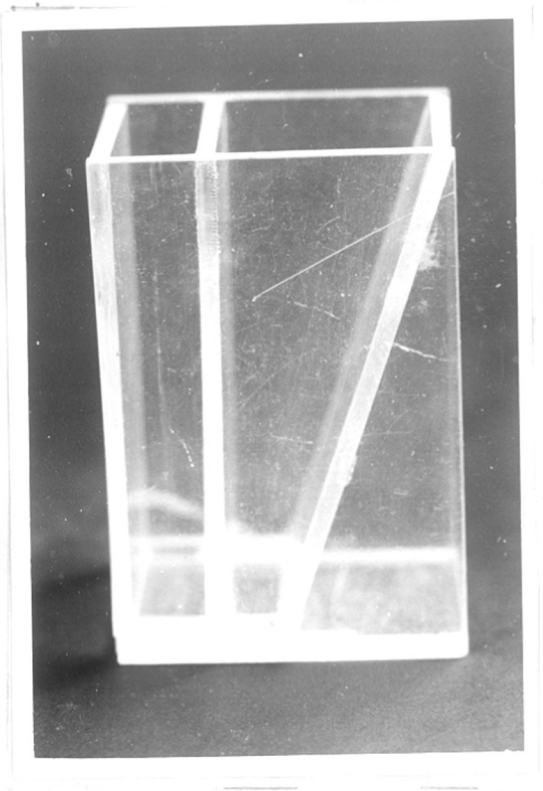


FIG. 2

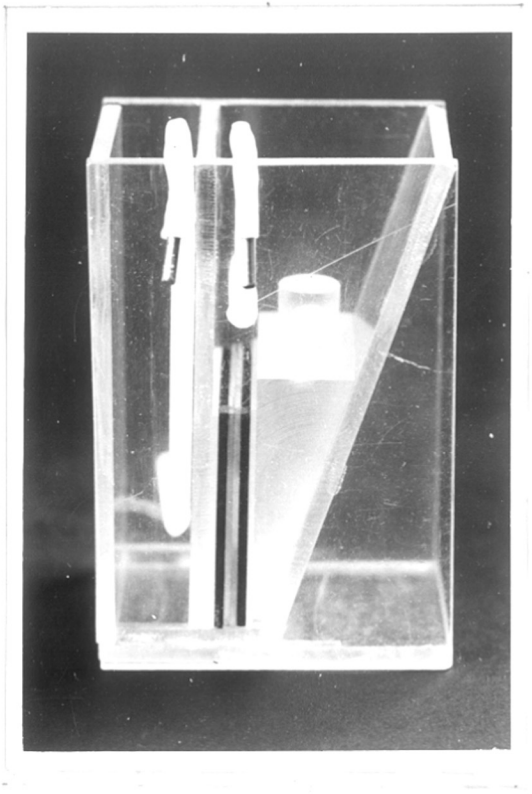


FIG. 3

Fig. 4 Dimensions of the empty electrophoresis unit

(a) Chamber with slanting wall, with 2 x 8.5 cm base.
(b) Second chamber, 3 x 8.5 x 19 cm
(c) Common middle wall, 8.5 x 18.2 cm height
(d) Two support strips each 5.5 x 0.5 x 1 cm
(e) 0.8 cm gap below the common middle wall
(f) Slanting wall of chamber 'a'
(g) Elevation of empty unit

Thickness of the perspex sheet (0.5 cm) used in construction of the unit is not shown in the figure.

Fig. 5 Dimensions of the assembled electrophoresis unit

(a-f) Same as in Figure 4

(g) Gel cassettes
(h) Wedge
(i) Spacer or spacers, if necessary, each 8.5 x 9.5 cm height, thickness from 0.3 to 0.5 cm
(j) Two 'L' shaped platinum electrodes
(k) Elevation of the assembled unit

Fig. 6 Wedge (with two circular openings and a handle on the top.

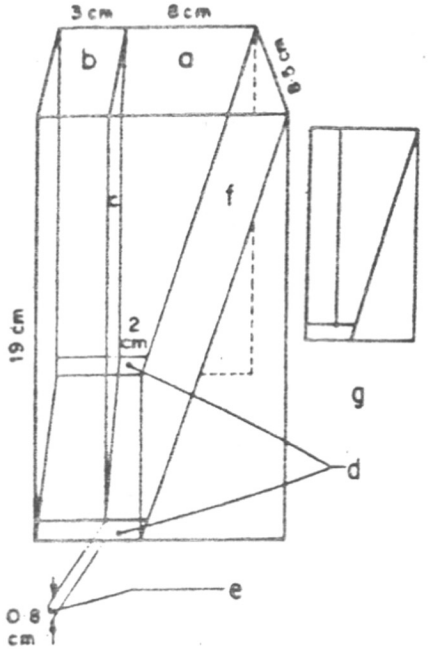


FIG. 4

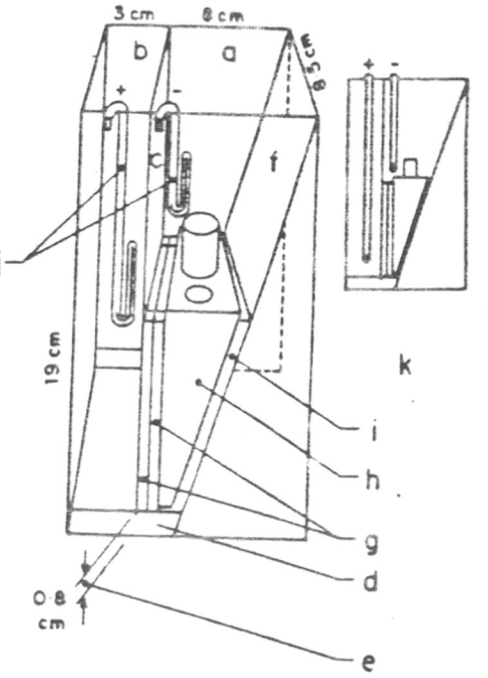


FIG. 5

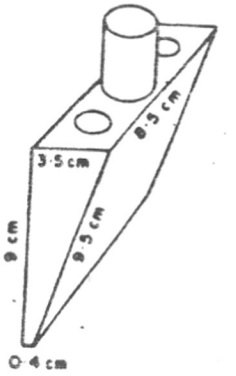


FIG. 6

The main apparatus consists of two vertical chambers 'a' and 'b' which form the electrode compartments. These chambers are separated by a common middle partition wall. A gap of 0.8 cm is kept at the base of the partition wall. One cm thick gel cast at the base of the chambers closes the gap (0.8 cm) kept below the partition wall. Thus, this basal 'contact gel' prevents buffer flow from one chamber to the other and also establishes an electrical contact between the two chambers. The wall opposite to the middle partition wall is made slanting for operation of the wedge (Fig. 6).

Gel cassettes (molds for casting slab gels) are placed above the contact gel in one of the chambers (chamber 'a') and are secured in position by the wedge (Fig. 5h and 6). Polyacrylamide slab gels are cast in the gel cassettes, both chambers are filled with electrode buffer and after loading the sample, <u>in situ</u> electrophoresis is carried out since both the chambers also form the two electrode chambers. Thus, out of two chambers one serves as a chamber for gel casting and both the chambers act as electrode compartments.

The apparatus described above is of standard size. However, for large scale preparative gel electrophoresis, which was carried out in the present work, a larger size

of the above apparatus was used. This larger size apparatus could accommodate slab gels of size 16.5 x 11.0 x 0.9 cm and 1.5 litre of electrode buffer in each chamber.

The compositions of separation gel and bath buffer were same as described earlier for cationic system C (at pH 7.6). The basal contact gel composition was 0.13 M Tris and 0.2 M boric acid, pH 7.8; 0.125% TEMED; 7.5% acrylamide; 0.2% Bis and 0.07% ammonium persulfate. In preparative gel electrophoresis a spacer gel was used. Spacer gel contained 0.1 M KOH, 0.2 M boric acid, pH 9.0; 5% acrylamide; 1% Bis; 0.05% TEMED; 5 µg/ml riboflavin and 20% sucrose.

Two slab gels (16.5 x 11.0 x 0.9 cm) were prepared. About 55 mg of protein with tracking dye (basic fuchsin) was loaded on each gel. A voltage of 80 volts and current of 50 mA was applied and electrophoresis was run for about 36 h until the tracking dye reached 1 - 2 cm above the bottom of gels. Between the two gel cassettes one empty cassettee which was sealed at the bottom, was placed. This acts as a cooling device for the two adjacent gel cassettes as it gets filled with the bath buffer. At the same time it does not convey current directly from one chamber to the other since it is sealed at the bottom.

After the run was over, gels were removed from cassettes. A vertical side strip (5 - 7 mm broad) of each slab gel was cut and stained with Xylene Cyanine Brilliant G dye prepared according to Blakesley and Boezi (249). This dye stains only proteins and background gel remains almost colourless. Since no destaining step is involved, a protein bands appear within 20 - 30 min. From this stained strip position of the enzyme could be determined (first band from the base corresponds to 'alkaline proteinase B' and third band from the base corresponds to 'alkaline proteinase A'), since in an analytical run the positions of both the enzymes were detected by slicing the gel tube and checking the slices for enzyme activity. Accordingly, the gels were cut to get two slices of 'alkaline proteinase A' and two slices of 'alkaline proteinase B'.

(b) Elution of the enzymes. Elution of the enzymes was carried out in an 'Elution apparatus' designed by Bodhe et al. (251) of this laboratory with some modification in the recovery cell assembly. Recovery cell of bigger size (7 x 3.5 x 1.5 cm) was used for elution of the enzyme. Gel slices of respective enzymes were placed on the recovery cells. The recovery cells contained foam pieces. Vertical downward electrophoretic

elution was carried out through the thickness of the gel. The foam piece in the recovery cell contained KOH-borate buffer, pH 7.6 and bath buffer was Tris-borate buffer, pH 7.8. The enzyme gets collected in the foam piece. The escape of enzyme from the foam is prevented by wrapping the recovery cell at the bottom with semipermeable membranes such as cellulose caseing. Since elution is carried out through the thickness of the gel, the distance travelled by proteins is very short. This results in rapid elution of the proteins. A current of 60 mA was applied and elution was carried out for 6 h. After the run, foam from the elution assembly was removed and squeezed out in a test tube. The foam was rinsed with a small amount of buffer (KOH-borate buffer, pH 7.6) and again squeezed out in the same test tube. The eluate was analyzed for enzyme activity.

Determination of isoelectric point

Isoelectric point (pI) of the purified enzyme was determined using 110 ml capacity electrofocusing column (LKB Produktor, AB, Bromma, Sweden) according to the method described by Vesterberg and Svensson (252). The density gradient was made with sorbitol using an automatic gradient mixer. Dense solution, light solution and

electrode solutions were prepared as given in LKB Instruction Manual. Ampholine carrier ampholytes and enzyme solutions were mixed in light and dense gradient solutions. First a pilot run was taken with ampholine carrier amplolytes of wide pH range (pH 3.5 - 10.0). Ampholine carrier ampholytes of pH range 9.0 - 11.0 at concentration of 1% (w/v) was then used to determine precise isoelectric point of the enzyme. Anode was at the top of the column. Electrofocusing was carried out at 5°C for 30 h.

At the end of run the voltage was steady at 400 V and current drop was from 20 mA to 1.0 mA which remained constant. After completion of electrofocusing the column was emptied at the rate of 30 - 40 ml/h and fractions of 1 ml were collected. Fractions were then processed for determination of pH (at 5° - 7°C), enzyme activity and protein.

Determination of molecular weight

(a) Gel filtration. Molecular weight of the enzyme was determined by gel filtration on Sephadex G-75 according to the method of Andrews (253). Sephadex G-75 was packed in a column (1.6 x 100 cm) and was equilibrated with 0.05 M phosphate buffer, pH 7.0. A calibration

curve was prepared by loading 0.05 ml of 0.05 M phosphate buffer, pH 7.0 containing 2 mg each of cytochrome c (MW 13 000), myoglobin (17 000), chymotrypsinogen A (25 000), ovalbumin (43 000) and BSA (67 000). Equilibration buffer served as elution buffer. Fractions of 2 ml were collected at a flow rate of 12 ml/h and were assayed for different proteins loaded. Cytochrome c was estimated at 550 nm by reducing it with dithionite at pH 8.0 (254). Myoglobin was estimated at 415 nm in 5 mM 2-mercaptoethanol at pH 8.0 (255). Chymotrypsinogen A, ovalbumin and bovine serum albumin were estimated by the method of Warburg and Christian (245) as described earlier. Molecular weight of the enzyme was calculated from the plot of the log molecular weight against elution volume (Ve/Vo).

(b) SDS-polyacrylamide gel electrophoresis. The SDS-phosphate continuous buffer system of Shapiro et al. (256) and Weber and Osborn (257) was used. The principle on which the method is based is that SDS forms complex with proteins and minimizes the native charge differences of the proteins as a result of which they migrate as anions. The rate of migration is proportional to the molecular weight.

Gels (0,6 x 10 cm) were prepared containing 10%

acrylamide, 0.13% Bis, 0.2% SDS, 0.05% TEMED, 0.1 M sodium phosphate, pH 7.0 and 0.07% ammonium persulfate. The electrode buffer contained 0.05 M sodium phosphate buffer, pH 7.0 and 0.1% SDS.

Molecular weight markers for SDS-gel electrophoresis (Sigma Chemical Co., Sweden, Cat. No. SDS-6) containing 13.5 mg mixture of BSA (66 000),;ovalbumin (45 000); pepsin (34 700); trypsinogen (24 000), β-lactoglobulin (18 400); lysozyme (14 300) and BPB was used as standard protein markers. This mixture was dissolved in 0.1 ml of 0.01 M sodium phosphate buffer, pH 7.0 containing 2.5% SDS and 5% β-mercaptoethanol and heated in boiling water bath for 5 min. Similar treatment was given to 50 µg of the enzyme. A 25 µl of standard protein marker mixture and 50 µl of enzyme sample were loaded in separate tube gels. 30 µl of 0.05% BPB was incorporated as tracking dye. In the samples, sucrose was added to make its concentration to 10%. Electrophoresis was carried out by applying 8 mA current per gel and was discontinued after about 8 h when tracking dye reached 1 - 2 cm from the bottom of the gel. Gels were stained with 0.1% Coomassie Blue R-250 (in 8% acetic acid, 25% ethanol) and destained with 8% acetic acid in 25% ethanol.

Molecular weight of the enzyme was calculated from a plot of log molecular weight against electrophoretic mobilities (Rf = distance of protein migration/distance of dye migration) of standard marker proteins.

Subunit detection

The presence of subunits in the enzyme was tested by its migration in SDS-polyacrylamide gel (256, 257) with and without treatment of enzyme with 1% SDS in combination with 1% β-mercaptoethanol and 0.5% iodoacetamide at 100°C for 5 min.

Amino acid composition

Samples of enzyme (0.5 to 1.0 mg) were hydrolyzed in 6N HCl at 110°C for 24, 48 and 72 h in evacuated, sealed pyrex types in a block heater. The hydrolysates were evaporated to dryness in a vacuum desiccator. Residual HCl in the hydrolysates was removed by dissolving the residue in about 2 ml of deionized water and then drying in vacuum desiccator. This was repeated twice. The residue was then dissolved in citrate buffer, pH 2.2 and aliquots were analyzed on a Beckman model 120B automatic amino acid analyzer by the method of Spackman *et al.* (258).

Proline, threonine, serine and tyrosine are partially destroyed during acid hydrolysis. Hence, a correction was applied by extrapolating to zero hydrolysis time from the data for 24, 48 and 72 h hydrolysis. Half cystine content was determined as cysteic acid after oxidation with performic acid (259) followed by hydrolysis and amino acid analysis as above.

Tryptophan is completely destroyed during the acid hydrolysis and thus could not be detected in amino acid analysis run. The quantity of tryptophan was determined by the method of Goodwin and Morton (260) by determining the absorbance of the protein at 294.4 nm, 257.15 nm and 280 nm.

Determination of glycoprotein nature

Glycoprotein nature of the enzyme was tested by Schiff's staining (261). After electrophoresis, the gels were immersed in 12.5% TCA for 30 min and then in 1% periodic acid (in 3% acetic acid) for 50 min. Excess of IO^{-3} ions were removed by stirring the gel tubes in water overnight with three changes. The gel tubes were then immersed in fuchsin sulfite stain in the dark for 50 min and washed with freshly prepared 0.5% potassium metabisulfite for 30 min. The gel tubes were rinsed in water for removing excess stain and stored in 7% acetic acid.

Preparation of S-2-AE-globin

(a) Preparation of globin. A clear solution of hemoglobin (1%) was poured into ten volumes of 0.5% ice cold HCl in acetone with rapid stirring. The precipitated globin was then removed by centrifugation and washed with HCl-acetone mixture once and then thrice with ice cold acetone. The procedure is repeated if the precipitate is coloured. The white globin was then dried in vacuum over NaOH pellets and stored in cold.

(b) Preparation of S-2-aminoethyl(AE)-globin. Free sulfhydryl and disulfide bonds in the globin were amino ethylated ($-S-CH_2-CH_2-NH_2$) according to the procedure described by Jones (262). 100 mg of globin was mixed with 2.6 g of deionized urea, 0.3 ml of 5% EDTA and 4.5 ml of 1 M Tris-HCl buffer, pH 8.5. This mixture was diluted to 7.5 ml with H_2O and was treated with 0.1 ml of β-mercaptoethanol. After 30 min, 0.2 ml of ethylene imine was added and the solution was incubated for another 30 min. The amino ethylated globin was thoroughly dialyzed first against tap water for 48 h and then against distilled water for 24 h with 5 - 6 changes, to remove urea and other low molecular weight substances. The insoluble amino ethylated globin was collected by centrifugation, dried in vacuum over NaOH pellets and stored at $-20^{\circ}C$.

Oxidation of lysozyme

The method of Sanger (263) was followed for oxidation of lysozyme. To 250 mg of lysozyme dissolved in 9.0 ml of formic acid, 1 ml of 30% (w/w) H_2O_2 was added. The mixture was allowed to stand at room temperature for 30 min. It was then diluted with 10 ml H_2O and evaporated in a vacuum desiccator over NaOH pellets to small volume (1 - 2 ml). Oxidized protein was then precipitated with large volume of acetone, centrifuged, washed with acetone till it was free of formic acid and dried in air.

Enzymatic hydrolysis of S-2-AE-globin, oxidized lysozyme and oxidized B-chain of insulin with subtilisin/ 'alkaline proteinase A'

10 mg of S-2-AE-globin was taken in a 10 ml beaker and suspended in 5 ml of water. pH of the solution was adjusted to 8.1 with 0.05 N NaOH and the protein was completely denatured by heating at 80°C for 8 min. The solution was kept in water bath at 37°C with constant stirring. Proteolytic enzyme was then added to the solution in an enzyme to protein ratio of 1:50 by weight. pH was maintained at 8.1 by adding 0.01 N NaOH. Uptake of alkali was observed for about 75 minutes. However, the reaction was continued for another three hours.

Reaction was stopped by lowering the pH to 6.4 with 0.05 N HCl and heating the hydrolysate in boiling water bath for two minutes. The solution was centrifuged and the clear supernatent (hydrolysate) was preserved at -20°C.

The hydrolysis of oxidized B-Chain of insulin and oxidized lysozyme was carried out for 1 h as described for AE-globin.

Finger printing

Finger printing of peptides generated from AE-globin, oxidized lysozyme and oxidised B-chain of insulin was carried out according to the procedure described by Ingram (264). The technique involved high voltage paper electrophoresis in one direction followed by ascending chromatography in a perpendicular direction. The enzymatic digest corresponding to 2 mg of protein was dried over NaOH pellets in a vacuum desiccator. Paper electrophoresis was carried out using Whatman No. 3 filter paper, cut into T-shape (34 x 30 cm with 13 cm long and 6 cm broad sleeves on either side of the length). Michl's buffer (265) was prepared by mixing pyridine: glacial acetic acid:water in the ratio of 10:0.4:90 by volume, pH was adjusted to 6.4 with glacial acetic acid and the electrophoresis chambers were filled with this

buffer. The filter paper was soaked in the buffer, blotted to remove excess buffer and was placed on horizontal glass plate. The dried hydrolysate was dissolved in 10 µl of H_2O and applied on the spot which was 3 cm above the edge of the paper at a distance of 23 cm from the edge of sleeve. The paper was covered with another glass plate and sleeves on either side were dipped in the chambers. After about 30 min equilibration, electrophoresis was started by applying a constant voltage of 1000 volts for 2 hours. After electrophoresis, the two sleeves were cut off and the paper was dried. The paper was then allowed to saturate in a chromatographic chamber over pyridine: n-butanol:acetic acid:water (20:30:6:24) (265) for 2 h and then dipped in the solvent system. Ascending chromatography was carried out for 12 h. The paper was then removed and dried. Peptide spots on the chromatogram were identified by spraying with 0.3% ninhydrin solution in acetone and then drying in air. Colour of the spots was intensified by heating the paper in oven at 60 - 65°C for 15 - 20 min. The colour was fixed by dipping the paper in a fixing reagent. (1% cupric nitrate and 0.05% nitric acid in acetone).

