CHARACTERIZATION OF EXTRACELLULAR PROTEASES OF PSYCHROTROPHIC PSEUDOMONADS

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CHARACTERIZATION OF EXTRACELLULAR PROTEASES OF PSYCHROTROPHIC PSEUDOMONADS

by

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ABSTRACT

For this study 8 cultures of P. fluorescens were selected. A protease was isolated from bacterial culture number T20 and was partially purified by a combination of ammonium sulfate precipitation, gel filtration on Sephadex G-200 and affinity column chromatography. The molecular weight of this protease was estimated to be 43,000 by gel filtration on Sephadex G-200. The proteases investigated in this study were active over a wide range of temperatures (5-45°C) with maximum activity at the temperature of 35°C. The pH optimum for T20 protease was 7.2; other proteases were also maximally active at neutral pH. All of the proteases investigated retained considerable activity after a heat exposure of ten minutes at 100°C. The T20 protease was stable over a wide range of pH (5.5-9.0) when stored at 10°C for four days. All of the proteases were found to be metalloproteases requiring divalent cations for activity and were inactivated slightly by sulfhydryl reagents. The synthesis of all proteases by the bacteria was stimulated by addition of milk to the growth medium. The protease activity of all isolates began to increase in early logarithmic phase and continued to increase till late stationary phase, and then began to decline. The proteases from various isolates were found to possess similar antigenic determinants. Each bacterial culture produced only one extracellular protease.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

CBZ-D-Phe-T	Carbobenzoxy-D-phenylalanyl-tetraethylenepentamine
DMSF	Dimethyl sulfonyl fluoride
EDTA	Ethylene-diamine-tetraacetic acid
EU	Enzyme unit
IgG	Immunoglobulin G
L-BAPA	α-N-Benzoyl-L-arginine-p-nitroanilide
LTI	Low temperature inactivation
MP	Skim milk powder
MSM	Minimal salt medium
Ps.	*Pseudomonas*
PCA	Plate count agar
PCMB	Para-chloromercuribenzoate
SDS	Sodium dodecyl sulfate
TAME	P-Toluenesulfonyl-L-arginine methyl ester
TCA	Trichloroacetic acid
TEP	Tetraethylene pentamine
Tris	Tris (hydroxymethyl) aminomethane
TSB	Trypticase soy broth
UHT	Ultra high temperature
Z-L-Phe-T	Carbobenzoxy-L-phenylalanyl-triethylene tetraminyl-Sepharose 4B

INTRODUCTION

The general advancement of modern science and technology has had its impact on the dairy industry and there has been a tendency in the dairy industry towards larger farms producing bulk quantities of milk. This transition has been associated with the requirement for storage of milk for longer periods of time. Milk is generally stored at cold temperatures. This storage of milk includes the storage at the farm, in transport, at the processing plant, in the supermarket and in the consumer's household. In all, the time period from the milk's production at the dairy farm till its consumption may extend upto 22 days (1).

The practice of cold storage of milk for extended periods before its processing has resulted in the problem of maintaining the shelf life of milk held at temperatures close to $0^{o}C$. Milk supplies a rich environment for the growth of the psychrotrophic organisms and production of their extracellular enzymes. Hence, these microorganisms have considerable spoilage potential. If allowed to multiply they may effect the quality of the milk and other dairy products. Therefore, precautionary measures must be taken to control their growth. For this we require a better understanding of the psychrotrophs and their enzymes. This involves studying the biochemical characteristics of the

extracellular enzymes as well as the study of the conditions under which these enzymes are produced (1,2).

In this study the biochemical characteristics of the extracellular proteases secreted from some psychrotrophic Pseudomonas fluorescens strains isolated previously in this laboratory from raw milk samples were investigated. The understanding of the properties of these proteases not only may lead us to a better knowledge of the spoilage of milk and other dairy products but it may also broaden our state of knowledge with respect to various aspects of extracellular proteolytic enzymes. A few reports available in literature on extracellular proteases from psychrotrophic pseudomonads deal mainly with their properties, such as heat stability in considerable detail and very few reports are available on purification and characterization of these proteases. Hence these investigations were undertaken with the main objective of characterization of some of the proteases in detail, so as to increase our knowledge of the biochemical properties of these proteases.

REVIEW OF LITERATURE

1. Psychrotrophs

a) Definition

The definition of microorganisms capable of growth close to 0°C has been an intriguing problem since 1887 when Forster first observed bacterial growth at 0°C (1). On the basis of their cardinal temperatures the bacteria are generally divided into three classes namely: thermophiles, mesophiles and psychrophiles. The thermophiles grow best at temperatures between 45° and 60°C, and the mesophiles grow best within a temperature range of 25-40°C (3). Schimdt-Nielson (1) first coined the term 'psychrophile', derived from the Greek words 'Psychros' which means cold, and 'philos', which means loving, implying that these organisms grow better at low temperatures. The terms thermophiles and mesophiles very clearly assign the microorganisms to one of these classes depending upon their optimum growth temperatures. However, the definition of psychrophilic microorganisms is not as simple since various investigators have defined psychrophiles using many different criteria including: a) optimum growth temperature; b) ability to grow at low temperature; c) the method of enumeration; and d) criteria which are independent of temperature (2). The finding that bacteria defined as psychrophiles did not have optimum growth at or close to 0°C led many investigators to

propose several other terms including: Glacial bakterien (4), the species name Psychrocarterien (5), Psychrotoleran (6), Eurythermic (7) and Thermophobe (8). Hucker (9) divided bacteria capable of growth at low temperatures into two categories, obligate and facultative psychrophiles. The former could grow at 0°C but not at 32°C, while the latter could grow at 0°C as well as 32°C. Ingraham and Stokes (10, 11) defined psychrophiles as organisms capable of rapid growth at 0°C within two weeks; and the term rapid growth implied a visible colony on a solid medium or a turbidity in a liquid medium visible to the naked eye. Eddy (12) favored the use of the term psychrotrophic for microorganisms capable of growth at 5°C and below, without mentioning their optimum temperature. It was further pointed out that bacteria should not be classified on the basis of their reactions to two temperatures lying as far apart as the minimum and optimum temperatures usually are; and psychrophiles should be defined according to optimum temperature (13).