Resolution of DL-phenylalanine and DL-phenylglycine

Procedure of Roper and Bauer (233) was used for the resolution of DL-phenylalanine and DL-phenylglycine. Automatic pH Stat Radiometer was used in this procedure.

N-acetyl-DL-phenylalanine methyl ester (221 mg) was suspended in about 5 ml of H_2O and the pH adjusted to 7.5 with 0.02 N NaOH. Purified 'alkaline proteinase A' (200 µg) was added with constant stirring. pH of the solution decreases as a result of release of protons due to hydrolysis of ester bonds. pH was readjusted to 7.5 and maintained at that value by addition of 0.02 N NaOH, until enzymatic action ceased after about 1 h. pH of the reaction mixture was not allowed to come down below 6.0 as the enzyme is destroyed at lower pH values.

The aqueous reaction mixture was extracted twice with 10 ml of dichloromethane. Combined organic extract was dried with unhydrous $MgSO_4$ and concentrated to give N-acetyl-D-phenylalanine methyl ester as a colourless solid.

The aqueous portion of the reaction mixture remained after organic solvent extraction was adjusted to pH 1.0, and extracted twice with 10 ml of ethylacetate. The

combined organic extracts were dried with unhydrous $MgSO_4$ and concentrated in vacuum to give a colourless solid which was recrystallized from water to give N-acetyl-L-phenylalanine.

CHAPTER III

ISOLATION AND PURIFICATION OF 'ALKALINE PROTEINASE A' FROM *CONIDIOBOLUS* sp. (NCL 82.1.1)

SUMMARY

The culture filtrate of Conidiobolus sp. (NCL 82·1.1) showed the presence of two alkaline proteinases in the proportion of 70:30. The major alkaline proteinase, 'alkaline proteinase A' was purified to homogeneity by (i) solvent precipitation; (ii) batchwise treatment with DEAE-cellulose at pH 7.0; (iii) ammonium sulfate precipitation (90% saturation); (iv) preparative polyacrylamide gel electrophoresis at pH 7.6; (v) ion-exchange chromatography on CM-cellulose at pH 7.0 and (vi) gel filtration on Sephadex G-50 column. A new versatile gel casting cum electrophoresis device was developed for preparative polyacrylamide gel electrophoresis and was used during purification of the enzyme.

The purified enzyme showed a single protein band in disc gel electrophoresis at two different pH values (pH 7.6 and 4.3), in SDS-gel electrophoresis, gel filtration and in isoelectric focusing analysis.

INTRODUCTION

Production and purification of alkaline proteinases from various species of bacteria, yeast and moulds is studied in detail. However, fungi belonging to phycomycetes have been little investigated for production and characterization of purified alkaline proteinases. Few reports are available on the production and characterization of purified alkaline proteinases from this group (121 - 126). The Conidiobolus sp. (NCL 82.1.1) culture broth showed high activity of alkaline proteinase. Complete understanding of various physicochemical properties, substrate specificity and mechanism of action is necessary before commercial exploitation of the enzyme. A limited knowledge of the alkaline proteinases from this group of fungi and presence of high activity of alkaline proteinase in the culture broth of Conidiobolus sp. (NCL 82.1.1) instigated us to study the physico-chemical properties of the purified enzyme. This Chapter describes the studies on purification of this enzyme. During these studies a simple and versatile device was developed for preparative polyacrylamide gel electrophoresis.

RESULTS

Preparation of crude enzyme extract

The extracellular proteinase activity was observed at 24 h and reached its maximum by 40 h after which it started declining. Increase in pH from 6.8 to 8.2 was observed during the production of enzyme. The culture was harvested after 40 h by filtering through Whatman No. 1 filter paper. The clear culture filtrate was used for purification of the enzyme.

Purification

'Alkaline proteinase A' was purified from the culture filtrate obtained. All operations were carried out at 0 - 4°C unless otherwise mentioned. The purification steps involved solvent precipitation, batchwise DEAE-cellulose treatment, salting out, preparative polyacrylamide gel electrophoresis, CM-cellulose chromatography and gel filtration.

Step I : Ethanol precipitation. The culture filtrate was concentrated by precipitation with ethanol. To one litre of culture filtrate, three litres of chilled ethanol was added slowly with constant stirring and allowed to stand for 2 h at 4°C. The filtrate

was collected by centrifugation at 3000 rpm for 20 min. Traces of ethanol were removed by drying the precipitate under vacuum in a desiccator. The dry powder was suspended in 50 ml of 0.01 M potassium phosphate buffer, pH 7.0 and dialyzed against the same buffer. The specific activity increased by 3-fold during this step with a recovery of 72%.

Step II : Batchwise DEAE-cellulose treatment (negative adsorption). The enzyme obtained in Step I was further purified by batchwise treatment with DEAE-cellulose (OH^- form). 20 g of DEAE-cellulose was added to 58 ml of the enzyme (protein 33 mg/ml) and volume was made to 700 ml with phosphate buffer, pH 8.0. Final molarity of the buffer was 0.01 M. After 2 h of occassional stirring, it was filtered through Whatman No. 1 filter paper. The filtrate was adjusted to pH 7.0 immediately to prevent autolysis. Alkaline proteinase was not adsorbed on DEAE-cellulose and remained in the filtrate, however, some protein and colour pigment impurities were adsorbed. The filtrate was assayed for protein and enzyme activity. It retained 83% of the activity, and specific activity of the enzyme increased to 20 020 PU/mg.

Step III : Ammonium sulfate precipitation. The enzyme obtained in Step II was concentrated by precipitation with ammonium sulfate. To 650 ml of the enzyme (protein 0.9 mg/ml), 390 g of ammonium sulfate was added slowly with stirring (90% saturation) and allowed to stand for 2 h. Precipitated enzyme was collected by filtration through Whatman No. 1 filter paper. The precipitate was suspended in 10 ml of 0.01 M potassium phosphate buffer, pH 7.0 and then dialyzed against the same buffer till it was free from ammonium sulfate. The dialyzed enzyme (volume 18 ml, 24.4 mg protein per ml) was used for further purification.

Step IV : Preparative polyacrylamide gel electrophoresis. Enzyme preparation from Step III when run on analytical disc gel electrophoresis and examined for proteinase activity (by slicing the gel tube and checking for enzyme activity) showed presence of two different proteinases. These two proteinases were separated by preparative polyacrylamide gel electrophoresis. Preparative polyacrylamide gel electrophoresis (PAGE) was carried out in an apparatus (Fig. 3) developed during this work (250) and is described in Materials and Methods. Two slab gels (16.5x11.0x0.9 cm)

were prepared. About 55 mg of enzyme protein (in about 2 ml) with basic fuchsin as a tracking dye was loaded on each slab gel. After completion of the run, gels were cut as described in Materials and Methods to get two portions from each slab gel containing proteinase activity. The two enzymes were designated as 'alkaline proteinase A' (third protein band from base) and 'alkaline proteinase B' (first band from base i.e. moving along with the tracking dye) (Fig 6a). The proteinases were eluted electrophoretically, through the thickness of the gel into polyurethane foams soaked in buffer (KOH-borate, pH 7.6) according to the method of Bodhe et al. (251). 'Alkaline proteinase A' (volume 27 ml, protein 1.2 mg/ml) and 'alkaline proteinase B' (volume 25 ml, protein 0.56 mg/ml) were obtained in separate fractions. in the proportion of 70:30 with respect to activity. Four such runs were carried out. The fractions containing 'alkaline proteinase A' were pooled and processed further. 'Alkaline proteinase A' at this stage was almost pure with minor impurities.

Step V : CM-cellulose ion-exchange chromatography. 'Alkaline proteinase A' from Step IV was concentrated by lyophilization, dissolved in 10 ml of 0.02 M phosphate buffer, pH 7.0 and then dialyzed for 6 h against the

Fig. 6a Disc gel electrophoresis at pH 7,6 of alkaline proteinase preparation from Step III

A. Alkaline proteinase A

B. Alkaline proteinase B

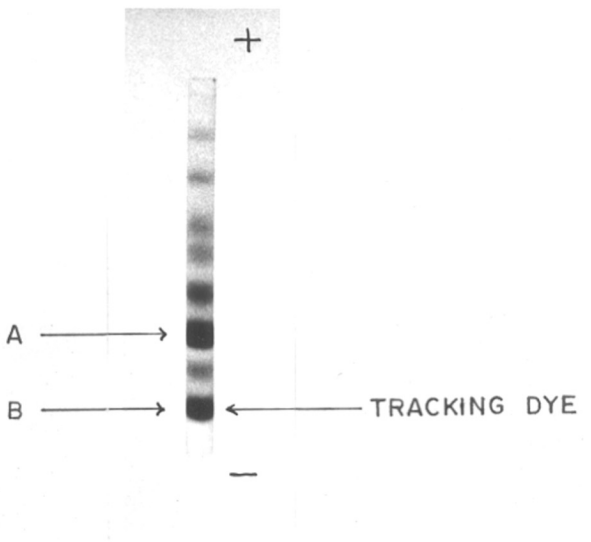


FIG. 6a

same buffer. One third of this enzyme solution (44 mg protein) was chromatographed on CM-cellulose column (1.2 x 15 cm) pre-equilibrated with 0.02 M potassium phosphate buffer, pH 7.0. 'Alkaline proteinase A' got adsorbed on CM-cellulose column. The column was washed with 120 ml of 0.02 M phosphate buffer, pH 7.0 to remove impurities and the enzyme was eluted with 120 ml of 0.06 M phosphate buffer, pH 7.0. Fractions of 6 ml were collected at the flow rate of 12 ml/h and assayed for protein and proteinase activity. The elution pattern is shown in Fig. 7. To purify the total 'alkaline proteinase A' from Step IV three CM-cellulose columns were operated. Fractions 17 - 24 of each column were pooled, concentrated by lyophilization and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0. After dialysis, 9 ml of the solution contained 90 mg of 'alkaline proteinase A' with specific activity of 25 710.

Step VI : Sephadex G-50 gel filtration. 'Alkaline proteinase A' was further purified by gel filtration on Sephadex G-50 column. 5.0 mg of the enzyme from Step V was loaded on Sephadex G-50 column (1.6 x 100 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.0 and eluted with the same buffer. Fractions of

Fig. 7 CM-cellulose ion-exchange chromatography of 'alkaline proteinase A' from Step IV. The enzyme was adsorbed on the column (1.2 x 15 cm) previously equilibrated with 0.02 M phosphate buffer, pH 7.0 and eluted with 0.06 M phosphate buffer, pH 7.0.

Sample : 44 mg protein

Flow rate : 12 ml/h

Symbols : △ , Proteinase activity

⊙ , Protein

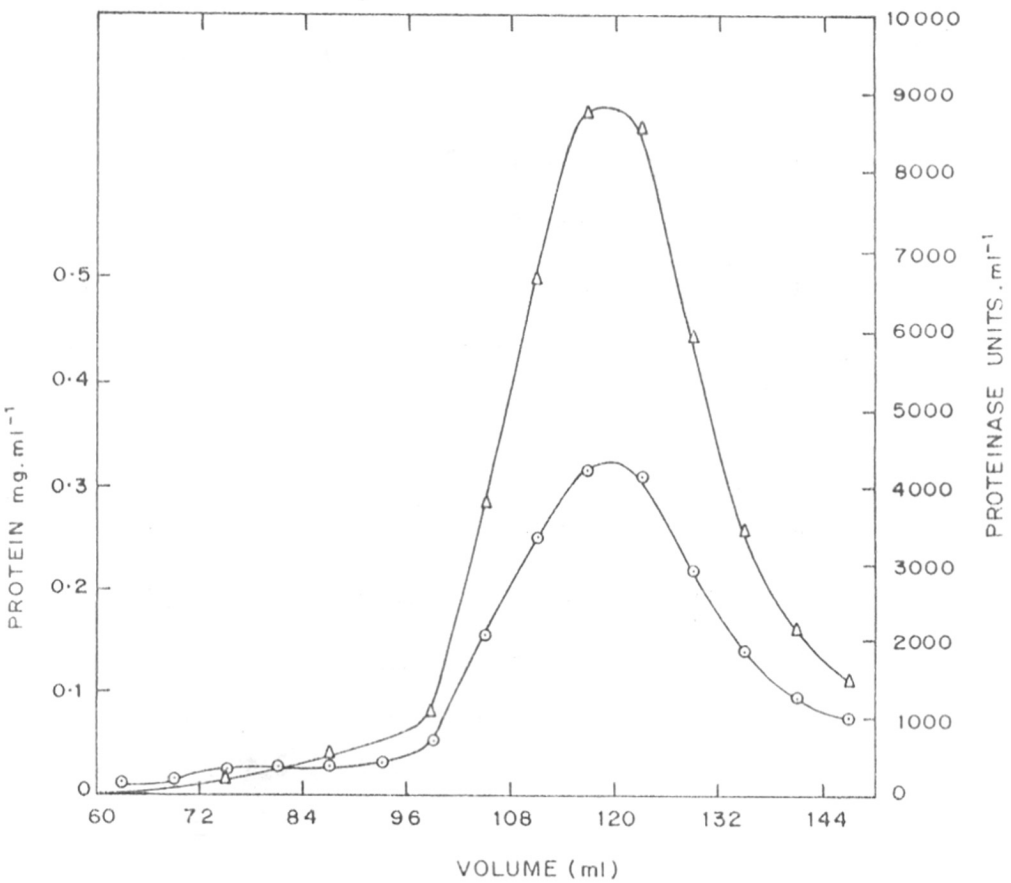

PROTEIN mg.ml⁻¹
0·5
0·4
0·3
0·2
0·1
0
PROTEINASE UNITS. ml⁻¹
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
60
72
84
96
108
120
132
144
VOLUME (ml)

FIG. 7

2 ml were collected at the flow rate of 12 ml/h and were assayed for protein and proteinase activity. The elution profile is shown in Fig. 8. Fractions (23 to 29) with specific activity of 27 000 - 28 500 were pooled, concentrated by lyophilization, dissolved in 0.01 M phosphate buffer, pH 7.0 and dialyzed for 6 h against the same buffer.

At this stage 'alkaline proteinase A' showed single band in disc gel electrophoresis at pH 7.6 and 4.3. Yield and specific activity of 'alkaline proteinase A' at different stages of purification is summarized in Table V.

Criteria of purity

The purified 'alkaline proteinase A' was found homogeneous as revealed by disc gel electrophoresis at pH 7.6 (cathodic run) and pH 4.3 (cathodic run) (Fig. 9). A strict coelution of protein and proteinase activity was observed on Sephadex G-50 (Fig. 8) and isoelectric focusing (Fig. 10). SDS-polyacrylamide gel electrophoresis showed only one protein band (Fig. 9).

Fig. 8 Sephadex G-50 gel filtration of 'alkaline proteinase A' from Step V.

Sample	:	5.0 mg protein in 0.5 ml
Column dimensions	:	1.6 x 100 cm
Buffer	:	0.05 M phosphate buffer, pH 7.0
Flow rate	:	12 ml/h
Symbols	:	△, Proteinase activity
		⊙, Protein

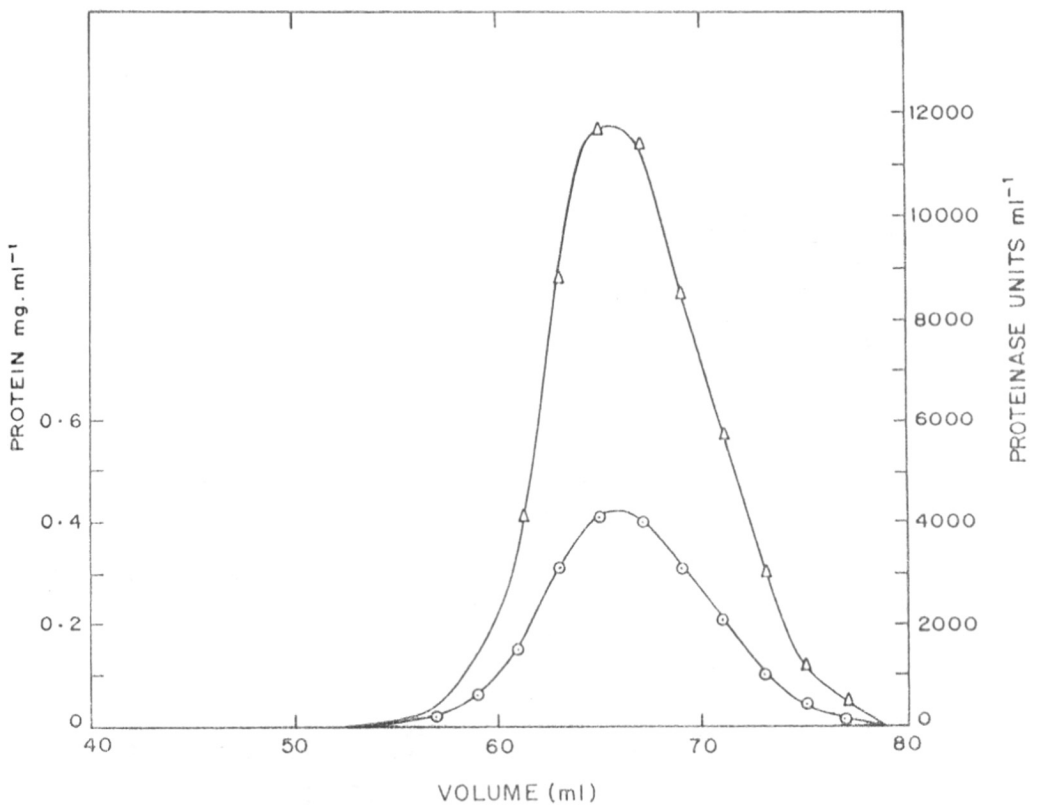

PROTEIN mg.ml⁻¹
0·6
0·4
0·2
0
40
50
60
70
80
VOLUME (ml)
12000
10000
8000
6000
4000
2000
0
PROTEINASE UNITS ml⁻¹

FIG. 8

TABLE V : PURIFICATION OF 'ALKALINE PROTEINASE A' FROM CONIDIOBOLUS sp. (NCL 82.1.1)

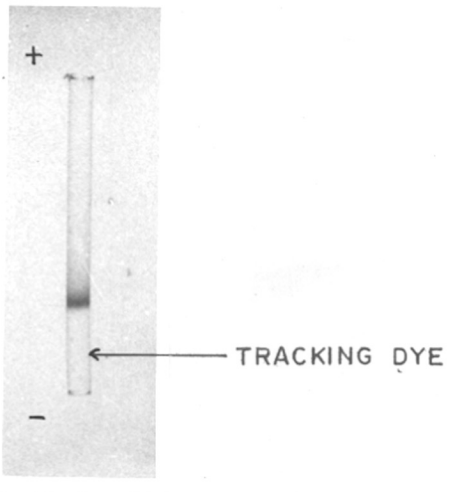
Step	Total protein (mg)	Total units (PU x 10^{-3})	Specific activity	Purification (fold)	Recovery (%)
I. Crude extract	8000	20000	2500	0	100
II. Ethanol precipitation	1920	14400	7500	3.0	72
III. DEAE-cellulose (negative adsorption)	597	11952	22020	8.0	59.8
IV. Ammonium sulfate precipitation (0.9 saturation)	439	9323	21237	8.5	46.6
V. Preparative PAGE					
'Alkaline Proteinase A'	130	3170	24385	9.8	15.8
'Alkaline Proteinase B'	56	1358	24250	9.7	6.8
VI. CM-cellulose chromatography of 'Alkaline Proteinase A'	90	2314	25710	10.3	11.6
VII. Sephadex G-50 gel filtration of 'Alkaline Proteinase A'	65	1850	28500	11.4	9.25

Fig. 9 Polyacrylamide gel electrophoresis of 'alkaline proteinase A'

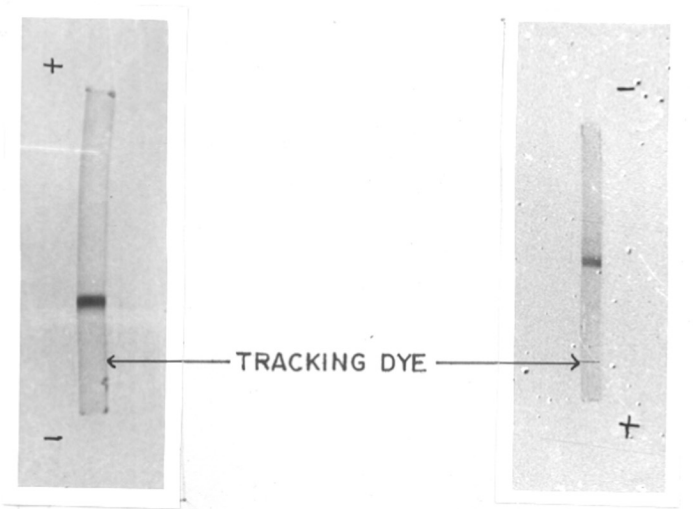
(a) Disc gel electrophoresis at pH 7.6 (cathodic run)

(b) Disc gel electrophoresis at pH 4.3 (cathodic run)

(c) SDS-gel electrophoresis when the enzyme was treated with 1% B-mercaptoethanol and 0.5% iodoacetamide.



a



b c

FIG. 9

Fig. 10 Isoelectric focusing profile of 'alkaline proteinase A' over a pH range of 9.0 - 11.0

Sample : 5.0 mg protein

Symbols : ⊙ , Proteinase activity

△ , Protein

• , pH

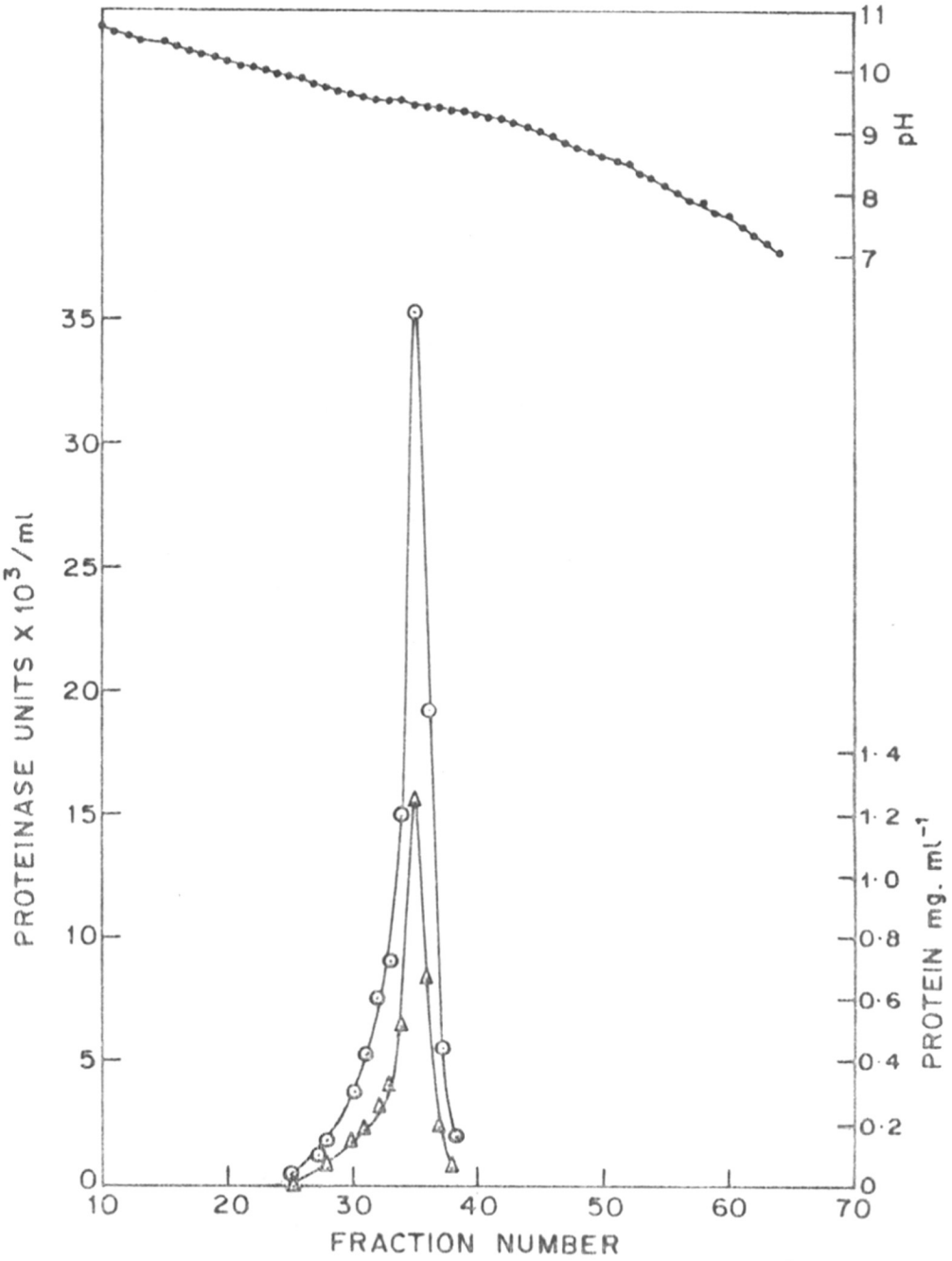

11
10
9
pH
8
7
35
30
25
20
15
10
5
0
PROTEINASE UNITS X 10³/ml
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
PROTEIN mg. ml⁻¹
10
20
30
40
50
60
70
FRACTION NUMBER

FIG. 10

DISCUSSION

'Alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was purified by solvent precipitation, fractionation with ammonium sulfate, preparative polyacrylamide gel electrophoresis, ion exchange chromatography and gel filtration. During precipitation of ethanol, considerable amount of impurities were eliminated in the supernatant. Acidic proteins and coloured impurities were removed by adsorption on DEAE-cellulose where alkaline proteinase remained unadsorbed. The batchwise process of purification used in this work will be advantageous for large scale purification of the enzyme.

'Alkaline proteinase A' and 'B' were separated by preparative PAGE. By using this procedure, substantial purification of the two proteinases was achieved by eliminating all other proteins moving differently in the gel. This step eliminates the elaborate purification procedures such as gradient ion-exchange chromatography. In some cases this step can be a one step purification procedure. However, in this procedure it is essential to remove the non-protein impurities of acrylamide and acrylate.

The apparatus used for preparative PAGE is simple and leakproof. Several other available models of vertical gel electrophoresis including commercial models possess one or more of the following disadvantages. The gels are made separately in gel casting device and then transferred to the electrophoresis apparatus, thus developing mechanisal stresses (266 - 270). Hydrostatic equilibrium is not attained (266 - 269, 271). Moreover to avoid leakage the above metnioned models and also those described by Blatter (272), Roberts and Jones (273) and Andrew et al. (274) require devices such as clamps, screws, O-rings, gaskets, melted agar, greese and plasticine clay.

In the apparatus developed in this work gel slabs of various thickness (0.04 cm to 1.0 cm) can be made and also more than one gel can be run simultaneously. Hydrostatic balance (266) is automatically adjusted due to the position of the two buffer chambers. Mechanical stress (266) on the gel is also avoided by in situ polymerization of the gel and subsequent electrophoresis in the same apparatus. Contact gel is reusable. The apparatus shows some resemblance with the Pharmacia gel making apparatus (Pharmacia Fine Chemicals, Sweden, Gel Slab Casting Apparatus GSC-8).

However, the introduction of one of the main vital modifications, the gap kept below the central partition, in our apparatus has made a vast difference making the unit a 'two-in-one' type i.e. the same apparatus can be used for gel casting and subsequent electrophoresis also. Introduction of one empty cassettee between the two slab gels provides the necessary cooling. The hollow wedge gets filled when chamber 'a' is filled with bath buffer which cools the gel (Fig. 6). Also the middle partition wall offers cooling by the bath buffer of chamber 'b'. The run was carried out at $4^{o}C$ in a cold room. During the run temperature rise in the bath buffer and in the gel was not more than $3^{o}C$ and $7^{o}C$, respectively.

Electrophoretic elution of the enzyme was caffied out across the thickness of the gel, hence distance travelled by the proteins for elution is very short. This resulted in rapid elution of the enzyme. During electrophoretic elution of the enzyme, some acrylamide impurities were carried along with the enzyme. Determination of protein by Lowry's method (239) or spectrophotometric method (241) became erroneous because of the interferrence due to acrylamide impurities. Hence in this case protein determinations

were carried out using Coomassie Brilliant Blue G-250 binding method (246) since acrylamide impurities did not interfere in this assay. These impurities and other traces of protein impurities were removed by dialysis and further purification on CM-cellulose and Sephadex G-50 column chromatography.

The procedure developed for purification of 'alkaline proteinase A' from Conidiobolus sp. (NCL 82.1.1) resulted in a 11.4-fold purification with 9% yield. The specific activity, yield and fold purification of some of the fungal alkaline proteinases isolated by other investigators is summarized in Table VI. The specific activities of alkaline proteinases from Conidiobolus sp. (NCL 82.1.1), A. oryzae (65), A. flavus (71), A. sydowi (75), A. sulphureus (81) and T. album (275) are comparable. Specific activity of 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) is about 50 times higher than that of Conidiobolus sp. reported by Tokuyama and Asano (125). It is worth noting that the fold purification of 'alkaline proteinase A' is considerably less (11.4-fold) when compared with other preparations (50-120 fold) but the resulting specific activity is high. This suggests that the ratio of 'alkaline proteinase A' to other proteins in the Conidiobolus sp. (NCL 82.1.1) culture broth is high.

TABLE VI : SPECIFIC ACTIVITIES, FOLD PURIFICATION AND YIELDS OF FUNGAL ALKALINE PROTEINASES

Organism	Specific activity[a] (PU/mg protein)	Purification (fold)	Yield (%)	Reference
Conidiobolus sp. (NCL 82.1.1)	28 500	11.4	9	Present work
Conidiobolus sp.	556	50.0	5	125
A. flavus	23 800	120.0	12	71
A. sulphureus	20 810	10.3	14	81
A. sojae	4 121	-	-	76
A. sydowi	24 000	4.5	9	75
A. oryzae[b]	31 000	55.0	14	65
T. album	29 090	15.0	28	275

[a] For comparision the specific activities mentioned here are converted into Kunitz units from the original data

[b] Aspergillopeptidase B

(-) indicates that the data is not cited in the reference

CHAPTER IV

PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF 'ALKALINE PROTEINASE A' AND APPLICATIONS OF ALKALINE PROTEINASE FROM *CONIDIOBOLUS* sp. (NCL 82.1.1)

SECTION I

PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES

SUMMARY

Physicochemical properties and substrate specificity of purified 'alkaline proteinase A' from Conidiobolus sp. (NCL 82.1.1) were studied. The enzyme is composed of a single polypeptide chain with molecular weight of 22 000. It is a basic protein with isoelectric point of 9.45. The enzyme does not contain carbohydrate moiety. 'Alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was found stable for a period of one year in solution form in the presence or absence of glycerol at low temperatures. However, it was unstable in powder form. The enzyme showed maximum stability at pH 6.5 - 7,0 and below 30°C. Optimum pH and temperature with casein as substrate were pH 10.0 and 40°C, respectively. The enzyme is classified as a serine proteinase with a possible role of histidine and aspartic acid or glutamic acid residue in the catalytic function of the enzyme.

'Alkaline proteinase A' hydrolyzed casein, denatured and native hemoglobin, oxidized lysozyme, oxidized B-chain of insulin, azocoll, azocasein and azoalbumin. Bovine serum albumin and ovalbumin were the least preferred substrates. The enzyme showed

esterase activity on ATEE, BTEE, TAME, BAEE and APME. Kinetic parameters for these substrates were determined. The 'alkaline proteinase A' of Conidiobolus sp.(NCL 82.1.1) exhibited low K_m values indicating higher affinity of the enzyme than subtilisins. However, the V_{max} values were lower for 'alkaline proteinase A' than those for subtilisins. This suggests that its deacylation rate is lower than subtilisins.

'Alkaline proteinase A' did not show any amidase activity on DL-leucinamide, benzoyl-L-tyrosinamide, BAPNA, BTPNA and L-leucine-p-nitroanilide.

Substrate specificity of 'alkaline proteinase A' was compared with that of subtilisin Carlsberg by using peptide mapping technique. The peptide maps of digests obtained from aminoethylated globin, oxidized lysozyme and oxidized B-chain of insulin by 'alkaline proteinase A' and subtilisin Carlsberg were comparable indicating broad specificity of 'alkaline proteinase A' as subtilisin Carlsberg. However, 'alkaline proteinase A' cleaved comparatively more peptide bonds indicating wider specificity than subtilisin Carlsberg.

INTRODUCTION

Extracellular alkaline proteinases from different groups of fungi are investigated in detail. However, few studies are reported on purification, characterization and substrate specificity of extracellular alkaline proteinases from fungi belonging to phycomycetes. Although the production of extracellular alkaline proteinases by Entomophthora (121 -123), Basidiobolus (121) and Conidiobolus (121, 124, 126) is studied, reports on their purification and characterization are rare. A report by Tokuyama and Asano (125) describes the physicochemical properties of purified alkaline proteinase from Conidiobolus sp., whereas Ishikawa et al. (126) have reported some properties of partially purified alkaline proteinase from Conidiobolus lamprauges.

The fungal alkaline proteinases, in general, and subtilisins are endopeptidases with broad specificity towards hydrolysis of proteins and possess esterolytic activity (278, 283). These are classified as serine proteinases since serine residue is located at their active site (50, 128, 138 -141, 164, 165). Involvement of histidine in the catalytic action is also demonstrated in some of these enzymes (141, 143 - 148, 166).

As described earlier (Chapter III) Conidiobolus sp. (NCL 82.1.1) secretes high amounts of 'alkaline proteinase A' which is purified to homogeneity. This Chapter describes the physicochemical properties and substrate specificity of 'alkaline proteinase A'. Its properties are compared with other fungal alkaline proteinases and subtilisins.

RESULTS AND DISCUSSION

(a) Physicochemical properties

1. Molecular weight

(i) Gel filtration. Molecular weight of 'alkaline proteinase A' was determined by comparing its elution volume with the elution volumes of marker proteins of known molecular weight from Sephadex G-75 column (1.6 x 100 cm). The molecular weight of 'alkaline proteinase A' as calculated from the graph of V_e/V_o against log molecular weight plotted according to Andrews (253) was 21 880 (Fig. 11).

(ii) SDS-gel electrophoresis. Molecular weight of 'alkaline proteinase A' and its subunit structure was determined from its migration in the SDS-polyacrylamide gel according to the method of Shapiro et al.(256) and Weber and Osborn (257). A plot of log molecular weight versus relative mobilities of marker proteins indicated the molecular weight of 'alkaline proteinase A' to be 22 000 (Fig. 12). Reduction and carboxyamidomethylation of the enzyme with 1% SDS, 1% β-mercaptoethanol and 0.5% iodoacetamide showed only one protein band in SDS-gel electrophoresis, with molecular weight corresponding to the native protein. This indicated that the enzyme is composed of a single polypeptide chain.

Fig. 11 Molecular weight determination of 'alkaline proteinase A' by gel filtration on Sephadex G-75 column (1.6 x 100 cm) calibrated with :

1. BSA (MW 67 000)
2. Ovalbumin (MW 43 000)
3. Chymotrypsinogen A (MW 25 000)
4. Myoglobin (MW 17 600)
5. Cytochrome c (MW 13 000)

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

V_o - Void volume V_e - Elution volume

Fig. 12 Molecular weight determination of 'alkaline proteinase A' by SDS-gel electrophoresis. Relative mobility was plotted against the log molecular weight of marker proteins. The marker proteins used were :

1. BSA (MW 66 000)
2. Ovalbumin (MW 45 000)
3. Pepsin (MW 34 700)
4. Trypsinogen (MW 24 000)
5. B-Lactoglobulin (MW 18 400)
6. Lysozyme (MW 14 300)

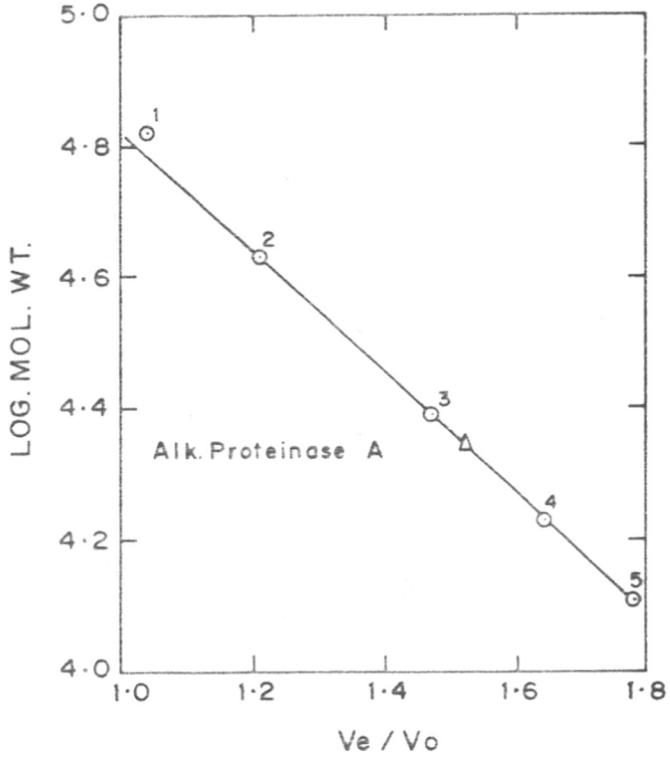

5·0
4·8
4·6
4·4
4·2
4·0
LOG. MOL. WT.
Alk. Proteinase A
1
2
3
4
5
1·0
1·2
1·4
1·6
1·8
Ve / Vo

FIG. 11

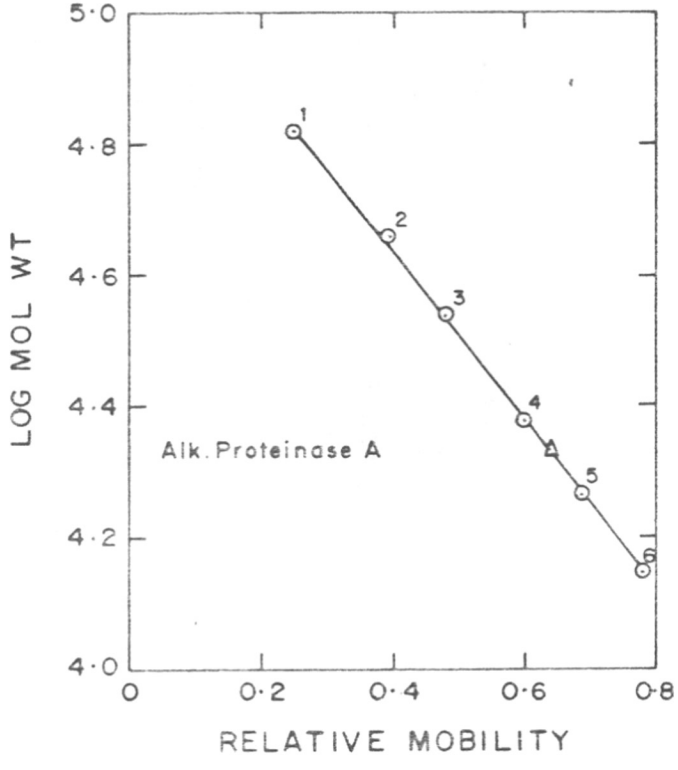

5·0
4·8
4·6
4·4
4·2
4·0
LOG MOL WT
Alk. Proteinase A
1
2
3
4
5
6
0
0·2
0·4
0·6
0·8
RELATIVE MOBILITY

FIG. 12

Molecular weight of 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) as revealed by gel filtration and SDS-gel electrophoresis, was 21 880 and 22 000, respectively whereas when calculated from the amino acid composition it was 25 450 (data presented in amino acid composition, Table VII) which is in close resemblance with other two methods. Most of the microbial extracellular alkaline proteinases exhibit molecular weight in the range of 20 000 - 28 000 (Table I). However, molecular weight of alkaline proteinases isolated from A. oryzae EI 212 (69), A. oryzae OUT 5038 (64), P. cyaneofulvum (85) and P. omnivorum (120) are 35 000, 52 000, 45 000 and 33 000,respectively. Rappaport et al. (61, 62) have reported a subtilisin like proteinase from a transformable strain of B. subtilis which has a molecular weight of 166 000 and probably consists of several sub units.