The difficulties encountered in defining these microorganisms led many investigators to make use of properties other than temperatures. The psychrophiles were implied to possess the following characteristics: Gram negative rods, asporogenous, resistant to penicillin, non-acid forming, motile and resistant to basic dyes, etc. (2). Although many psychrophiles do possess the above characteristics there are some exceptions. Not all of these organisms are gram negative rods; sporeforming

bacteria of the Bacillus species are also found to grow at low temperatures (14).

It is apparent that there is a clear distinction between psychrophiles and psychrotrophs. As far as industrial application is concerned, the term psychrotroph is used more often since this term defines these organisms in such a way which has direct application to the dairy industry. Keeping these facts in mind, the definition given by Eddy (12) seems to serve the purpose of defining these organisms in a practical way. The term psychrotroph, in the dairy industry refers to those organisms which are mesophilic by nature, but are able to adapt and become active during refrigerated storage of raw and pasteurized milk. The psychrotrophic species found in milk grow over a wide range of temperatures. Growth slows noticeably at the extremes. Counts made following incubation temperature of $3^{o}C$ or $35^{o}C$ are significantly lower than those made following incubation at 5 or $32^{o}C$ respectively (15).

b). Psychrotrophic microorganism

Psychrotrophs are found almost everywhere in nature and may include bacteria, yeasts and molds (16). However, the majority of investigations have been mainly concerned with bacteria. The different types of psychrotrophic bacteria that have been isolated include gram negative as well as gram positive, sporeforming or non-sporeforming, aerobic, facultative anaerobic and anaerobic, long or short rods,

cocci or vibrios (1). Characteristics that have been used to identify psychrotrophic isolates include: gram reaction, cell shape, motility, oxidative fermentation of carbohydrates by Hugh-Liefson methods, production of ammonia from arginine, arginine reaction according to Kovac's test, lipolysis, proteolysis and action on litmus milk (17). Most of the psychrotrophic bacteria isolated from milk and dairy products are gram negative and belong to such genera as Achromobacter, Aeromonas, Alcaligenes, Chromobacterium, Citrobacter, Cytophaga, Enterobacter, Escherichia, Flavobacterium, Klebsiella, Pseudomonas, Serratia and Vibrio (1). However, the genus Pseudomonas is most common not only in milk and other dairy products but also in meats, fish and eggs (2).

Gram positive psychrotrophic bacteria have also been isolated from milk, however, they are present in smaller numbers than gram negative bacteria (1). The gram positive genera commonly encountered in milk and other dairy produc include: Arthrobacter, Bacillus, Clostridium, Corynebacterium, Lactobacillus, Micrococcus, Microbacterium, Sarcina, Staphylococcus and Streptococcus. The species of Micrococcus, Bacillus and Arthrobacter are most common among this group. The isolation of gram positive bacteria is of serious concern as some of these organisms can form spores which survive the heat treatments given to the product and may cause spoilage of these products on subsequent storage. Chang and Canon (18) investigated the characteristics of

psychrotrophic sporeforming bacteria isolated from raw milk and observed that some of the milk samples contained 2-900 spores per ml. These sporeforming bacteria were found to be species of Bacillus and had a lag phase of 8-14 days. Bacillus species were also the most common bacteria isolated in laboratory pasteurized milk (19).

Psychrotrophic yeast and molds do not present a major problem to the dairy industry. Nevertheless, species of Candida, Saccharomycess, Rhodotorulla, Torulopsis, Trichosporon, Aspergillus, Cladosporium, Geotrichum and Penicillium have often been associated with spoilage of milk and other dairy products (1).

c) Biochemical Changes:

The growth and activity of psychrotrophic microorganisms in dairy products at low temperature causes biochemical changes in the products, although at a very slow rate. For example biochemical changes such as fermentation of carbohydrates with acid and gas production, proteolysis and lipolysis causing bitter taste and off flavors in milk may require many months incubation (11). Therefore, apparent signs of spoilage of food are dependent upon the storage time of these products. Other biochemical changes, such as hydrolysis of urea and starch and reduction of nitrate to nitrite may take place at a slow rate (11). Lipolysis and proteolysis are the major causes of spoilage

of milk and other dairy products stored at low temperatures and result in a number of quality problems (1, 15, 20).

i) Lipolysis

Many psychrotrophic bacteria produce lipases in amounts sufficient to cause off flavors in milk and other dairy products. Many of these lipases are found to be heat resistant and cannot be destroyed completely by normal heat treatments given to these products (21). Kishonti (22) reported that a heat treatment at 150°C was required to inactivate a lipase in milk. The development of off flavors in milk is usually due to the hydrolysis of milk fats. Suhren (23) observed that about 20% of 200 pasteurized milk samples developed free fatty acids in amounts sufficient enough to be called rancid. Some of the psychrotrophs produce phospholipases as well as lipases and cause the release of fats from micelles followed by their hydrolysis (24). Phospholipase C, a phosphodiesterase was found to be responsible for hydrolysis of lecithin and cephalin with subsequent release of phosphorylcholine and phosphorylethanolamine from these phospholipids, causing the off flavors in cream (21). The lipases produced by Pseudomonas species; P. fluorescens, P. putida and P. fragi and species of Acinetobacter, Aeromonas and Hydrophilia were found to be wholly or partially active after a heat exposure at 63°C for 30 minutes. Moreover two strains further tested

for their heat stability by a heat treatment of 10 minutes at 100°C, retained 20-25% of their activity (25).

ii) Proteolysis:

Proteolysis of caseins and whey proteins, by extracellular enzymes produced by psychrotrophic microorganisms is one of the major causes of milk spoilage. Proteolysis results in release of various nitrogenous compounds, such as amino acids, peptides and proteoses and peptones causing off flavors in milk and other dairy products. Many of these proteolytic enzymes are heat stable and active at refrigeration temperatures, the temperatures at which milk and other dairy products are generally stored (1).