2. Isoelectric point (pI)

Isoelectric focusing of 'alkaline proteinase A' was carried out using ampholine carrier ampholytes of a pH range 9.0 - 11.0. A pilot run was performed by using wide range (pH 3.5 - 10.0) ampholine carrier ampholytes. Isoelectric point of the enzyme was

9.45 (Fig. 10). Subtilisins and an alkaline proteinase of A. tenuissima (94) are reported to have isoelectric points in the alkaline pH range (Table I). However, pI of alkaline proteinases from A. sojae (76) and A. candidus (79) were 5.1 and 4.9, respectively (Table I).

3. Glycoprotein nature

Glycoprotein nature of 'alkaline proteinase A' was examined by Schiff's staining (261). The purified enzyme did not show a purple-red band corresponding to protein band in the simultaneoulsy run polyacrylamide gel. This indicated that 'alkaline proteinase A' does not contain any covalently bound carbohydrate moiety.

Aspergillopeptidase B of A. oryzae (163) and an alkaline proteinase of A. flavus (71) have been reported to contain small amounts of carbohydrates.

4. Amino acid composition

Amino acid composition of 'alkaline proteinase A' is given in Table VII. The enzyme contained about 266 amino acid residues per mole. Its amino acid analysis showed high content of neutral amino acids (glycine and alanine) followed by hydrophilic amino acids (serine and threonine). Among hydrophobic amino acids valine content was considerably higher than isoleucine

TABLE VII : AMINO ACID COMPOSITION OF 'ALKALINE PROTEINASE A' OF CONIDIOBOLUS sp. (NCL 82.1.1)[a] AND SUBTILISINS

Amino acid	Alkaline Proteinase A (present work)	Subtilisin Carlsberg (129)	Subtilisin BPN'/Novo (132 /134)
	Residues/mole	Residues/mole	Residues/mole
Lysine	11	9	11
Histidine	5	5	6
Arginine	3	4	2
Aspartic acid[b]	38	28	28
Threonine[c]	26	19	13
Serine[c]	30	32	37
Glutamic acid[b]	5	12	15
Proline	7	9	14
Glycine	38	35	33
Alanine	43	41	37
Valine[d]	22	31	30
Methionine[e]	2	5	5
Isoleucine[d]	10	10	13
Leucine[d]	12	16	15
Tyrosine	5	13	10
Phenylalanine	6	4	3
Tryptophan	2	1	3
Cysteine[f]	1	0	0
Total	266	274	275
Molecular weight	25 454	27 287	27 532

[a]The calculated number of residues per mole of 'Alkaline proteinase A from Conidiobolus sp. (NCL 82.1.1) are based upon molecular weight of 22 000.

[b]Values of aspartic acid and glutamic acid include aspargine and glutamine values, respectively.

[c]Extrapolated to xero time

[d]72 h value is reported

[e]Determined as methionine sulfone after performic acid oxidation

[f]Determined as cysteic acid after performic acid oxidation

and leucine. Aromatic amino acids content was low. Aspartic acid content was about 7.5 times higher than glutamic acid. The values of aspartic acid and glutamic acid also account for aspargine and glutamine, respectively, which are deaminated during acid hydrolysis. Presence of one cysteine and two methionine residues was observed. Half cystine content was determined as cysteic acid after oxidation with performic acid (259) followed by hydrolysis and amino acid analysis. Unidentified ninhydrin sensitive peak was not observed in the amino acid analysis profile of 'alkaline proteinase A' hydrolysate.

High contents of glycine, alanine, and serine in 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) resembles with subtilisins (129, 132, 134). Alkaline proteinases of A. sojae (76) and A. tenuissima (94)(Table II) also contain about same number of serine residues as in 'alkaline proteinase A'. The number of isoleucine residues in most of the alkaline proteinases is similar, whereas leucine content in subtilisins (129, 132, 134) is higher than 'alkaline proteinase A'. Glutamic acid (glutamic acid + glutamine) in 'alkaline proteinase A' is significantly lower (5 residues) as compared to other proteinases (12 to 19 residues, Table II). Among aromatic amino acids number of tyrosine residues in 'alkaline proteinase A' is about half of those in subtilisins (129, 132, 134). Presence of one cysteine residue in 'alkaline proteinase A'

is unusual since subtilisins and most of the fungal alkaline proteinases are devoid of cysteine (Table II). Subtilisins contain more methionine residues (5 residues) than 'alkaline proteinase A' (2 residues) and other fungal proteinases (1 to 2 residues). Valine residues in 'alkaline proteinase A' are less than those in subtilisins and more than other fungal proteinases (Table II).

In conclusion, amino acid composition of 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) resembles more with subtilisin Carlsberg than other alkaline proteinases. However, significant difference is observed in the contents of sulfur containing amino acids of 'alkaline proteinase A' and subtilisin Carlsberg.

(b) Enzymatic properties

The rate of proteolysis by 'alkaline proteinase A', under standard assay conditions, was linear upto 5.0, 3.0, 5.0 and 2.0 µg of enzyme protein with casein, native and denatured hemoglobin and bovine serum albumin as substrates, respectively (Fig. 13). The enzyme showed milk clotting activity but the clot was dissolved on prolonged incubation. Qualitative analysis showed that the enzyme did not clot fibrinogen, however, the fibrin clot formed from fibrinogen by thrombin was dissolved by 'alkaline proteinase A'.

Fig. 13 Rate of proteolysis by 'alkaline proteinase A' of :

1. Casein
2. Hemoglobin (denatured)
3. Hemoglobin (native)
4. BSA
5. Ovalbumin

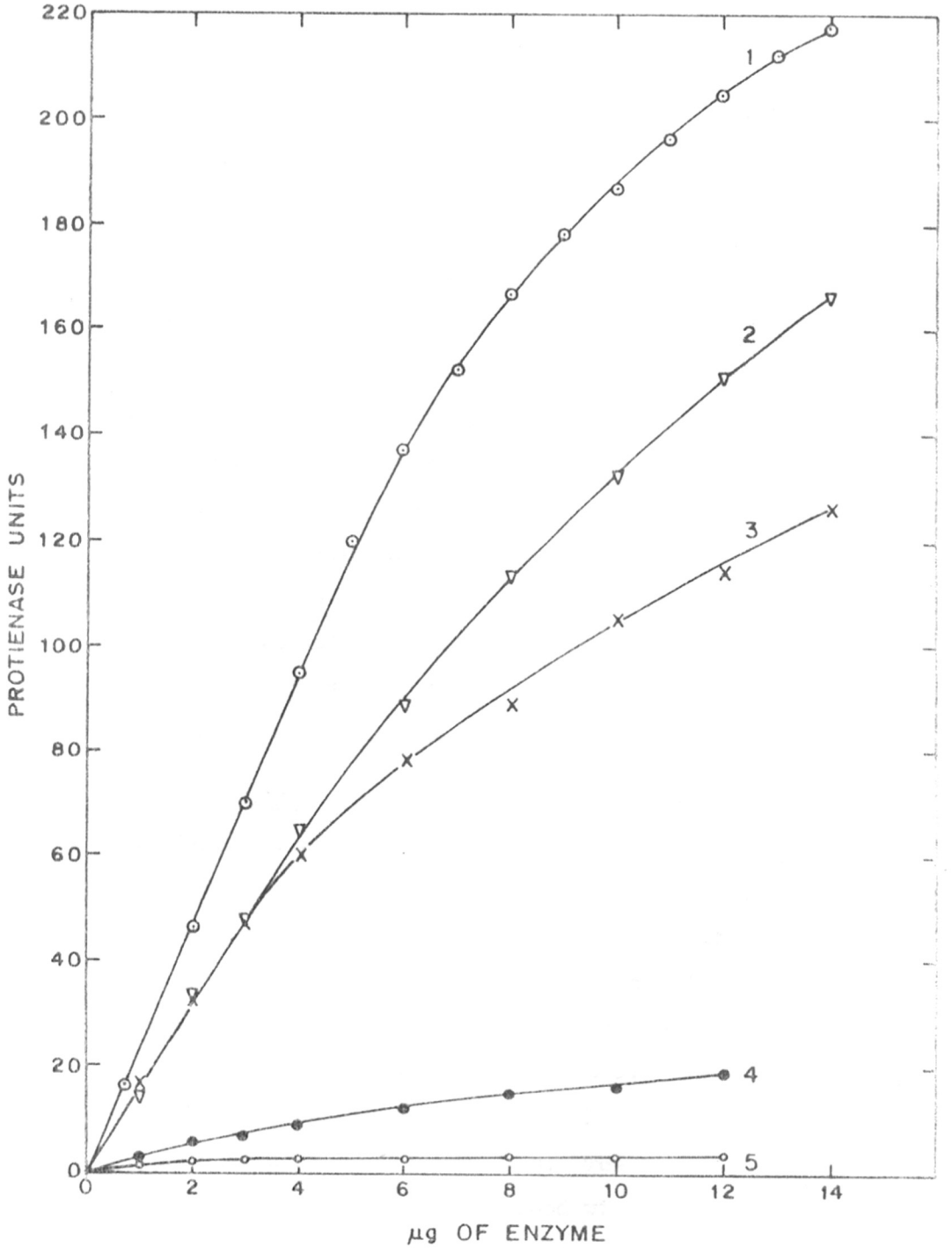

220
200
180
160
140
120
100
80
60
40
20
0
PROTIENASE UNITS
0
2
4
6
8
10
12
14
µg OF ENZYME
1
2
3
4
5

FIG. 13

(1) Enzyme stability

The stability studies of the enzyme are essential since alkaline proteinases are used industrially and in various products. The purified enzyme was stable when stored at -20°C between pH 6.0 - 9.0 in 10% glycerol solution. No significant loss of activity was observed over a period of one year. It was also observed that the enzyme retained atleast 95% of its activity when stored in solution form at a concentration of at least 1 mg/ml at pH 6.0 - 9.0, whereas the enzyme in powder form lost its activity substantially within a few months. The purified 'alkaline proteinase A' lost considerable activity on repeated freezing and thawing.

(i) Effect of pH. Effect of pH on stability of 'alkaline proteinase A' was studied by incubating the enzyme in the pH range of 4.5 - 9.5. Different buffers used to cover the pH range were sodium citrate-citric acid (pH 4.5), sodium acetate-acetic acid (pH 5.0), potassium phosphate (pH 5.5 - 7.5), Tris-HCl (pH 8.0 - 8.5) and sodium carbonate-bicarbonate (pH 9.0 - 9.5). 16 µg of 'alkaline proteinase A' in 0.4 ml of 0.05 M buffer was incubated at 50°C for 10 min. The residual proteinase activity was determined with 0.1 ml aliquot from the incubation mixture by caseinolytic assay and

compared with the control kept at 4°C, pH 7.0. 'Alkaline proteinase A' was most stable at pH 6.5 - 7.0 (Fig. 14). The activity was lost almost completely below pH 4.5 and above pH 9.5.

(ii) Effect of temperature. 'Alkaline proteinase A' (16 µg in 0.4 ml of 0.05 M phosphate buffer, pH 7.0) was incubated at various temperatures ranging from 0° to 60°C. After one hour 0.1 ml aliquot was estimated for the residual proteinase activity. Figure 15 shows the thermostability curve of 'alkaline proteinase A'. The enzyme was found to be most stable upto 30°C. 50% of the activity was retained at 50°C, however, the enzyme completely lost its activity above 55°C. Bovine serum albumin offers no special protective effect against heat inactivation.

Most of the fungal alkaline proteinases are stable in the pH range of 5.0 - 10.0 at low temperatures (Table III). They exhibit maximum stability at neutral pH and are inactivated completely below pH 5.0 and above pH 10.0. A report by Tokuyama and Asano (125) describes that an alkaline proteinase of Conidiobolus sp. is stable upto pH 12.0 when kept at 4°C for 24 h. 'Alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was found most stable at pH 6.5 - 7.0 when incubated at 50°C for 10 min and

Fig. 14 pH Stability of 'alkaline proteinase A' The enzyme (16 ug) was incubated at 50°C for 10 min in 0.4 ml of 0.05 M buffer in the pH range of 4.5 - 9.5. An aliquot (0.1 ml) from the incubation mixture was assayed for residual activity by caseinolytic assay procedure.

Fig. 15 Thermal stability of 'alkaline proteinase A' The enzyme (16 ug) in 0.4 ml of 0.05 M phosphate buffer, pH 7.0 was incubated at different temperature for 1 h. 0.1 ml aliquot was estimated for the residual proteinase activity by caseinolytic assay procedure.

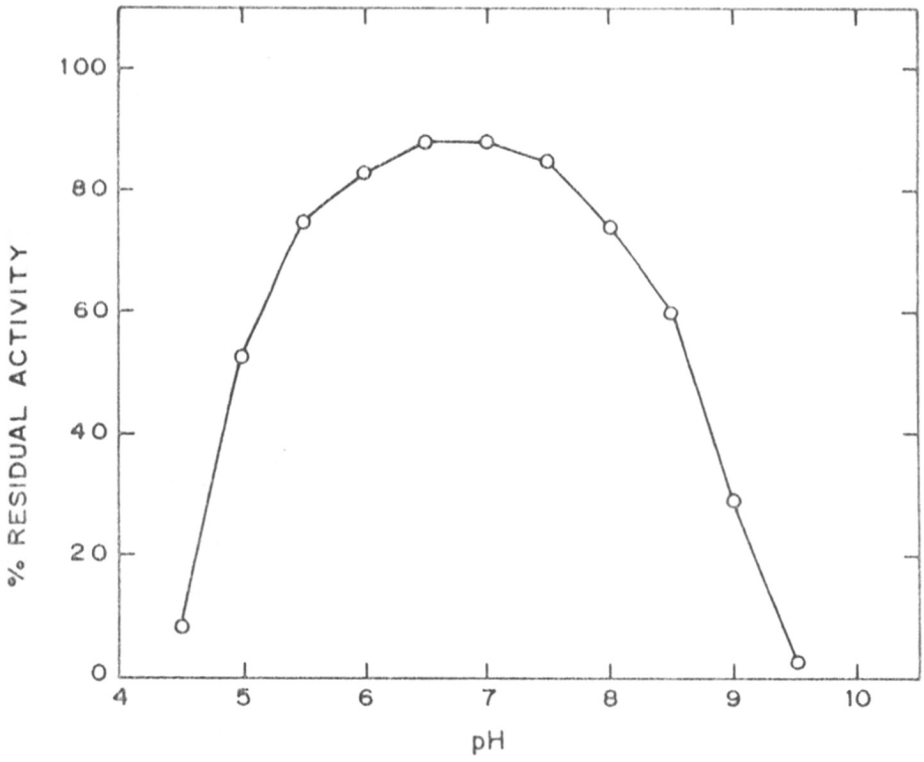

% RESIDUAL ACTIVITY
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

FIG. 14

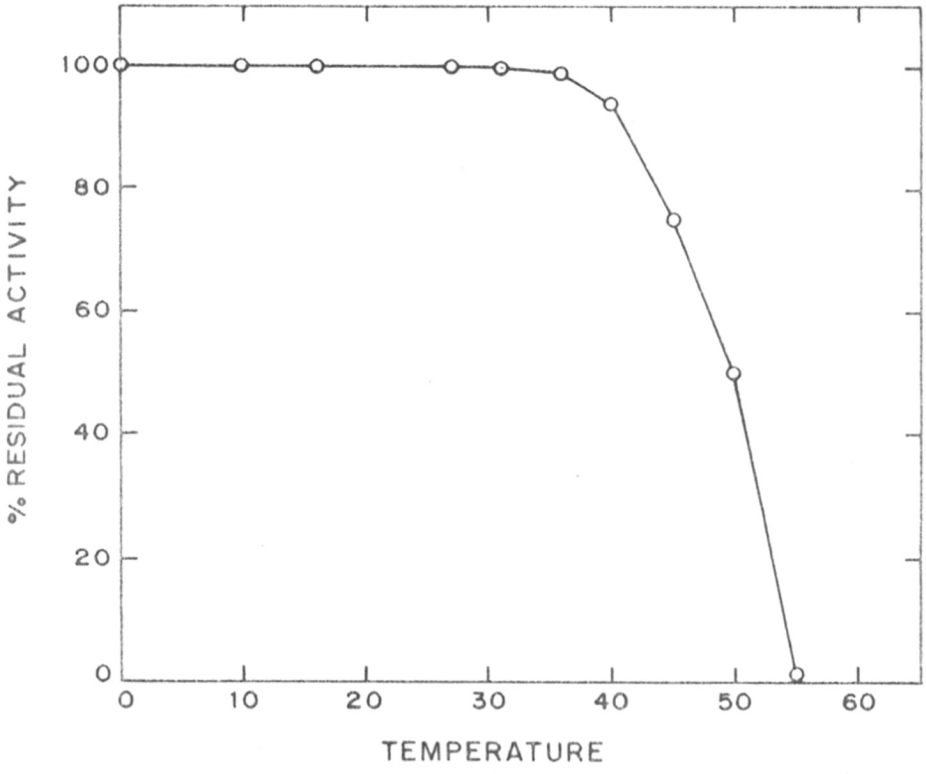

% RESIDUAL ACTIVITY
100
80
60
40
20
0
0
10
20
30
40
50
60
TEMPERATURE

FIG. 15

lost its activity completely below pH 4.5 and above pH 9.5. However, when kept at -20°C, the enzyme did not show significant loss in the activity for a period of one year at pH 6.0 - 9.0. Subtilisin Carlsberg, BPN' and Novo are most stable at pH 5.3 - 6.5 than at alkaline pH values (pH 8.0 - 9.5). Their susceptibility to alkaline pH is attributed to autolysis. These enzymes loose their activity below pH 5.0. However, they are stable for several months in glycerol and at -10°C in lyophilized state.

Fungal proteinases, in general, are unstable at temperatures above 55° - 60°C (Table III). Alkaline proteinase of Conidiobolus sp. (125) is reported to exhibit stability upto 40°C at pH 8.0 but above 40°C inactivation rate increases sharply. 'Alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was found to be most stable upto 30°C and was inactivated completely above 55°C. Replacement of aspargine at position 218 by serine in a genetically engineered subtilisin increased its stability by four fold at 65°C in the presence of $CaCl_2$ and three fold at 45°C with EDTA (276).

2. Optimum pH

Proteinase activity of 'alkaline proteinase A' was determined at different pH values (6.0 - 11.0) by the caseinolytic assay. Various buffers used were potassium phosphate (pH 6.0 - 7.5), Tris-HCl (pH 8.0 - 8.5) and sodium carbonate-bicarbonate (pH 9.0 -19.5). Figure 16 shows the enzyme activity profile at different pH values. 'Alkaline proteinase A' showed maximum activity at pH 10.0.

Alkaline proteinases, as the name indicates, show a broad pH optima in the pH range of 7.0 - 11.0 with casein as substrate (Table III). Alkaline proteinase of A. fumigatus (74) shows bimodal pH activity profile with maximum activity at pH 7.5 and pH 10.0.

3. Optimum temperature

Activity of .'alkaline proteinase A' with casein as a substrate was determined at pH 10.0 using various temperatures (25°C - 55°C). Optimum temperature for the proteolytic activity of 'alkaline proteinase A' was found to be 40°C (Fig. 17). Alkaline proteinases from various fungal sources exhibit optimum temperatures between 40°C and 50°C (Table III).

Fig. 16 Optimum pH of 'alkaline proteinase A'

The proteinase activity was determined at indicated pH values (6.0 - 11.0) by the caseinolytic assay as described in the text.