2. Heat Stable Protease

Many psychrotrophic gram negative rods including the species of genus Pseudomonas, Acinetobacter, Aeromonas, Achromobacter, Enterobacter, Escherichia, Flavobacterium, Xanthomonas, Cytophaga and Proteus produce extracellular proteases (20). However the enzymes produced by Pseudomonas species are the best studied.

a) Isolation and purification

Some of the extracellular proteases from pseudomonads have been purified and characterized. Morihara (26), using a combination of ammonium sulfate, acetone precipitation and

chromatography on a DEAE Cellulose column separated the culture medium of P. aeruginosa into three fractions, containing a neutral, semialkaline and alkaline protease. The semialkaline fraction was crystallized and was found to be an elastase with a molecular weight of 38,000. Peterson and Gunderson (27) separated dialysed culture medium of P. fluorescens into two proteolytic fractions. The pH and temperature optimum of these two fractions led them to suggest that more than one extracellular protease is produced by P. fluorescens. The extracellular proteolytic enzyme produced by P. fluorescens strain R-12 was purified by gel filtration and ion exchange chromatography (28). The molecular weight of this enzyme was estimated by gel filtration and was found to be 37,000. The molecular weight of a protease from P. fluorescens strain AR-11 was found to be 38,000 as determined by gel filtration (29). However, the molecular weight of protease produced by P. fluorescens strain P-26 was found to be quite different from that of the above two strains (30). This enzyme had a molecular weight of only 23,000 and was purified to homogeniety by ammonium sulfate precipitation and gel filtration. Interstrain variation in the properties of protease from pseudomonds was demonstrated by Richardsoq and Whaitii (31). Most of the psychrotrophic strains produced one protease with an electrophoretic mobility between 0.15 to 0.22. However, one strain produced two proteases, which differed in their electrophoretic mobility (0.17 and 0.30 respectively). A 60

fold purification of P. fragi protease was achieved by a combination of ammonium sulfate precipitation, chromatography on Bio-gel p-100 and DEAE Cellulose (32). This enzyme was purified to homogeniety as revealed by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The molecular weight was estimated to be 50,000 by SDS-gel electrophoresis and 40,000 by gel filtration. A serine protease from P. maltophillia was partially purified by ammonium sulfate precipitation, gel filtration and ion exchange chromatography. This enzyme had a molecular weight of 35,000 and an isoelectric point of 9.3. This partially purified enzyme when run in gel electrophoresis, showed a minor protein band immediately following the enzyme protein band. The increase in staining of this band with longer period of storage of enzyme led Boethling (33) to suggest that the enzyme was subject to autodigestion.

b) pH and temperature optimum

The psychrotrophic extracellular proteases are active over a wide range of pH and temperatures. Some of these proteases are optimally active in the neutral to alkaline region. Moreover, some of these proteases remain active at a temperature as low as 0°C. The proteases produced by various psychrotrophic pseudomonads, whose temperature optimum was found to be 40°C, showed different pH optima at two temperatures (34). Although the pH optimum of a protease from P. maltophillia was 10, it retained about 50%

of its maximum activity at pH 6 and 12 (33), while protease from P. fluorescens AR-11 with a pH optimum of 6.5, had very little activity at pH 9 when assayed using isoelectrically precipitated casein (29). On the other hand protease of strain R-12 was almost equally active in a pH range of 6.5-10 (28). P. fluorescens B52 protease was also found to retain about 70% of its activity in the range of 6 to 10.5 (35), but Pseudomonas MC 60 protease had a pH optimum between pH 7 and 8 (36). Although the temperature optima of P. fluorescens strains MC 60 and B52 were almost similar, both being maximally active at a temperature of 45°C (34, 36), the protease of AR-11 had a much lower temperature optimum and was maximally active at about 35°C (29).

c) Inhibition studies

Hartley (37) divided the proteolytic enzymes into 4 major classes based upon their mechanism of action. The classes are a) serine proteinases b) thiol proteinases c) acid proteinases and d) metal proteinases. Although some studies have been done on the effect of known protease inhibitors on psychrotrophic extracellular proteases, further work is needed to classify all known psychrotrophic proteases into one of the above classes.

i) Metal chelating agents

Many of the extracellular proteases of pseudomonads are metalloproteases requiring divalent cations, mostly Ca^{2+}

or Zn^{2+}. Ca^{2+} or other divalent cations were required to stabilize the P. fragi protease (32). This conclusion was drawn from the investigations on inhibition of the enzyme by EDTA. This enzyme was completely inactivated within a few minutes when treated with 10 mM EDTA in the absence of calcium ions. The enzyme activity was not lost to a great extent if $CaCl_2$ at a concentration of 5 mM was added soon after the addition of EDTA. However, the stabilization effect of calcium was lost with the increase in the time following addition of $CaCl_2$ after EDTA treatment. Only 17% activity remained after 60 minutes time gap. Other metal ions such a Sr^{2+}, Co^{2+}, Ba^{2+}, were equally effective in the stabilization of enzyme activity.

The P. fluorescens R-12 protease was completely inactivated when dialysed extensively against EDTA (28). At a concentration of 1mM, Ca^{2+} and Mn^{2+}, were mildly effective in the restoration of the enzyme activity. However, Zn^{2+} or Co^{2+} were much more effective, and 0.5 mM concentration of Zn^{2+} and Co^{2+} restored the enzyme activity to almost 100% of the initial activity. Further evidence of involvement of Zn^{2+} or Co^{2+} for protease activity was provided by inhibition of this protease by o-phenanthroline, which is more specific for Zn^{2+} than Ca^{2+} (28). When a number of heavy metal ions at a concentration of 10 mM were added to a reaction mixture containing P. fluorescens AR-11 protease, the enzyme was completely inactivated (29). EDTA, at a concentration of 1 mM without addition of any metal ion

slightly stimulated the enzyme activity probably by removing the traces of inhibitory ions. This was consistent with the finding that at concentrations higher than 0.5 mM Zn^{2+} had an inhibitory effect on P. fluorescens R12 protease (28). 0.2 mM EDTA also stimulated the activity of P. fluorescens B 52 protease activity but at 1.3 mM it inactivated the enzyme completely (35). However, only 0.3 mM O-phenanthroline was sufficient to cause 100% inactivation. This observation together with the finding that the enzyme lost the ability to bind to carbo-benzoxy-D-phenylalanyl-tetraethylene pentamine (CBZ-D-Phe-T) sepharose 4 B in the presence of 5 mM tetraethylene pentamine (TEP), which binds Zn^{2+} strongly, led Richardson (35) to suggest that Ca^{2+} has a role in stabilization of the enzyme and it is zinc which is involved in enzyme activity.

ii) Sulfhydryl reagents

When the effect of various sulfhydryl reagents on protease activity was investigated, enzymes from different Pseudomonas species showed different results. The P. fluorescens AR-11 protease was inhibited by dithiothreitol, iodoacetic acid, iodoacetamide, N-ethylmaleiamide and parachloromercuribenzoate (29). On the other hand the R-12 and B-52 proteases were not inhibited by sulfhydryl reagents (28, 35).

d) Heat stability

Many of the psychrotrophic proteases possess a remarkable capacity to survive heat treatments, even beyond the time temperature combinations used for ultrahigh temperature (UHT) sterilization. The heat stability of the extracellular protease from P. fluorescens was first reported by Mayerhoffer (30), who observed that for complete inactivation of protease from P. fluorescens p-26 15 hours at 62.8°C, 8 hours at 71.4°C or 9 minutes at 121°C were required. Skim milk, whey or 2.5% casein seemed to have a protective effect on protease from heat denaturation. When heat denaturation of ten Pseudomonas proteases was investigated, most of them retained about 70% of their activity after a heat exposure of 10 seconds at 149°C (36). The protease produced by Pseudomonas MC 60 was found to be the most heat resistant enzyme in these studies. When the heat denaturation of this enzyme at 149°C was compared with the destruction of spores used to establish UHT sterilization parameters, it was found to be 400 times more heat resistant than spores of putrefactive anaerobe3679, and more than 4000 times more heat resistant than Bacillus stearothermophilus spores. This enzyme was also stable over a temperature range of 110-150°C. The Z value (change in temperature yielding 10-fold change in rate of inactivation) for this protease was 32.5°C and the D value (time required for a 90% decrease in enzyme activity) at 149°C was 90 seconds (36).

The protease produced by P. fluorescens strain AR-11 was also found to be heat stable (29). For a 50% inactivation, heating for 25 seconds at 130°C, or 17 seconds at 140°C or 8.5 seconds at 150°C was required. Twelve other pseudomonads isolated from raw milk also produced heat stable proteases (34). Griffith (38) investigated the heat resistance of proteases produced by many fluorescent and non-fluorescent pseudomonads and observed that about 55-65% of their activity was retained after heat treatment for 17 seconds at 77°C and 20-40% activity was retained after 5 seconds at 140°C. The only Pseudomonas species producing a heat labile protease was P. maltophilia. Gram positive genera including the species Enterobacter, Serratia, Alcaligenes, Flavobacterium, Moraxella, Acinetobacter, Aeromonas and Achromobacter were also observed to produce extracellular heat stable proteases.

A psychrotrophic yeast, Rhodotorula, isolated from laboratory pasteurized milk retained 25% of its activity after heating at 80°C for 10 minutes and boiling for 20 minutes was required for complete inactivation of this enzyme (39). Other genera of yeast and fungi producing extracellular proteases are also reported including the species of Aureobasidium, Cephalosporium, Endomycopsis, Kluyveromyces and Candida (40).

i) Mechanism of heat stability

Barach and Adams (41) compared the heat stability of

thermolysin and Pseudomonas MC-60 protease. The inactivation of thermolysin took place in two stages, this biphasic inactivation curve suggested that in the first rapid phase autodigestion was the major cause of inactivation, while in the second phase, first order inactivation kinetics were observed. In contrast to thermolysin, a linear inactivation curve for MC 60 protease suggested autodigestion was not the major cause of inactivation of this enzyme at ultrahigh temperature. Lack of cysteine in both of these enzymes may provide flexibility to the enzyme and renaturation after heat treatment may take place easily. The percentage of hydrophobic side chains is similar in the two enzymes and both contain Ca^{2+} and Zn^{2+}. On the basis of these findings it was concluded that the enzymes can withstand extremes of temperatures due to the structural flexibility of these proteins and their metal ion contents (41).