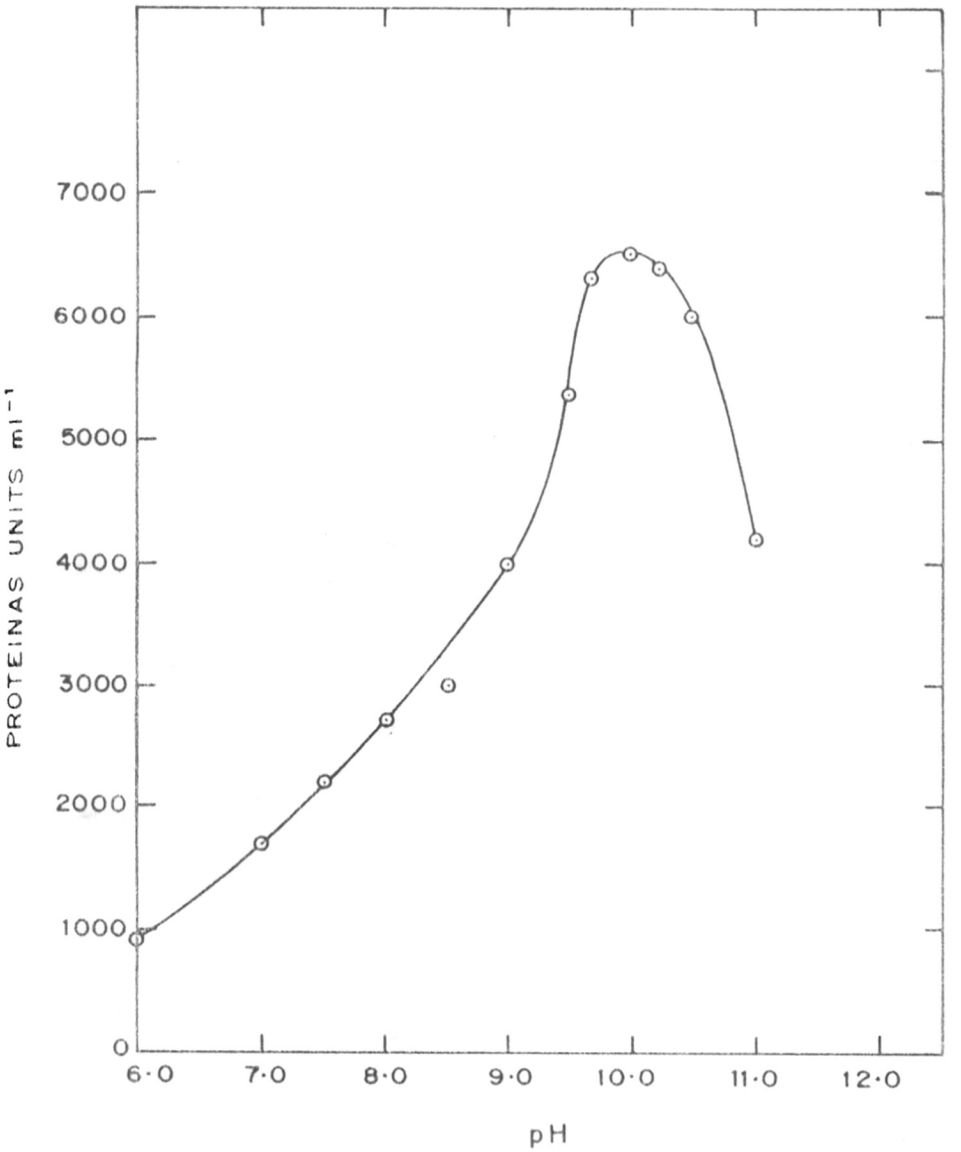

PROTEINAS UNITS ml⁻¹
7000
6000
5000
4000
3000
2000
1000
0
6·0
7·0
8·0
9·0
10·0
11·0
12·0
pH

FIG. 16

Fig. 17 Optimum temperature of 'alkaline proteinase A' The proteinase activity was determined at pH 10 with casein as a substrate at indicated temperatures.

Fig. 18 Arrhenius plot showing effect of temperature on the rate of proteolysis of casein by 'alkaline proteinase A' under standard assay conditions.

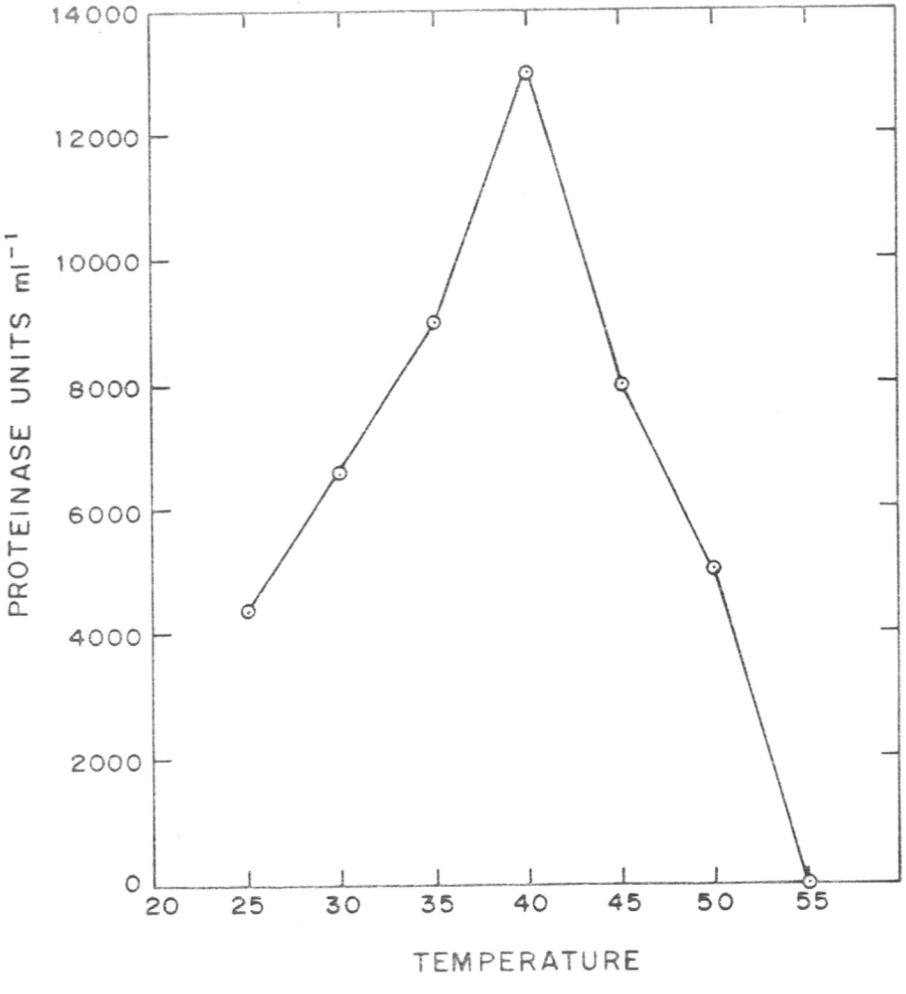

14000
12000
10000
8000
6000
4000
2000
0
PROTEINASE UNITS ml⁻¹
20
25
30
35
40
45
50
55
TEMPERATURE

FIG. 17

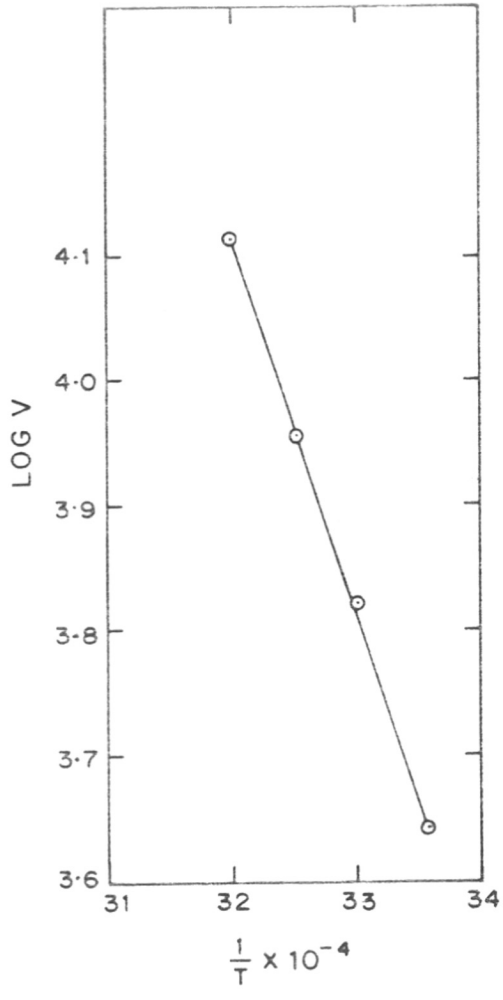

4·1
4·0
3·9
3·8
3·7
3·6
LOG V
31
32
33
34
1/T x 10⁻⁴

FIG. 18

TABLE VIII : EFFECT OF METAL IONS ON 'ALKALINE PROTEINASE A' ACTIVITY[a]

Compound	Relative activity (%)
None	100
$ZnSO_4.7H_2O$	96
$CuSO_4.5H_2O$	106
$FeSO_4.7H_2O$	219
$MnCl_2.4H_2O$	132
$CoCl_2.6H_2O$	131
$MgSO_4.7H_2O$	115
$BaCl_2.2H_2O$	145
$CaCl_2.2H_2O$	155
$Na_2WO_4.2H_2O$	100
$HgCl_2$	3

[a] The enzyme (6 µg) was incubated with the metal ion at 30°C and pH 8.4 for 15 min. The final concentration of metal ion was 1 x 10^{-3}M in the incubation mixture.

The energy of activation of 'alkaline proteinase A' with casein as a substrate, as calculated from Arrhenius plot (Fig. 18) was 12.785 kcal/mol (58 KJ).

4. Effect of metal ions on proteinase activity

Effect of various metal ions on proteinase activity of 'alkaline proteinase A' was determined by pre-incubating the enzyme with various metal ions. 6.0 µg of the enzyme in 1.0 ml of 0.1 M Tris-HCl buffer, pH 8.4, containing 1 x 10^{-3}M metal ion was incubated at 30°C. After 15 minutes the reaction was started by adding 1.0 ml of casein (1% in 0.1 M Tris-HCl buffer, pH 8.4). Reaction was carried out at 35°C for 30 min. Enzyme activity was compared with the control incubated without metal ion under similar conditions. Tris-HCl buffer, pH 8.4 was used for this experiment since some of the metal salts get precipitated in carbonate buffer, pH 10.0.

Effect of different metal ions on proteolytic activity of purified 'alkaline proteinase A' is summarized in Table VIII. Fe^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, and Ca^{2+} caused increase in the enzyme activity by 119, 32, 31, 45, and 55 percent, respectively. Hg^{2+} showed strong inhibition (97%) of the proteinase activity. A number of other alkaline proteinases e.g. purified enzymes from

A. oryzae (127), A. sydowi (75), A. sojae (162), P. cyaneofulvum (85) and Conidiobolus sp. (125) are inhibited by Hg^{2+}.

Metal ion inactivation of fungal alkaline proteinases is well documented and Zn^{2+}, Hg^{2+}, Ni^{2+} and Cu^{2+} are common metal ion inhibitors. Alkaline proteinase isolated by Tokuyama and Asano (125) from Conidiobolus sp. was inhibited by Ba^{2+}, Ag^{2+}, Hg^{2+}, Al^{2+} and Cu^{2+}. 'Alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was inhibited by Hg^{2+}, however, Ba^{2+} and Cu^{2+} did not inhibit the enzyme. Zn^{2+} was found inhibitory to some of the alkaline proteinases (63, 81, 85, 127) but it did not show any effect on the proteolytic eactivity of 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1). Cu^{2+} also did not show any effect on 'alkaline proteinase A'. However, it is reported to inhibit the alkaline proteinases of A. sojae (162), A. sulphureus (81), A. oryzae (63) and aspergillopeptidase C of A. oryzae (127). Aspergillopeptidase B (163) also showed no effect of Zn^{2+} and Cu^{2+} similar to 'alkaline proteinase A'.

5. Effect of EDTA, cysteine hydrochloride and proteinase inhibitor of subtilisin

A sample of 'alkaline proteinase A' (4 µg in 1.0 ml of sodium carbonate-bicarbonate buffer, pH 10.0) was incubated at 30°C with EDTA (20 mM), cysteine hydrochloride (50 mM) and proteinase inhibitor of subtilisin from

Dolichos biflorus (289) (4×10^{-5} mM i.e. inhibitor to enzyme ratio 1:4 mole/mole). After 20 min the reaction was initiated by adding 1 ml of 1% casein (in 0.1 M sodium carbonate-bicarbonate buffer, pH 10) and the caseinolytic assay was performed. The percent activity retained was calculated by comparing residual activity with the controls incubated under similar conditions without the additives.

There was no effect of EDTA or cysteine hydrochloride on 'alkaline proteinase A' activity. Alkaline proteinases of other fungal sources were also not inhibited by EDTA (68, 74, 81, 85, 127) with few exceptions such as an alkaline proteinases of Conidiobolus sp. (125) and P. omnivorum (119, 120) which are inhibited by EDTA. Cysteine hydrochloride is reported to have no inhibitory effect on most of the fungal alkaline proteinases (74, 75, 81, 85, 98, 127, 162). A specific subtilisin inhibitor showed 50% inhibition of proteolytic activity of 'alkaline proteinase A'.

6. Effect of active site group specific reagents on proteinase activity

Effect of various active site group specific reagents on the 'alkaline proteinase A' was studied. Samples of 'alkaline proteinase A' (80 µg) were incubated at indicated reagent concentration and time interval (Table IX) in 1.0 ml of 0.05 M Tris-HCl buffer, pH 8.4 at 30°C; except for 1-cyclohexyl-3-(2-morpholinoethyl)-

TABLE IX : EFFECT OF ACTIVE SITE GROUP SPECIFIC REAGENTS ON 'ALKALINE PROTEINASE A' ACTIVITY

Specific Reagent	Concentration (mM)	Incubation time (Min)	Activity (%)
Serine			
(a) Control			100
(b) Phenylmethane sulfonyl fluoride (PMSF)	0.1	15	0
Arginine			
(a) Control			100
(b) Phenylglyoxal	10.0	60	102
(c) 2,3-Butanedione	50.0	90	100
Histidine			
(a) Control			100
(b) N-Tosyl-L-lysine chloromethyl ketone (TLCK)	2.0	30	102
(c) N-Tosyl-L-phenyl-alanine chloro-methyl ketor (TPCK)	2.0	30	107
Tyrosine			
(a) Control			100
(b) Iodine	1.0	90	3
(c) N-acetylimidazole	10.0	90	71
Glutamate/Aspartate			
(a) Control			100
(b) 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMC)	10.0	90	54

carbodiimide metho-p-toluene sulfonate (CMC) and N-acetylimidazole which were incubated in 1.0 ml of 0.05 M potassium phosphate buffer, pH 7.0 at 4°C. The treated enzyme was then dialyzed against the incubation buffer for 5 h to remove excess of the reagent. An aliquot (0.1 ml) from this dialyzed mixture was assayed for the enzyme activity. Controls were also run under similar conditions in the absence of modifying reagents.

Table IX shows the effect of various active site group specific reagents on the proteinase activity of 'alkaline proteinase A'. The enzyme was completely inactivated by PMSF even at concentration of 0.1 mM. Sulfonylation by PMSF occurs exclusively at the active site serine residue and it does not appear to react outside the reactive site under normal conditions of pH and temperature even when high concentrations are applied for extended periods (277). Inactivation of 'alkaline proteinase A' by PMSF suggests the involvement of serine residue in the catalytic function of the enzyme. 'Alkaline proteinase A', therefore, appears to be a serine proteinase. Involvement of serine residue in the active site of subtilisins is well established (50, 128, 138 - 141). Inactivation of fungal alkaline proteinases by DFP or PMSF indicating the role of

active serine in the mechanism of action of these enzymes is also documented (278).

Phenylglyoxal reacts with the guanidino group of arginine residue under mild conditions (279). The reaction also occurs with ϵ-amino group of lysine residues when proteins are treated with high concentrations of phenylglyoxal for a long period. The arginine residue could also be modified near neutral pH with 2,3-butanedione (280). The reaction was carried in dark to avoid possible photochemical effects. Proteinase activity of 'alkaline proteinase A' was not affected by phenylglyoxal or 2,3-butanedione. This suggests that arginine may not be essential for catalytic function of the enzyme.

The enzyme activity was not altered by TLCK or TPCK which are the specific inhibitors for histidine. This indicates that histidine is not involved in the active site of 'alkaline proteinase A'. However, the hydrolysis of BAEE by 'alkaline proteinase A' at 37°C showed a sigmoid pH-velocity profile over pH range of 4.5 to 9.0 with pK'_a of 6.65. Data regarding this is presented in the synthetic substrate studies. This pK'_a of 6.65 is in close resemblance with pK' value of imidazole to be 6.58 at 37°C. These studies suggest

the involvement of histidine in the catalytic action of 'alkaline proteinase A'. This is in accordance with subtilisins which are also not inhibited by TLCK or TPCK but they are reported to have involvement of histidine in the catalytic action (141, 144, 147, 148). Turkova (166) on the basis of kinetic studies, has suggested the involvement of active histidine in the action of alkaline proteinase of A. flavus.

Iodine and N-acetylimidazole at stoichiometric concentrations did not inactivate the enzyme indicating that tyrosine may not be essential for the enzyme activity. However, excess amount of iodine and N-acetylimidazole showed about 97% and 29% inactivation of the enzyme, respectively. Tyrosine, under mild conditions, is iodinated to mono and diiodo derivatives and to a lesser extent histidine is also modified (281). Iodination of tyrosine residues changes its hydrophobicity which probably alters the enzyme substrate interactions. Role of hydrophobicity in binding of synthetic substrates has been reported by Glazer (144). N-acetylimidazole principally acetylates the tyrosine residues of proteins. However, acetylation of amino group has also been observed. N-Acetylimidazole has shown only 29% inactivation of'alkaline proteinase A'. Strong

inactivation effect of iodine could be because of secondary modification of active site histidine or due to structural alteration of the enzyme which prohibits the substrate binding. To solve the discrepancy observed in tyrosine inactivation, it needs more experimentation by using protective modification approach. Protection from modification of the enzymatic activity by iodination in the presence of competitive inhibitor will demonstrate location of tyrosine at the active site.

Water soluble carbodiimides like CMC is known to react specifically with side chain carboxylic group of aspartic or glutamic acid residues in a protein (282). 'Alkaline proteinase A' showed 54% residual activity after modification with CMC, which suggests the possible essentiality of free carboxylic group for the catalytic function of the enzyme. Role of aspartic acid in the mechanism of action of subtilisins has been suggested by Polgar and Bender (177).

7. Substrate specificity

Substrate specificity of 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) was studied by using various synthetic and protein substrates. It hydrolyzed a variety of proteins including casein, native and

denatured hemoglobin, aminoethylated globin, oxidized lysozyme, oxidized B-chain of insulin, azocasein, azoalbumin, azocoll, and fibrin clot. The enzyme also showed esterase activity on various benzoyl- or acetyl-L-amino acid esters indicating its broad specificity. However, it did not show amidase activity.

(i) Protein substrates. Linearity of the enzyme action on casein, native and denatured hemoglobin, BSA and ovalbumin is shown in Fig. 13. Casein was the most preferred substrate among the tested protein substrates for 'alkaline proteinase A' followed by denatured hemoglobin and native hemoglobin. The enzyme was least active on BSA and ovalbumin. Specific activities of 'alkaline proteinase A' on various protein substrates are given in Table X.

Alkaline proteinases of A. sojae (171), P. cyaneofulvum (85), S. brevicaulis (98) hydrolyze preferentially casein and hemoglobin. However, ovalbumin was a poor substrate for alkaline proteinases of P. cyaneofulvum (85) and S. brevicaulis (98). Alkaline proteinase of P. cyaneofulvum (85) could hydrolyze BSA but that of S. brevicaulis (98) could not. Hydrolysis of azocoll by 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) indicates the presence of collagenase like

TABLE X : SPECIFIC ACTIVITY OF 'ALKALINE PROTEINASE A' FOR VARIOUS PROTEIN SUBSTRATES

Substrate	Specific activity (PU/mg of enzyme)	Assay method
Casein	28 500	a
Hemoglobin (native & denatured)	16 000	a
Bovine serum albumin	2 750	a
Ovalbumin	$<$1 000	a
Azoalbumin	21 390	b
Azocasein	18 700	b
Azocoll	73 330	b

[a]Caseinolytic assay.

[b]Method of Ansari and Stevens (240).

activity. Aspergillopeptidase C (127) and alkaline proteinase of A. sojae (171) are also reported to exhibit collagenolytic activity. 'Alkaline proteinase A' showed milk clotting activity but the clot was dissolved on prolonged incubation. Alkaline proteinases of A. fumigatus (74), P. cyaneofulvum (85) and S. brevicaulis (98) are also reported to coagulate milk and protease of A. fumigatus (74) was found to dissolve the clot. 'Alkaline proteinase A' did not show a thrombin like activity on fibrinogen, however, it could dissolve the fibrin clot rapidly. Alkaline proteinases of A. sojae (171) and S. brevicaulis (98) were found to hydrolyze both fibronogen and fibrin.