Autodigestion was found to be the major cause of heat inactivation of P. fluorescens B-52 protease (35). However since this enzyme also contains Ca^{2+} and Zn^{2+}, it was suggested that the presence of Ca^{2+} ions allows the maintenance of native conformation of this enzyme. Furthermore, both the enzymes from Pseudomonas MC 60 and B-52 have high glycine content whose small side chains would minimize steric hindrance and allow structural flexibility during heat treatment.

ii) Low temperature inactivation

Although most of the Pseudomonas extracellular proteases can survive ultrahigh temperature exposure, some of these enzymes are rapidly inactivated at lower temperatures. More than 90% inactivation of MC 60 protease was observed at 55°C within 10 minutes, while it lost only 24% of its activity after a heat exposure of 10 seconds at 149°C (42). This inactivation at 55°C was about 10 times greater than the expected value based upon its D value at 150°C. A crude sample of protease was found to be more resistant to low temperature inactivation (LTI) in milk than purified enzyme in milk or a crude sample of protease in buffer. Barach et al. (42) suggested that purification or heating in buffer may have altered the heat resistance due to the loss of associated factor(s) or protein required for heat resistance. When this enzyme was subjected to LTI, it was rapidly autolysed as shown by an increase in trichloroacetic acid soluble nitrogen and disappearance of native enzyme protein band in SDS electrophoresis; whereas in presence of 3.5% sodium caseinate, the inactivation was very low (43). Purified C^{14}-labelled protease, when heated in milk at 55°C for 1 hour, aggregated with casein. Due to this aggregation, reversible loss of enzyme activity took place. Partial restoration of activity was observed when LTI was performed in presence of 6M urea with high enzyme concentration. A complete restoration of activity was observed when the enzyme concentration was low. On the

basis of the above findings it was concluded that LTI is a two step process. In the first step, heating at 55°C results in a conformational change in the enzyme molecule which causes its autodigestion; and in the second step, this residual altered protein forms a complex with casein and results in loss of enzyme activity. Heating up to 55°C caused the nonpolar side chains of proteins to be exposed to the solvent which favors the aggregate formation with casein. This thermodynamically favored aggregate would be stable even upon cooling. However, at temperatures above 60°C, these hydrophobic interactions are no longer favored.

e) Hydrolysis of Milk Proteins

Many investigators have studied the effect of various proteolytic enzymes on milk proteins using either a crude preparation of the enzyme or by incubating the bacteria responsible for the production of these enzymes. When Adams et al. (44) inoculated Pseudomonas MC 60 into the milk, k-casein was found to be the most proteolysis-prone fraction of casein after 7 days' incubation at 5°C. There was a complete loss of this fraction after 13 days' incubation under similar conditions. The amount of β-casein also decreased rapidly. The whey proteins were not hydrolyzed by this isolate. In contrast to the above findings, micrococci isolated from cheddar cheese preferentially hydrolyzed the αs_1-casein. However, the intracellular protease from the same bacteria degraded β-casein preferentially (45). Other

investigators (46) observed that isolated αs_1 and β-casein were hydrolyzed more efficiently than in whole casein mixture. β-casein was found to be more susceptible to proteolysis at lower temperatures. αs_1-casein became more susceptible to proteolysis when the micellar structure was disrupted by removal of calcium ions. Richardson and Whaitii (31) observed that all fractions of casein are attacked by the Pseudomonas proteases. They also observed that milk protein degradation began with hydrolysis of k and α-caseins followed by degradation of β and αs_1-casein.

f) Control of protease production

Several psychrotrophic extracellular proteases have been studied in detail. However, very little is known about regulation of the synthesis and secretion of these enzymes. Daatselaar and Harder (47) isolated bacteria from the gills of a fish and found that amino acids seem to induce the production of proteolytic enzymes from this bacterial isolate. Moreover, glucose, when present in the growth medium, completely inhibited the protease production. It was also observed that proteolytic activity in the culture medium increased at the end of the logarithmic growth phase. Enzyme production stopped when the stationary phase was reached. It was also observed that the addition of chloramphenicol during logarithmic phase immediately stopped the production of the protease. All these findings led Daatselaar and Harder (47) to suggest that the synthesis of

extracellular proteolytic enzymes of the organism studied is controlled by an efficient regulatory mechanism in which growth rate is an important parameter. In contrast, Adams et al. (44) observed that Pseudomonas MC 60 produced the extracellular protease throughout the exponential growth phase at 4°C. Juffs (48) investigated the effect of varying the temperature and composition of the medium on production of proteases by P. fluorescens and P. aeruginosa isolated from raw milk. Protease production by P. fluorescens decreased gradually in Peptone-Yeast Extract broth when the temperature was reduced from 20°C to 5°C. At 30°C although there was heavy growth in the same medium, a very small amount of protease was produced. On the other hand protease production by P. aeruginosa was maximal at 30°C. Both glucose and lactate decreased the protease production by these organisms via catabolic repression (48).

MATERIALS AND METHODS

1. Materials

All chemicals were of analytical grade and most of them were purchased from Sigma Chemical Co. (St. Louis, MO.). Soluble casein was obtained from British Drug House Ltd. (Toronto, Ont). The affinity chromatography material, carbobenzoxy- L-phenylalanyl-triethylenetetraminyl-Sepharose 4B was supplied by Pierce Chemical Co. (Rockford, Ill.). Sephadex G-200 and some of the proteins used as standard were purchased from Pharmacia Fine Chemicals (Uppasla, Sweden). The majority of electrophoresis reagents and those used in immunological procedures were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Ont.) and the rest from Sigma Chemical Co., (St. Louis, MO).