Studies on digestion of AE-globin, oxidized lysozyme and oxidized B-chain of insulin by 'alkaline proteinase A' were carried out using finger printing technique as described in Materials and Methods. A comparison was made between the peptide maps of digests prepared under similar conditions by hydrolyzing the proteins with subtilisin Carlsberg and 'alkaline proteinase A'. Figures 19a and 19b show the peptide maps of AE-globin hydrolyzed by subtilisin Carlsberg and 'alkaline proteinase A', respectively. The two maps show significant similarity. Peptide map of AE-globin hydrolyzed by subtilisin Carlsberg shows

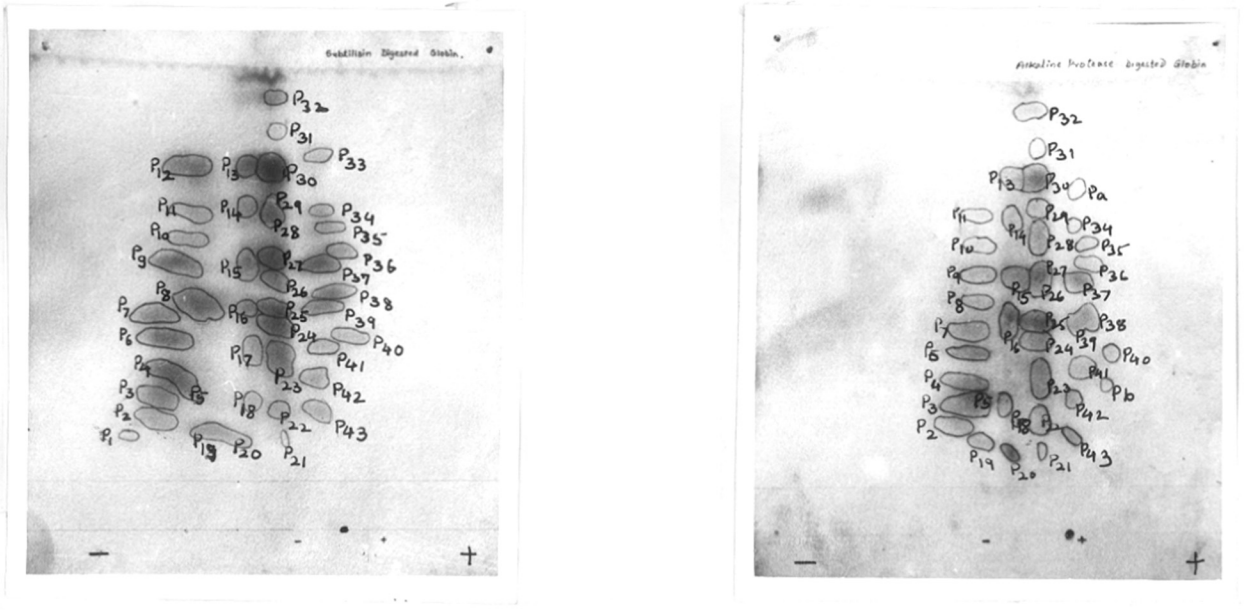
Fig. 19 Peptide maps of AE-globin digested by

(a) Subtilisin Carlsberg

(b) 'Alkaline proteinase A'

The peptides are numbered as P_1, P_2, ...P_{43} in both the peptide maps and the two additional peptides in the peptide map of AE-globin digested by 'alkaline proteinase A' are designated as Pa and Pb.

Negative sign (-) indicates the absence of corresponding peptide.


Subtilisin Digested Globin.
P32
P31
P33
P12
P13
P30
P11
P14
P29
P34
P28
P35
P10
P9
P15
P27
P36
P37
P8
P26
P38
P7
P16
P25
P39
P6
P24
P40
P17
P41
P4
P23
P42
P3
P5
P18
P22
P43
P2
P1
P19
P20
P21
+
Alkaline Protease digested Globin
P32
P31
P33
P34
P35
P36
P37
P38
P39
P40
P41
P42
P43
+

a b

FIG. 19

total 43 peptides indicating the cleavage at 42 peptide bonds, whereas that by 'alkaline proteinase A' shows 39 peptides suggesting the cleavage of 38 points. Four peptides (P_1, P_{12}, P_{17}, P_{33}) from peptide map of subtilisin Carlsberg were absent in the peptide map of 'alkaline proteinase A'. However, the additional peptides (Pa, Pb) were detected in the peptide map of 'alkaline proteinase A'. The position of peptide P_8 in the two maps is slightly different with respect to other peptides. This might be because of the difference of few residues in their amino acid compositions. The similarities between both the peptide maps indicate that mode of action of 'alkaline proteinase A' and subtilisin Carlsberg on AE-globin is more or less similar. The difference in the two peptide maps could be because of multiple cleavages due to the steric effect of the residues adjacent to the cleavage points.

Peptide maps of oxidized lysozyme digested by subtilisin Carlsberg and 'alkaline proteinase A' are shown in Fig. 20a, and 20b, respectively. Total 22 peptides were detected on the peptide map of subtilisin Carlsberg digest which indicated that subtilisin Carlsberg cleaved 21 peptide bonds whereas 29 peptides

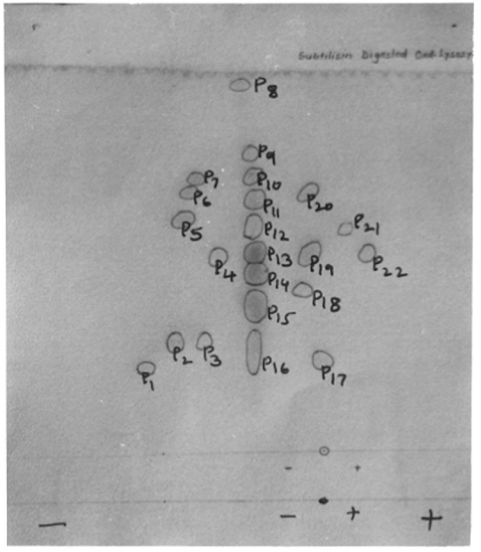
Fig. 20 Peptide maps of oxidized lysozyme digested by

(a) Subtilisin Carlsberg

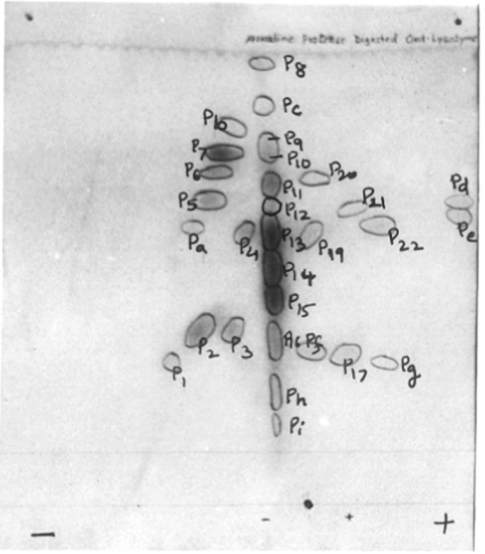
(b) 'Alkaline proteinase A'

The peptides are numbered as P_1, P_2 ....P_{22} in both the peptide maps and the nine additional peptides in the peptide map of oxidized lysozyme digested by 'alkaline proteinase A' as Pa, Pb....P_i.

Negative sign (-) indicates the absence of corresponding peptide.


P8
P9
P7
P10
P6
P11
P20
P21
P5
P12
P13
P19
P22
P4
P14
P18
P15
P2
P3
P16
P17
P1

a


P8
Pc
Pb
Pa
P9
P7
P10
P6
P30
P11
P21
Pd
P5
P12
Pe
P4
P13
P19
P22
P14
P15
P2
P3
Ac
P5
P17
Pg
P1
Ph
Pi

b

FIG. 20

were detected on the peptide map of 'alkaline proteinase A' digest indicating cleavages at 28 points. The peptides detected on peptide map of subtilisin Carlsberg, with an exception of P_{18}, were also identified on the peptide map of 'alkaline proteinase A'. Thus, the bonds cleaved by subtilisin Carlsberg were also cleaved by 'alkaline proteinase A'. In addition to these, nine other peptides (Pa - Pi) were identified on the peptide map of oxidized lysozyme digested by 'alkaline proteinase A'. Alkaline proteinase A', therefore, shows wider specificity than subtilisin Carlsberg.

The peptide maps of oxidized B-chain of insulin digested by subtilisin Carlsberg (Fig. 21a) and 'alkaline proteinase A' (Fig. 21b) show significant resemblance. Total 20 peptides were detected in the peptide map of oxidized B-chain of insulin digested by subtilisin Carlsberg indicating proteolytic cleavage at 19 peptide bonds whereas 22 peptides were detected in the peptide map of 'alkaline proteinase A' suggesting cleavage at 21 sites. Position of most of the peptides in the two maps are similar with some exceptions. Peptide P_2 in the peptide map of subtilisin Carlsberg is absent in the peptide map of

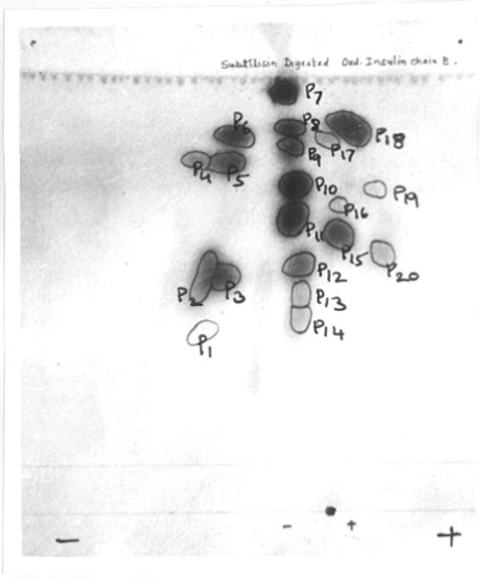
Fig. 21 Peptide map of oxidized B-chain of insulin digested by

(a) Subtilisin Carlsberg

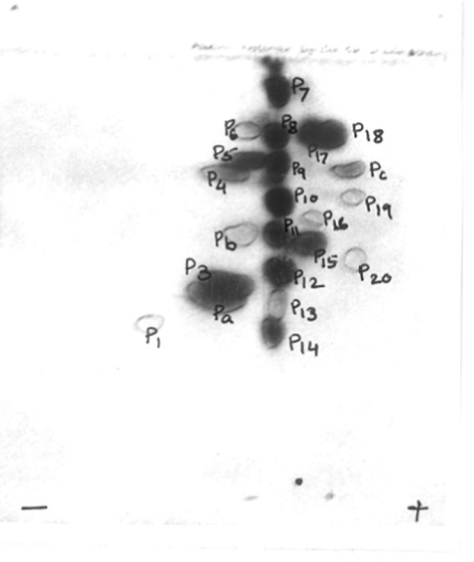
(b) 'Alkaline proteinase A'

The peptides are numbered as P_1, P_2 ....P_{20} and the additional peptides in the latter peptide map are designated as Pa, P_b and P_c.

Negative sign (-) indicates the absence of corresponding peptide.


Subtilisin Digested Oxd. Insulin chain B.
P7
P6
P8
P18
P17
P4
P5
P10
P9
P16
P11
P15
P20
P12
P2
P3
P13
P14
P1
–
+

a


P7
P6
P8
P18
P5
P17
P9
P4
P10
P19
P16
P11
P15
P20
P12
P3
P13
P14
P1
–
+

b

FIG. 21

'alkaline proteinase A'. Additional three peptides (P_a, P_b, P_c) were located in the map of 'alkaline proteinase A' Peptides P_{17} in the map of 'alkaline proteinase A' is masked by peptide P_{18}. Resemblance between the two peptide maps suggests the broad specificity of 'alkaline proteinase A' similar to subtilisin Carlsberg. However, 'alkaline proteinase A' shows wider specificity than subtilisin Carlsberg towards oxidized lysozyme.

The studies on digestion of protein substrates reveal the broad specificity of 'alkaline proteinase A' similar to that of subtilisin Carlsberg. It is worth noting that the hydrolysis of AE-globin is more random by subtilisin Carlsberg (cleavage at 42 peptide bonds) than by 'alkaline proteinase A' (cleavage at 38 peptide bonds), whereas 'alkaline proteinase A' showed more random hydrolysis of oxidized lysozyme and B-chain of insulin as compared to subtilisin Carlsberg. During the amino ethylation, free sulfhydryl and disulfide bonds of the globin are modified which provides the structure ($-S-CH_2-CH_2-NH_2$) similar to side chain of lysine. Subtilisin Carlsberg showed more random cleavage of AE-globin than 'alkaline proteinase A' which suggests that the specificity of alkaline proteinase A' for lysine is less than that of

subtilisin Carlsberg.

(ii) Synthetic substrates. 'Alkaline proteinase A' cleaved ester bonds of BAEE, TAME, ATEE, BTEE and APME. However, it could not hydrolyze p-nitroanilide substrates such as BTPNA, BAPNA, LPNA and also amide bonds of DL-leucinamide and benzoyl-L-tyrosinamide.

Kinetics. Kinetics of hydrolysis of various amino acid esters by 'alkaline proteinase A' were studied and compared with subtilisin Novo and Carlsberg. Esterolysis of BAEE, TAME, ATEE and APME was carried out by potentiometric titration at constant pH 8.0 with 0.02 N NaOH using a pH stat Radiometer. Esterase activity of the enzyme on BTEE was determined spectrophotometrically at 256 nm (10 mm light path) in a Shimadzu spectrophotometer model UV-240.

Michaelis parameters (K_m and V_{max}) for various substrates were determined by studying the reaction rate at different substrate concentrations. Initial velocities were determined for initial 5% hydrolysis which can be calculated from the alkali uptake. Velocity of the reaction was expressed in terms of µmoles of product formed per minute under the experimental conditions for given concentration of the enzyme.

K_m and V_{max} values were calculated from the Lineweaver-Burk plots (Fig. 22 - 26). The V_{max} values were calculated per mole of enzyme assuming its molecular weight as 25 000.

K_m and V_{max} values of 'alkaline proteinase A' and subtilisins for various synthetic ester substrates are summarized in Table XI. A critical analysis of the results leads to the following conclusions:

(i) K_m values of 'alkaline proteinase A' for all substrates were lower as compared to subtilisin Novo and Carlsberg (241). This indicates that 'alkaline proteinase A' has higher affinity for all the above synthetic substrates than subtilisin Carlsberg and Novo.

(ii) K_m value of 'alkaline proteinase A' for ATEE was 2.7 times lower than that for TAME. On the other hand K_m value of subtilisin Carlsberg for ATEE is 2.25 times higher than that for TAME (241).

(iii) Similarly, affinity of ATEE was more (i.e. lower K_m) as compared to APME for 'alkaline proteinase A' whereas affinity for ATEE is less (i.e. higher K_m) than that for APME with both subtilisin

Fig. 22 Lineweaver-Burk plot of BAEE hydrolysis by 'alkaline proteinase A' under standard assay conditions.

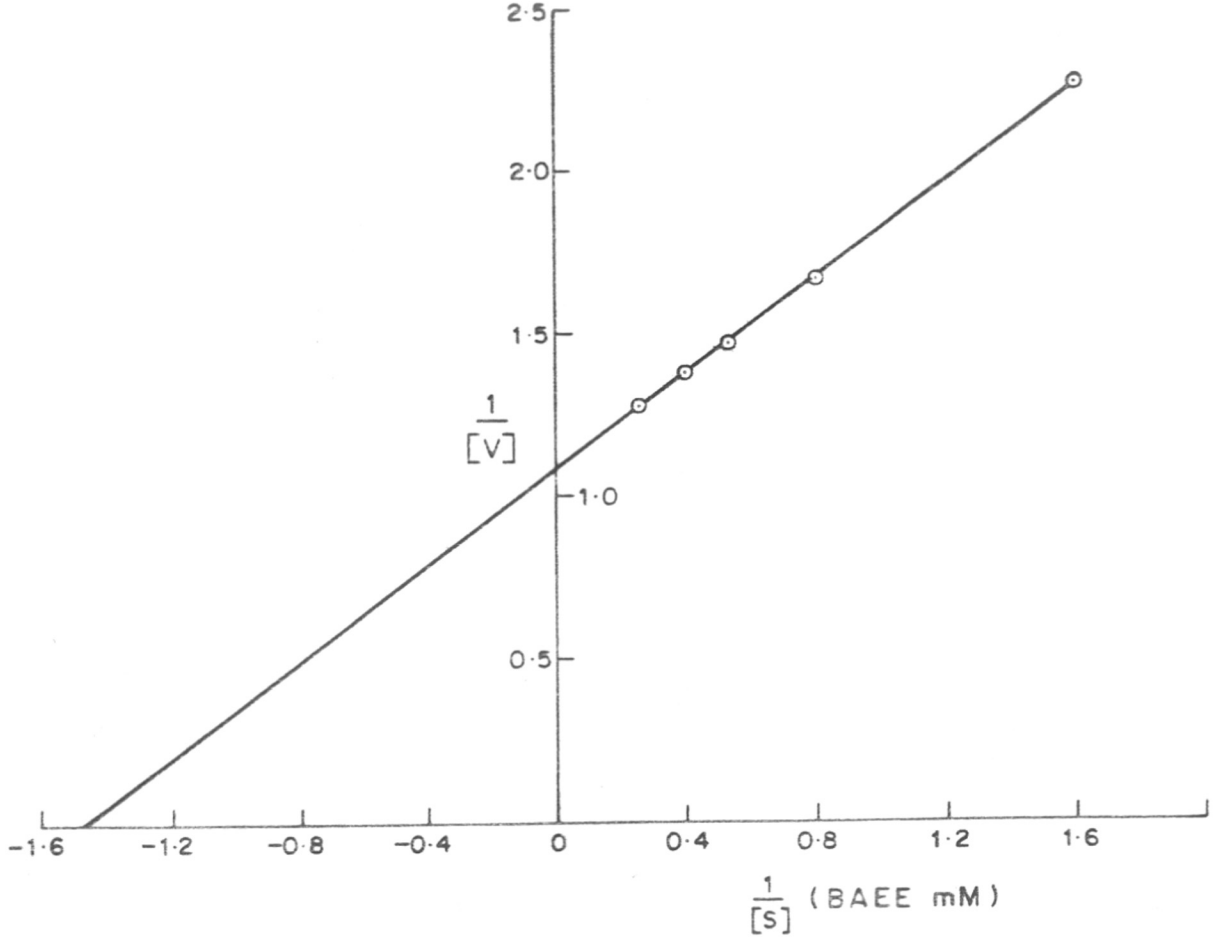


FIG. 22

Fig. 23 Lineweaver-Burk plot for the hydrolysis of TAME by 'alkaline proteinase A' under standard assay conditions.

Fig. 24 Lineweaver-Burk plot for the hydrolysis of ATEE by 'alkaline proteinase A' under standard assay conditions.