2. Cultures

The psychrotrophic pseudomonads were isolated previously in our laboratory as described in a recent report (49). For isolation of these bacteria raw milk samples obtained from a local dairy farm were streaked on plate count agar (PCA) containing two percent (w/v) skim milk powder. These plates were incubated at 7°C for 10 days. The isolated colonies showing clear zone of proteolysis were picked up and streaked on fresh medium (PCA plus milk) and incubated at 7°C for two days and then served as stock

cultures. These bacterial cultures were then identified using both biochemical and microbiological tests (50, 51).

3. Preparation of crude extract

A summary of the procedure employed for crude enzyme preparation is depicted in Fig. 1. In some studies this crude enzyme preparation was used without any further treatment. However, in most instances the crude dialyzed extract was concentrated by freeze drying and/or by ultrafiltration on Amicon Ultrafiltration unit with Millipore filter membrane of 10,000 molecular weight exclusion limit.

4. Protease assay.

a) Casein or other proteins as substrates

Method of Hull (52) was used to determine the proteolytic activity. The assay mixture contained 0.1 M Tris (hydroxymethyl) amino-methane (Tris-HCl) buffer, pH 7.5, 1% soluble casein (w/v, 0.5 ml) dissolved and dialyzed against the above mentioned buffer, and enzyme, 0.1-0.2 ml. The total volume of the assay mixture was 2.0 ml. The assay tubes were incubated for 20 minutes in a water bath maintained at 25°C. After the incubation period the reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA). A control tube was also included with each test in which either the enzyme or

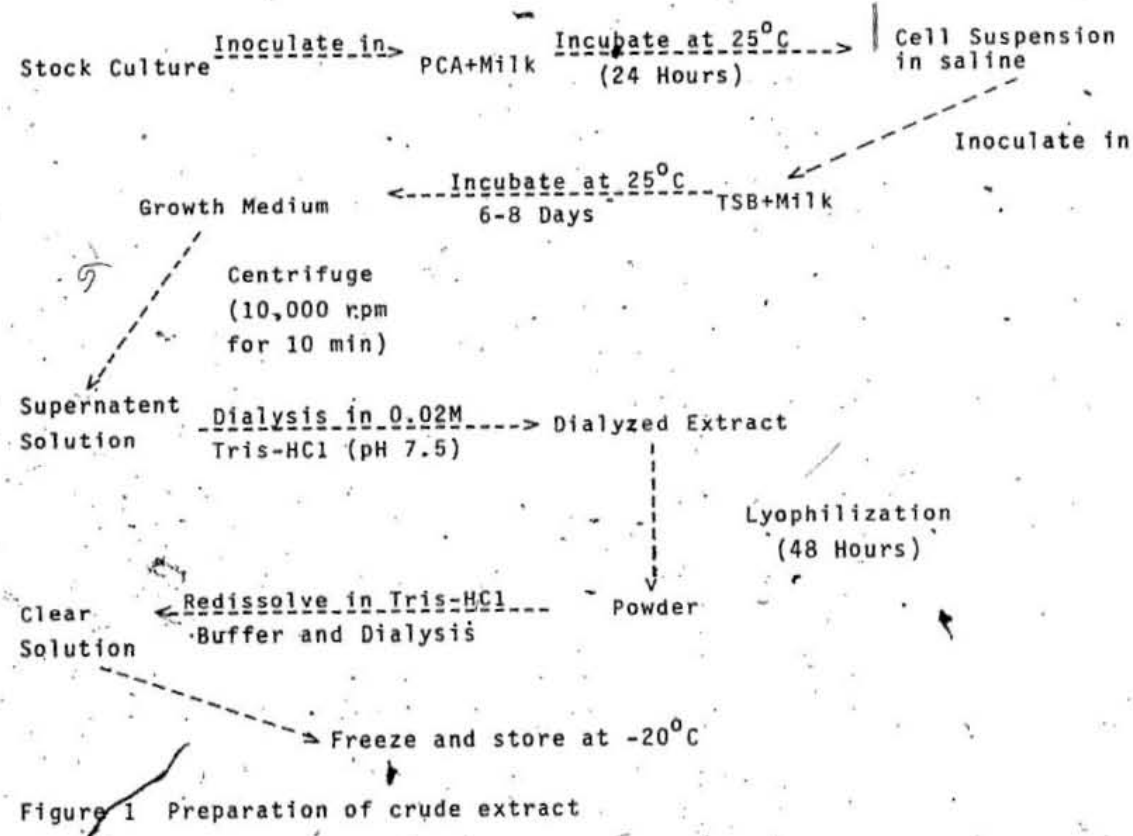


Figure 1 Preparation of crude extract

substrate was added after the addition of TCA. Following the separation of precipitated proteins by centrifugation for 10 minutes at 10,000 rpm in a Sorval Centrifuge, the TCA soluble aromatic amino acids released by hydrolysis of proteins were determined directly by measuring absorbance at 280 nm. An enzyme unit (EU) is defined as the amount of enzyme required to release 1 umol of tyrosine equivalence (absorption of equal amount of tyrosine and other aromatic amino acids) per ml per minute at 25°C. The specific activity is EU per mg protein. All of the assays were performed in duplicate or triplicate and presented as the average of two or more readings.

b) Proteolytic activity on milk plates

Aliquots (10 ml) of 1% agar containing 1% skim milk powder and 0.02% sodium azide (w/v) were poured in petri plates (100 x 15 mm) and allowed to solidify after which they were stored in the cold (10°C). Wells with a diameter of 5 mm were punched in solidified agar and 10-15 ul of enzyme was applied per well. The plates were incubated at 25°C for 18-24 hours. The diameter of the true clear zone (allowing for well diameter) was measured after flooding the plates with acid sublimate solution ($HgCl_2$ + HCl) according to the method of Foissy (53). A standard curve was constructed using various dilutions of the protease, Subtilisin BPN' purchased from Sigma Chemical Company.

c) Protease activity using various synthetic substrates

The substrates used were N-Benzoyl-L-tyrosine-p-nitroanilide, Glutaryl-L-phenylalanine-p-nitroanilide, α-N-Benzoyl-L-arginine-p-nitroanilide, p-Toluenesulfonyl-L-arginine methyl ester and Glycyl-L-phenylalanine-β-naphthylamide. The proteolytic activity using these substrates was determined by measuring absorption changes according to the methods given in Worthington Enzyme Manual (54) and according to the method of Erlanger et al. (55).

5. Determination of Proteins

Protein concentrations in solutions were determined by the method of Lowry et al. (56) using bovine serum albumin as a standard protein.

6. Partial purification of protease

a) Ammonium sulfate precipitation

To 90 ml of crude lyophilized extract 35.1 g ammonium sulfate was added slowly to give a 60% saturation, the pH was adjusted to neutrality and stirred at 10°C for 40 hours. The precipitated proteins were separated by centrifugation at 10,000 rpm for 10 minutes and the supernatant discarded. The precipitated proteins were redissolved in 30 ml of 0.02 M Tris-HCl buffer pH 7.5, and dialyzed for 24 hours against the same buffer to remove the salts. The dialyzed solution

was centrifuged and the clear supernatant solution obtained was concentrated to 21 ml by ultrafiltration.

b) Gel filtration

A concentrated sample (20 ml, specific activity 0.171) was applied to a column (2.5 x 81 cms) packed with Sephadex G-200. The column was equilibrated with 0.02 M Tris HCl buffer (pH 7.5) containing 0.02% (w/v) sodium azide. The proteins were eluted with the same buffer at a flow rate of 18 mls per hour and fractions approximately 5.2 ml per tube were collected. The protein in the fractions was estimated spectrophotometrically at 280 nm. The protease activity was measured by Hull's method (52). Peak fractions containing maximum proteolytic activity were pooled together and concentrated to 21 ml by ultrafiltration.

c) Affinity column chromatography

The pooled fractions from the gel filtration column were further purified by affinity chromatography on a column (1 x 8 cms) packed with Carbo-benzoxy-L-phenylalanyl-triethylenetetraminyl-Sepharose 4B (Z-L-Phe-T-Sepharose) according to the method of Cuatrecasas et al. (57). The column was equilibrated with 0.025 M sodium acetate buffer (pH 5.7) containing 0.1 M sodium chloride, 0.01 M calcium chloride and 0.02% (w/v) sodium azide. The concentrated pooled fractions (about 20 ml) from the gel filtration column were dialyzed against the same buffer for 24 hours

and applied on affinity column. The column was then washed with the above mentioned buffer to remove the unbound proteins. The protein bound to the column was eluted using 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M sodium chloride, 0.01 M calcium chloride and 0.02% (w/v) sodium azide. Fractions (3.1 ml) were collected at the flow rate of 12 ml per hour. The protein was determined spectrophotometrically by measuring the absorption at 280 nm and proteolytic activity was determined by Hull's method (52). Fractions containing maximum protease activity were pooled together and gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed according to the method of Weber and Osborne (58), or according to the method of Davis (59).

7. Properties of the Protease

The protease activity was assayed by Hull's method (52) as described on page 23, varying only one parameter at a time unless otherwise stated.

a) Effect of enzyme concentration

In order to determine the volume of lyophilized extract (redissolved in the Tris-HCl buffer) required for protease assay in Hull's method (52) various volumes (25-300 ul) of T20 extract were used keeping other conditions constant as described previously.

b) pH optimum

Soluble casein, 1% (w/v) was dissolved in various buffers to cover pH range 5.5-10.5 and the proteolytic activity was measured by Hull's method (52). The buffers used were 0.1 M citrate-phosphate buffer, 0.1 M Tris-HCl buffer and 0.1 M glycine-sodium hydroxide buffer.

c) Effect of temperature

After a preincubation of the tubes containing enzyme plus buffer (Tris-HCl) for 5 minutes the reaction was started by the addition of substrate calibrated to the required temperature as enzyme and buffer.

d) Time course of protease activity

The protease assay was performed in separate tubes and at various time intervals the reaction was stopped by addition of TCA.

e) Effect of substrate concentration

Solutions of α, β and soluble casein (1% w/v), dialyzed against the Tris-HCl buffer were used to cover a wide range of concentration. The Km was determined for α and β-casein by Lineweaver Burke Plot (60) and straight line was obtained by regression analysis.

f) Substrate specificity

The proteins were dissolved in and dialyzed against the Tris-HCl buffer except α, β, γ and k casein which were dissolved in 0.001 N NaOH and dialyzed against the same buffer. The final concentration of each protein was 2.5 mg/ml assay mixture, total volume, 2 ml.

g) pH stability

Crude extracts (Sp activity 0.121) concentrated by lyophilization and redissolved in 0.02 M Tris-HCl buffer (pH 7.5) were used for this experiment. One ml of this protease solution was added to 4 ml of the buffers covering a pH range between 2.5-11.5. The buffers used were similar to those used in pH optimum studies. These diluted enzyme solutions were then stored at 10°C for four days. The enzyme solutions were then subjected to centrifugation (10,000 rpm for 10 minutes) to remove the precipitated proteins, and the clear supernatant solutions were used for protease assay by Hull's method (52).