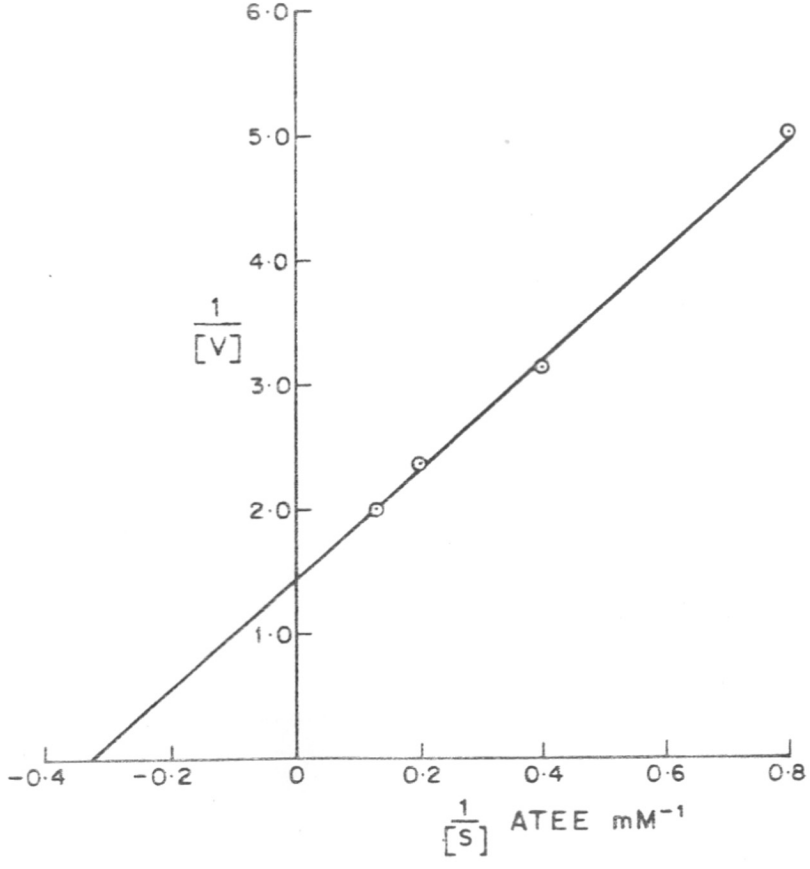

6·0
5·0
4·0
3·0
2·0
1·0
1/[V]
-0·4
-0·2
0
0·2
0·4
0·6
0·8
1/[S] ATEE mM⁻¹

FIG. 24

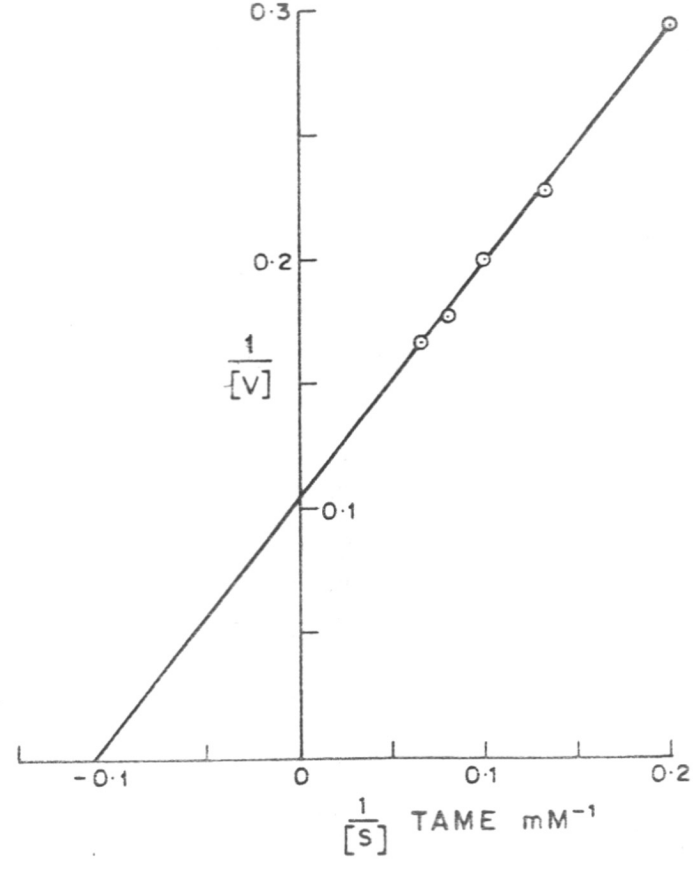

0·3
0·2
0·1
1/[V]
-0·1
0
0·1
0·2
1/[S] TAME mM⁻¹

FIG. 23

Fig. 25 Lineweaver-Burk plot for the hydrolysis of BTEE by 'alkaline proteinase A' under standard assay conditions.

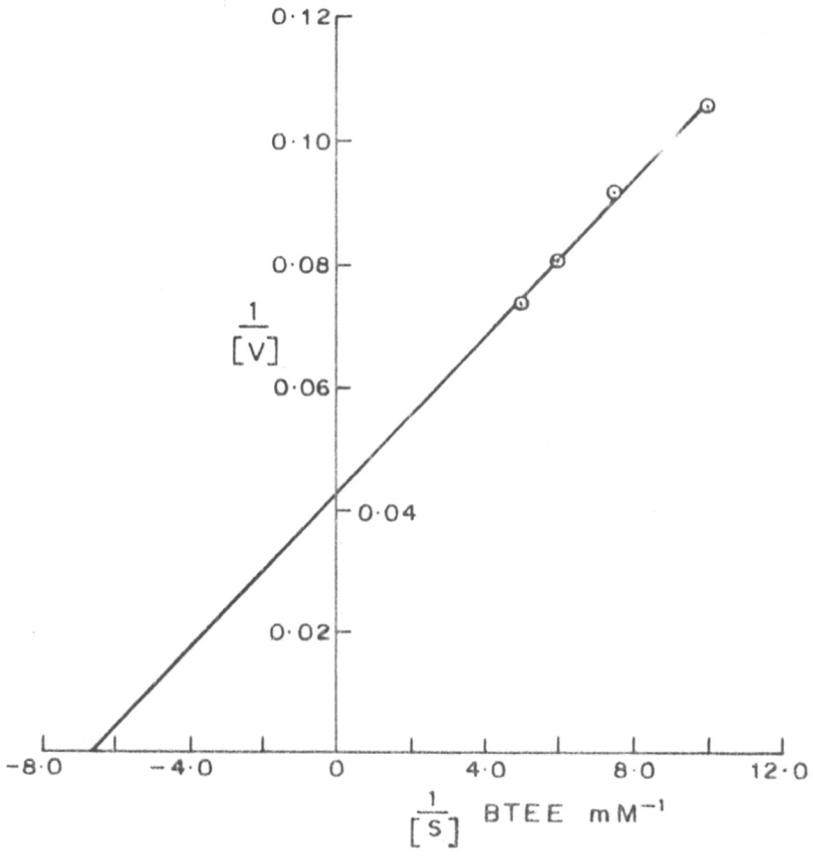


FIG. 25

Fig. 26 Lineweaver-Burk plot for the hydrolysis of APME by 'alkaline proteinase A' under standard assay conditions.

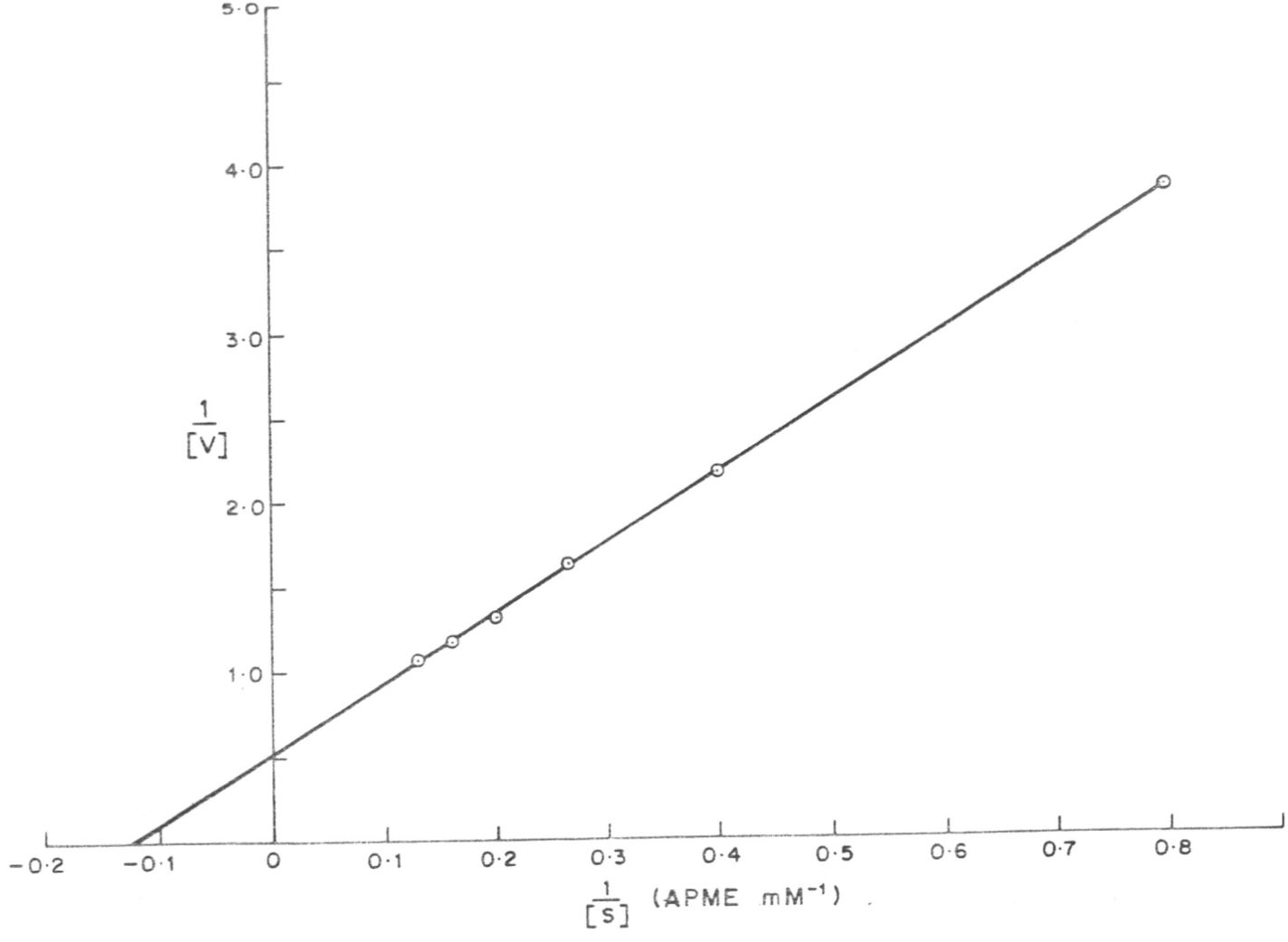


FIG. 26

TABLE XI : KINETIC PARAMETERS FOR HYDROLYSIS OF AMINO ACID ESTERS BY 'ALKALINE PROTEINASE A', SUBTILISIN CARLSBERG AND SUBTILISIN NOVO

Amino acid ester substrate	'Alkaline Proteinase A' (Present work)		Subtilisin Carlsberg (241)		Subtilisin Novo (241)	
	K_m (mM)	V_{max} (sec^{-1})	K_m (mM)	V_{max} (sec^{-1})	K_m (mM)	V_{max} (sec^{-1})
BAEE	0.6	2.14	7.0	16.1	7.0	3.9
TAME	9.0	13.6	40.0	68.6	70.0	15.5
ATEE	3.3	206	90.0	1316	70.0	731
BTEE	0.15	6454	-	-	-	-
APME	8.3	92	30.0	765	60.0	415

(-) indicates that the data is not cited in the reference

Novo and Carlsberg (241).

(iv) The V_{max} values of 'alkaline proteinase A' for all the tested synthetic substrates were lower as compared to subtilisin Novo and Carlsberg (241). However, the V_{max} values of 'alkaline proteinase A' and subtilisin Novo (241) were fairly comparable for BAEE and TAME. The V_{max} values indicate that the deacylation rates of the tested substrate for the three enzymes is in the following order: Subtilisin Carlsberg Substilisin Novo 'Alkaline proteinase A' .

Effect of pH on hydrolysis of BAEE. Effect of pH on the hydrolysis of BAEE was examined at 37°C and substrate concentration (0.025 M) exceeding its K_m value by 40 fold. The reaction was monitored by potentiometric titration procedure as described in Materials and Methods. A plot of relative velocity against pH (Fig. 27) showed a sigmoid curve over pH range of 4.5 to 9.0. The value of pK'_a calculated from the curve was 6.65 which is in close resemblance to the pK' of imidazole group (pK'_a 6.58). The resemblance of pH-velocity profile for hydrolysis of BAEE by 'alkaline proteinase A' at 37°C with the titration curve for imidazole group (pK'_a of 6.58)

Fig. 27 Effect of pH on the hydrolysis of BAEE (0.025 M) by 'alkaline proteinase A' at 37°C.

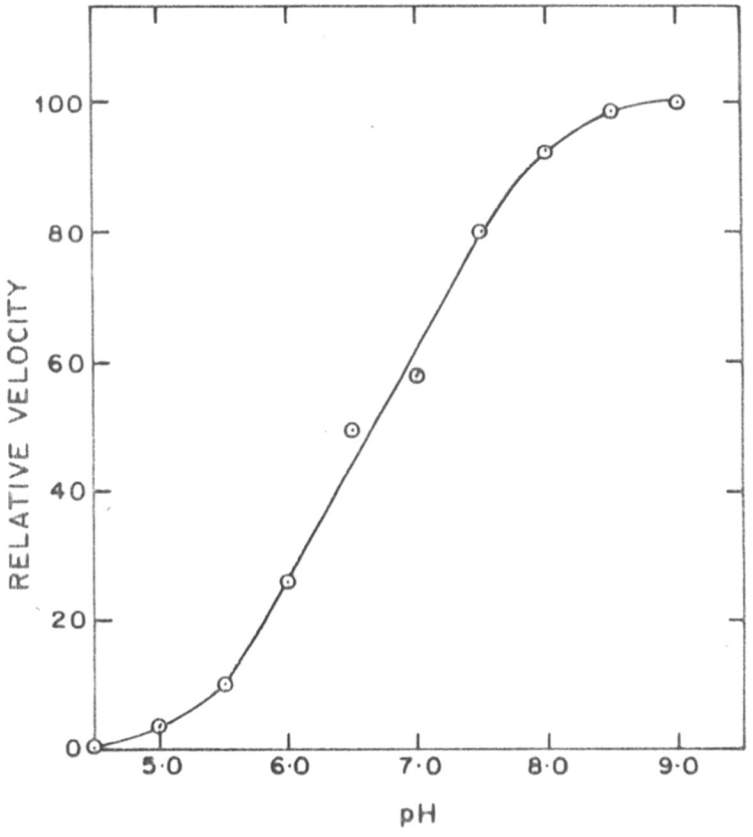


FIG. 27

suggests that protonation ofan imidazole group in the active site of alkaline proteinase A' results in inactivation of the enzymes. The role of imidazole group in the catalytic function of subtilisins and an alkaline proteinase of A. flavus, is suggested (144, 166).

SECTION II

APPLICATIONS

SUMMARY

Alkaline proteinase of Conidiobolus sp. (NCL 82.1.1) was studied for its possible industrial and clinical applications. The purified 'alkaline proteinase A' was used for resolving (D) and (L) isomers of phenylalanine and phenylglycine from a racemic mixture of their esters. A crude preparation of alkaline proteinase could replace trypsin in animal cell cultures for (i) dissociation of cells from monolayer cell cultures; (ii) dissociation of cells from tissues for primary cell cultures and (iii) treatment of metaphase chromosome preparations for the production of G-bands preparations. A crude preparation of the enzyme was also used for preparation of protein hydrolysates from soya meal and casein. The extent of hydrolysis is comparable to that of Alcalase (Novo Industries, Denmark) and Bacterial protease (Sarabhai Chemicals, India).

INTRODUCTION

High content of alkaline proteinase in the culture filtrate of Conidiobolus sp. (NCL 82.1.1), its high productivity, physicochemical properties and wider substrate specificity of the major component,'alkaline proteinase A', encouraged us to examine its possible use in commercial processes. This section describes the successful use of alkaline proteinase from Conidiobolus sp. (NCL 82.1.1) in resolution of (D,L)-phenylalanine and (D,L)-phenylglycine which is useful in the synthesis of important drugs, in animal cell culture as a substitute for trypsin and in preparation of protein hydrolysates from soya meal and casein.

RESULTS AND DISCUSSION

(a) Resolution of (D,L)-phenylalanine and (D,L)-phenylglycine

'Alkaline proteinase A' specifically hydrolyzed the ester bond at carboxyl group of the (L) isomers of derivatized (N-acetylated and C-esterified) phenylalanine and phenylglycine. The ester of D-isomer remained unchanged by the enzymatic treatment and was obtained as N-acetyl-D-phenylalanine and N-acetyl-D-phenylglycine ethyl ester by extracting with organic solvent. From the remaining portion L-isomers were obtained as N-acetyl-L-phenylalanine and N-acetyl-L-phenylglycine, respectively. These can then be refluxed with HCl to yield (L)-phenylalanine and (L)-phenylglycine. The unchanged D-isomer can be epimerized with base to give (D,L)-mixture which can be further recycled.

Purified 'alkaline proteinase A' was tested for its utility in the resolution of D,L-phenylalanine and D,L-phenylglycine. N-acylated esters of these compounds were used. Resolution was carried out at a constant pH 7.5 using an automatic pH stat Radiometer according to the method of Roper and Bauer (233). N-Acetyl-D,L-phenylalanine methyl ester (221 mg) was

suspended in about 5 ml of H_2O and pH adjusted to 7.5 with 0.02 N NaOH. Purified 'alkaline proteinase A' (200 µg) was added and the reaction mixture was incubated at 30°C with efficient stirring. pH of the reaction mixture was maintained at 7.5 with 0.02 N NaOH till the alkali uptake ceased (about 1 h). About 27 ml of 0.02 N NaOH was consumed. Original insoluble-N-acetyl-D,L-phenylalanine methyl ester was solubilised completely after the reaction with the enzyme. N-Acetyl-D-phenylalanine methyl ester was extracted with dichloromethane (2 x 10 ml) from the reaction mixture which was then dried with unhydrous $MgSO_4$ and concentrated in vaccuo to get colorless solid.
Yield : 73 mg (66%); m.p. 85°C.

The remaining aqueous portion was extracted with ethyl acetate (2 x 10 ml) at pH 1.0. The organic extract was dried with unhydrous $MgSO_4$ and concentrated in vaccuo to give a colorless solid of N-acetyl-L-phenylalanine.
Yield : 95 mg (91%); $|\alpha|_D = 41°$; optical purity : 82%; m.p. 170°C.

Resolution of D,L-phenylglycine was achieved by treating N-acetyl-D,L-phenylglycine ethyl ester (221 mg) with 'alkaline proteinase A' (200 µg) by

following the above mentioned procedure. The D-isomer was extracted from the reaction mixture with dichloromethane as unhydrolyzed N-acetyl-D-phenylglycine ethyl ester.
Yield : 81 mg (73%).

The L-isomer was obtained by extracting the remaining aqueous portion of reaction mixture with ethyl acetate at pH 1.0. It was obtained as N-acetyl-L-phenylglycine.
Yield : 93 mg (90%); $|\alpha|_D$ = 118°C; optical purity: 79%.

A report by Roper and Baur (233) describes the use of subtilisin Carlsberg for resolution of D,L-phenylalanine. They could get N-acetyl-L-phenylalanine with 96% yield and 98% optical purity and N-acetyl-D-phenylalanine methyl ester with 98% yield. Yields and optical purity obtained by using 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) were comparable to those obtained by using subtilisin Carlsberg (233).

Major use of (D) and (L)-phenylalanine is for the production of chloramphenicol and aspartame, respectively, while D-phenylglycine is used for the production of amphicillin. Resolution of the isomers

by the conventional chemical methods is laborious and costly while enzymatic method is simple, specific and can be carried out at room temperature. Moreover, by using immobilized alkaline proteinase, the enzyme can be repeatedly used with considerable reduction in the cost.

(b) Applications in animal cell culture

Trypsin, is commonly used in animal cell culture. Attempts were made to replace trypsin by alkaline proteinase of Conidiobolus sp. (NCL 82.1.1). The results have been published (283).

Animal cells and tissues. The methods described by Paul (285) were used for primary cell culture and cell line maintenance. Cell lines of chinese hamster ovary (CHO), african green monkey kidney (Vero) and human cervix carcinoma (HeLa) were maintained on minimum essential medium (Earle's base), supplemented with 10% goat serum. ATC-15 (Aedes albopictus) cell line was maintained on MM-medium (286) supplemented with 5% goat serum. Golden hamster kidney and 8-9 days old chick embryos were used for primary cultures.

Enzymes. Crude preparation of alkaline proteinase from Conidiobolus sp. (NCL 82.1.1) with proteinase

activity of 0.625 Anson units per mg at pH 7.2 (i.e. about 2000 PU per mg at pH 10.0) was used. Trypsin (Difco product) with an activity of 0.67 Anson units per mg was used for comparison. One Anson unit corresponds to 1 µmole of tyrosine produced per minute from 1% Hammerstein casein at pH 7.2 and 35°C.

1. Dissociation of monolayers

Two sets each of Vero, HeLA and CHO cell lines were maintained. One set was treated with TPVG solution containing 100 mg (67 Anson units) trypsin, 10 mg EDTA and 50 mg glucose per 100 ml of calcium-magnesium-free phosphate buffered saline and the other set with APVG solution wherein trypsin was replaced by 430 mg (19 Anson units , i.e. 62 700 PU at pH 10.0) of alkaline proteinase.

In preliminary experiments it was found that alkaline proteinase could dissociate the cells from monolayers and that these cells were viable as revealed by trypan blue dye exclusion. Optimum enzyme concentration was determined for cell dissociation by taking various concentrations of alkaline proteinase as a function of time (Fig. 28). It was observed that time required for dissociation

Fig. 28 Monolayer cell dissociation profiles for Chinese Hamster Ovary (CHO) by 'alkaline proteinase (AP) and trypsin.

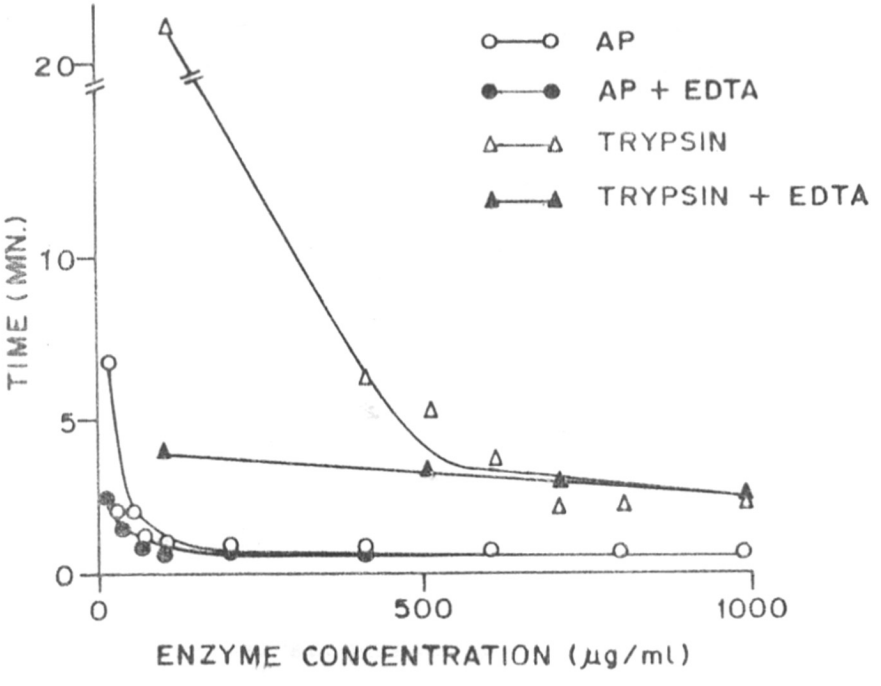

AP
AP + EDTA
TRYPSIN
TRYPSIN + EDTA
20
10
5
0
TIME (MIN.)
0
500
1000
ENZYME CONCENTRATION (µg/ml)

FIG. 28

of cells from monolayers decreased with increase in enzyme concentration upto 15 µg of enzyme/ml after which the dissociation time remained constant. EDTA (10 mg/ml) increased the cell dissociation rate at lower concentration of alkaline proteinase. Trypsin, as such and also in the presence of EDTA showed similar profile (Fig. 28). However, time required by alkaline proteinase to dissociate cells from monolayer was less as compared to that by trypsin.

On the basis of these profiles (Fig. 28) TPVG and APVG solutions were formulated as described above. CHO and Vero cell lines were maintained in serial passages using these TPVG and APVG solutions. There was no significant difference in the morphological appearance and growth properties of cells maintained using TPVG and APVG.

2. Dissociation of tissues

Two sets of primary chick embryo fibroblast cultures were prepared by using trypsin and alkaline proteinase for cell dissociation. Enzyme concentrations of 500 and 2000 µg/ml were used in each set. Cell dissociation was carried out at 37°C for 30 min. The cells were washed and viable cell count was obtained in a haemocytometer using 0.01%

trypan blue. The cells (10 ml suspension) were then set in a 4 oz. culture bottles at seeding density of 1 million cells per ml in the medium (M + Hanks base) supplemented with 2% goat serum. Similarly, two sets of primary cultures of golden hamster kidney were prepared using trypsin and alkaline proteinase. In this case, however, cell dissociation was carried out for 2 h at 37°C over magnetic stirrer and cultures were set at seeding density of 0.5 million cells per ml in medium 199 (Hanks base) with 10% goat serum.

The total and viable cell yields obtained from primary dissociation of chick embryos and hamster kidneys were plotted (Fig. 29). Comparable number of cells were dissociated from chick embryo fibroblast when concentration of 500 µg/ml of alkaline proteinase or 2000 µg per ml of trypsin was used. Cell viability in all the four sets was comparable. The above results indicated the high efficiency of alkaline proteinase over trypsin in dissociation of chick embryo fibroblast tissues.

For hamster kidneys, higher cell yields were obtained with 2000 µg than with 500 µg of both,

Fig. 29 Total and viable cell yields from primary cell dissociation of chick embryos (CEC) and hamster kidneys (HK) by alkaline proteinase (AP) and trypsin (T). Figures in the bars represent percent viability.

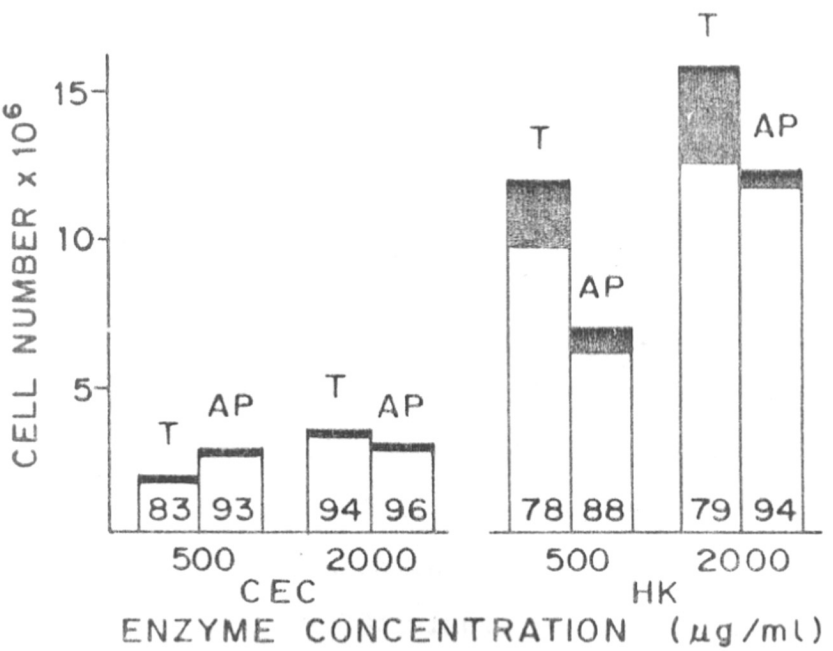

CELL NUMBER x 10^6
15
10
5
T
AP
T
AP
T
AP
T
AP
83
93
94
96
78
88
79
94
500
2000
CEC
500
2000
HK
ENZYME CONCENTRATION (µg/ml)

FIG. 29

alkaline proteinase and trypsin. The viable cell yields obtained with 2000 µg of alkaline proteinase and trypsin were comparable (11.5 million and 12.5 million, respectively although the total cell yields obtained with alkaline proteinase were less than those by trypsin.

3. Cytotoxicity evaluation

Cytotoxicity of alkaline proteinase was studied and compared with that of trypsin. ATC-15 cells were dissociated with alkaline proteinase and trypsin and then suspended in these enzyme solutions (1 mg/ml) for 30 min. Viability of these cells was determined in a haemocytometer using 0.01% trypan blue. Alkaline proteinase and trypsin yielded about 85% and 70% viable cells, respectively. These cells even after 30 min of enzyme treatment showed ability to grow into confluent sheets similar to the stock line. This indicated the lesser toxicity of alkaline proteinase than trypsin.

4. Chromosome preparations and G-banding

The method of Wang and Fedoroff (287) was used for G-banding. Actively growing HeLa and CHO cell cultures were used for c-metaphase chromosome preparations. The preparations were rinsed in

0.15 M NaCl and treated with 0.005% alkaline proteinase (0.033 Anson units /ml i.e. about 110 PU per ml, pH 10.0) in 0.15 M NaCl, for 30 - 120 seconds at room temperature. For comparison, slides were similarly treated with 0.005% trypsin (0.031 Anson units/ml) in 0.15 M NaCl. The slides were again rinsed in 0.15 M NaCl and then stained with Giemsa stain.

Banding patterns on metaphase chromosomes of CHO and HeLa cells after treatment with trypsin and alkaline proteinase were examined. Chromosome identification was done as described by Deaven and Petersen (288). Several metaphases were recorded for critical comparison of the banding pattern produced by trypsin and alkaline proteinase. It was observed that the G-bands produced by alkaline proteinase were comparable to those by trypsin (Fig. 30). Moreover, similar to trypsin, treatment of alkaline proteinase also resulted in excessive swelling and ghost-staining of the chromosomes.

The above studies show that alkaline proteinase of Conidiobolus sp. (NCL 82.1.1) can be used efficiently in the dissociation of cells for primary

Fig. 30 Some 'Z' group chromosomes of Chinese Hamster Ovary (CHO) showing G-banding after trypsin pretreatment (T) and alkaline proteinase pretreatment (AP).

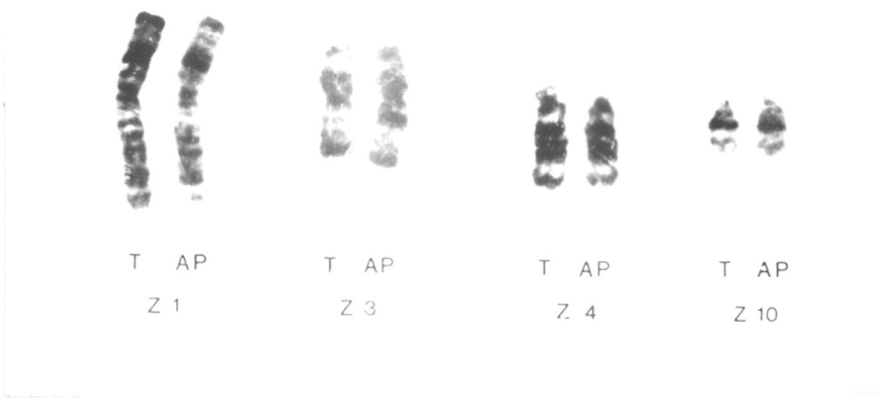


FIG. 30

cell cultures, maintenance of cell lines and in the production of G-bands on metaphase chromosomes. It was also found to be less toxic to the cells than trypsin.

(c) Preparation of protein hydrolysates

Attempts were made to prepare protein hydrolysates from casein and soya meal by using crude preparation of alkaline proteinase from Conidiobolus sp.(NCL 82.1.1) Preparations of Bacterial protease (Sarabhai Chemicals, India) and Alcalase (Novo Industries, Denmark) were also used for comparison. Hydrolysis was carried out at pH 9.5, 40°C for 4 h.

1. Casein hydrolysate preparation

50 g casein was suspended in 200 ml water and pH was adjusted to 9.5 with NaOH. Alkaline proteinase (250 mg, 1.12×10^6 PU) was added to the above suspension (200:1 protein to enzyme ratio by weight) and the mixture was stirred occassionally. The pH decreased during the reaction which was adjusted and maintained at pH 9.5 by adding NaOH. Alkali uptake was observed for first 2 h after which pH remained constant. Hydrolysis was continued for another 2 h,

and stopped by adjusting the pH to 5.0. The enzyme was completely inactivated by boiling the reaction mixture for 5 min. It was then centrifuged and supernatant was collected (202 ml). Hydrolysis of 50 g casein by using Bacterial protease (250 mg, 1.1×10^6 PU) was carried out under similar conditions (hydrolysate volume 224 ml). Soluble solid content of hydrolysates was determined by drying the hydrolysates. Casein hydrolysates obtained by using alkaline proteinase and Bacterial proteinase showed solid content of 43 g (86%) and 43.5 g (87%), respectively.

2. Soya meal hydrolysate preparation

Digestion of soya meal was carried out by using alkaline proteinase, Bacterial proteinase and Alcalase was described above. 75×10^4 proteinase units (at pH 10.0) of each enzyme were used for respective hydrolysis experiments. After stopping the hydrolysis by bringing down the pH to 5.0, reaction mixture was squeezed through nylon cloth. Filtrate was boiled to destroy the enzyme activity, centrifuged and supernatant was collected. Volumes of hydrolysates obtained by using alkaline proteinase, Bacterial proteinase and Alcalase were 211 ml, 214 ml and 207 ml with total soluble solid contents of 15.3 g (31%), 16.7 g (33%) and 17.0 g (34%), respectively.

Amino nitrogen of hydrolysates was determined by formal titration. To 10 ml of hydrolysate, 40 ml water was added and pH was adjusted to 8.0. 8 ml of 40% formaldehyde was added. After about 5 - 10 min titration was carried out at pH 8.0 for 30 min using 0.1 N NaOH. Amino nitrogen was calculated from the alkali uptake. Total amino nitrogen contents in the hydrolysates obtained by using alkaline proteinase, Bacterial proteinase and Alcalase were 106 mg, 108 mg and 109 mg, respectively.

The comparable results of soluble solid and amino nitrogen content in the soya meal hydrolysates obtained by using alkaline proteinase, Bacterial proteinase and Alcalase indicated that extent of hydrolysis by all the above enzymes was similar.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Extracellular alkaline proteinases from different groups of fungi are studied in detail. However, alkaline proteinases from fungi belonging to phycomycetes have not been explored. Although, the production of extracellular alkaline proteinases by Entomophthora (121 - 123), Basidiobolus (121) and Conidiobolus (121, 124, 126) is studied, reports on their purification and characterization are lacking. A report by Tokuyama and Asano (125) describes the physiochemical properties of purified alkaline proteinase from Conidiobolus sp., whereas Ishikawa et al. (126) have reported some properties of partially purified alkaline proteinase from Conidiobolus lamprauges.

A saprophytic strain of Conidiobolus, isolated from plant detritus in our laboratory and designated as NCL 82.1.1, showed ability to produce an alkaline proteinase in high amounts. It is secreted when Conidiobolus sp. is grown on the medium containing casein, peptone, sucrose, salts and metal ions under submerged conditions. A preliminary report on production of the enzyme has been published (235). Present work describes the purification, study of physicochemical and enzymatic properties, substrate

specificity and some applications of alkaline proteinase from Conidiobolus sp. (NCL 82.1.1).

Culture filtrate of Conidiobolus sp. (NCL 82.1.1) showed the presence of two alkaline proteinases in the proportion 70:30. The major alkaline proteinase designated as 'alkaline proteinase A' was purified to homogeneity by using methods such as solvent precipitation, DEAE-cellulose treatment, preparative polyacrylamide gel electrophoresis, electrophoretic elution, ion-exchange chromatography on CM-cellulose and gel filtration on Sephadex G-50. The purified enzyme was homogeneous as revealed by disc gel electrophoresis at pH 7.6 and 4.3 (both cathodic runs), SDS-polyacrylamide gel electrophoresis, gel filtration and isoelectric focusing analysis.

A simple and versatile gel casting cum electrophoresis device was developed for preparative polyacrylamide gel electrophoresis and was used during purification of the enzyme. In this apparatus gel slabs of various thickness (0.04 to 1.0 cm) can be made and also more than one gel can be run simultaneously. Hydrostatic balance (266) is automatically adjusted due to the position of the two buffer chambers. Mechanical stress on the gel is also avoided by in situ

polymerization of the gel and subsequent electrophoresis in the same apparatus.

'Alkaline proteinase A' is composed of a single polypeptide chain with a molecular wieght of 22 000. It is a basic protein with isoelectric point of 9.45. Physicochemical and enzymatic properties of the enzyme are summarised in Table XII. Its amino acid composition (Table VII) shows much more resemblance with that of subtilisin Carlsberg with the difference in content of sulfur containing amino acids. Mn^{2+}, Co^{2+}, Fe^{2+}, Ba^{2+} and Ca^{2+} increased the proteolytic activity of 'alkaline proteinase A',however, Hg^{2+} inhibited the enzyme. EDTA and cysteine hydrochloride did not alter the enzyme activity.

'Alkaline proteinase A' hydrolyzed casein, native and denatured hemoglobin, oxidized lysozyme and B-chain of insulin, azocoll, azocasein and azoalbumin. BSA and ovalbumin were the least preferred substrates. The enzyme showed milk clotting activity but the clot was dissolved on prolonged incubation. Qualitative analysis showed that the enzyme did not clot fibrinogen, however, the fibrin clot formed from fibrinogen by thrombin was dissolved by 'alkaline proteinase A'. The enzyme exhibited esterase activity on BAEE, TAME, ATEE, BTEE

TABLE XII : PHYSICOCHEMICAL AND ENZYMATIC PROPERTIES OF 'ALKALINE PROTEINASE A'

Property	Numerical value
Molecular weight	
- SDS PAGE	22 000
- Gel filtration	21 880
- Amino acid composition	25 454
Isoelectric point (pI)	9.45
Extinction coefficient ($E^{1\%}_{280\ nm}$)	10.0
Optimum pH (40°C)	10.0
Optimum temperature (pH 10.0)	40°C
Stability pH (50°C, 10 min)	6.5-7.0
Stability temperature (pH 7.0, 1 h)	upto 30°C
Activation Energy (casein as a substrate)	12.785 kcal/mole (68 KJ)

and APME. However, it did not show amidase activity on DL-leucinamide and benzoyl-L-tyrosinamide and on BAPNA, BTPNA and L-leucine-p-nitroanilide. Kinetic parameters for hydrolysis of amino acid ester substrates were determined (Table XI). 'Alkaline proteinase A' exhibited low K_m values indicating higher affinity of the enzyme than subtilisins. However, the V_{max} values were lower for 'alkaline proteinase A' than those for subtilisins, which suggests that its deacylation rate is lower than subtilisins.

The peptide maps of the digests obtained from AE-globin, oxidized lysozyme and oxidized B-chain of insulin by 'alkaline proteinase A' and subtilisin Carlsberg were comparable suggesting the broad specificity of 'alkaline proteinase A' similar to subtilisin Carlsberg.

'Alkaline proteinase A' was completely inactivated by PMSF even at concentration of 0.1 mM suggesting involvement of serine residue in the catalytic function of the enzyme. 'Alkaline proteinase A', therefore, appears to be a serine proteinase. Subtilisins and most of the fungal alkaline proteinases are serine proteinases (278, 283). Inability of TLCK and TPCK, which are specific inhibitors of histidine, to alter

the activity of 'alkaline proteinase A' suggests that histidine is unlikely to be involved in the catalytic function of the enzyme. However, the hydrolysis of BAEE by 'alkaline proteinase A' at 37°C showed a sigmoid pH-velocity profile over the pH range of 4.5 to 9.0 with pK_a' of 6.65 (Fig. 27). This pK_a' value is in close resemblance with pK_a' value of imidazole (pK_a' 6.58 at 37°C). This study suggests the involvement of histidine in the catalytic action of 'alkaline proteinase A'. This is in accordance with subtilisins which are also not inhibited by TLCK and TPCK but are reported to have histidine at their active site (141, 144, 147, 148). A possible role of carboxylate group of aspartate or glutamate is also suggested since 'alkaline proteinase A' is inactivated by CMC (46% inactivation under experimental conditions).

Although the possible role of histidine and aspartate or glutamate is suggested in the catalytic function of 'alkaline proteinase A', detailed studies are required to examine the involvement of these amino acid residues in the catalytic function of the enzyme.

Alkaline proteinase of Conidiobolus sp.(NCL 82.1.1) is shown to have potential applications in industry

and in animal cell cultures. The purified 'alkaline proteinase A' resolved (D) and (L) isomers of phenylalanine and phenylglycine from a racemic mixture of their esters. Major use of (D) and (L)-phenylalanine is for the production of chloramphenicol and aspartame, respectively, while (D)-phenylglycine is used for the production of ampicillins. A crude preparation of alkaline proteinase could efficiently replace trypsin in animal cell cultures for dissociation of cells from monolayer cell cultures, dissociation of cells from tissues for primary cell cultures and for treatment of metaphase chromosome preparations for the production of G-band preparations. Protein hydrolysates were prepared from soya meal and casein by using crude preparation of the enzyme. The extent of hydrolysis is comparable to that by Alcalase (Novo Industries, Denmark) and Bacterial protease (Sarabhai Chemicals, India).

In conclusion, 'alkaline proteinase A' of Conidiobolus sp. (NCL 82.1.1) showed resemblance with subtilisin Carlsberg with respect to physicochemical and enzymatic properties and substrate specificity. Moreover a specific subtilisin inhibitor from

Dolichos biflorus (289) inhibited proteolytic activity of the enzyme which supports its resemblance with subtilisin Carlsberg.

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