h) Heat stability

In order to investigate the effect of heating on protease activities of various isolates the following experiments were performed. In these experiments, the heat treatments were followed by subsequent storage of the proteases in the frozen state prior to protease assay. Therefore, a cumulative effect of heat exposure and cold

storage (freezing) was investigated. However, for simplicity the term heat stability is used.

i) Heat exposure of various proteases at different temperatures

Lyophilized extract dissolved in the Tris-HCl buffer was divided into various aliquots (2 ml per tube) and heated in water baths maintained at various temperatures between 50 and 100°C. The extracts were heated at these various temperatures and were cooled immediately by immersion in ice water and stored at -20°C till further use. Just before the protease assay, the heat treated extracts (stored at -20°C) were thawed in a water bath (maintained at 25°C) and proteolytic activity was determined by Hull's method (52).

ii) Time course of heat exposure at 100°C

Lyophilized extract dissolved in the Tris-HCl buffer (20 ml) was poured into a flask in a water bath maintained at 100°C. Samples (1 ml) were withdrawn at different time intervals and cooled immediately and treated as described above (i).

iii) Heat exposure in presence of calcium chloride

Lyophilized extracts dissolved in the Tris-HCl buffer were added to 1 ml of the same buffer containing different concentrations of calcium chloride (between 5 to 1000 u moles/ml). These enzyme solutions with calcium chloride were

subjected to heating as described above (i), except that clear supernatant solutions were dialyzed against the Tris-HCl buffer prior to protease assay.

8. Enzyme modulation studies

Various inhibitors/stimulators (50 mM) were dissolved in distilled water except 8-hydroxyquinoline and parachloromercuribenzoate (PCMB), which were dissolved in dimethyl sulfonyl fluoride (DMSF). Various modulators (50 ul and 100 ul) were added to tubes containing the Tris-HCl buffer and enzyme to give final concentration of 1.25 mM and 2.5 mM respectively. The protease and modulators were allowed to react for 10 minutes in a water bath maintained at 25°C in the absence of substrates. The reaction was initiated with the addition of 0.5 ml of 1% soluble casein. With each test a control tube was included in which the substrate was added after the addition of TCA. For each isolate one more control was also included in which protease activity was measured in the presence of DMSF.

9. Metal ions requirement

a) Effect of metal ions on the protease activity

The following metal salts were used for this experiment; calcium chloride, cobaltous chloride, cupric sulfate, manganese chloride, magnesium sulfate, zinc sulfate, mercuric chloride and nickel sulfate. Aliquots

(100 ul and 200 ul) of metal salt solution (20 mM) in distilled water were added to the tubes containing enzyme and the Tris-HCl buffer. The final concentrations of metal salts were 1 and 2 mM. The salts and the enzyme in buffer were allowed to react in cold ($10^{o}C$) for 15 minutes. After a further 5 minute preincubation at $25^{o}C$, the reaction was initiated by addition of substrate solution. With each test a control tube was also included, treated similarly as test except that the substrate solution was added after the addition of TCA.

b) Restoration of protease activity after Ethylenediamine-tetracetic acid (EDTA) treatment

Lyophilized extract dissolved in the Tris-HCl buffer was dialyzed extensively (more than 24 hours) against the Tris-HCl buffer containing 0.1 M EDTA to completely inactivate the protease. This EDTA treated extract was redialyzed against the Tris-HCl buffer without EDTA. The protease assay was then performed in the presence of various metal ions (2 mM) as described above (a). The following metal salts were used in addition to the above mentioned salts: lithium chloride, strontium chloride, stannous chloride, aluminium sulfate, ammonium molybdate, barium chloride, cadmium chloride, lead nitrate, potassium chloride and sodium phosphate.

10. Enzyme synthesis

a) Growth curve and protease production

TSB (25 ml) was inoculated with a single colony isolated from PCA containing 2% skim milk powder. The flask was incubated at 25°C for 24 hours. The cells were separated from TSB by centrifugation (10,000 rpm for 10 minutes) and washed (3 times) in physiological saline (0.85%, w/v NaCl). This washed cell suspension was then inoculated into 125 ml of TSB containing 2% skim milk powder. The flask was incubated in a shaker bath maintained at 25°C. At different time intervals, 4 ml samples were withdrawn and absorbance at 600 nm was measured. A 3 ml sample was centrifuged and the clear supernatant solution obtained was dialyzed against 0.02 Tris-HCl buffer (pH 7.5) and protease assay was performed using dialyzed extract. The remaining 1 ml sample was used for viable count after appropriate dilutions.

b) Induction of proteases

A washed bacterial cell suspension of various isolates obtained from growth on PCA was inoculated into 50 ml Mineral Salt medium, with or without 0.5% (w/v) soluble casein and various other carbon sources (10 mM). The carbon sources other than casein were pyruvate, citrate, succinate, glutamine, glucose and lactose. The initial pH and absorption at 600 nm was measured and the flasks were

incubated in a shaker water bath maintained at 25°C. After five days incubation the pH and the absorption were measured again. A sample (5 ml) was centrifuged and dialyzed against 0.02 Tris-HCl buffer (pH 7.5). This dialyzed sample was used for protease assay and protein determination.

The above experiment was repeated except that one more set of flasks containing 1% (w/v) skim milk powder with various carbon sources was also included.

In another experiment MSM with various carbon sources (0.1 M) were inoculated with an extensively washed cell suspension (1 ml). The rest of the procedure was similar to the method described above.

11. Extracellular nature of the protease

Trypticase soy broth (50 ml) with 2% skim milk powder (w/v) was inoculated with 5 ml of cell suspension from various isolates in physiological saline and incubated for 5 days at 25°C. The medium was then centrifuged (10,000 rpm for 10 minutes) and the clear supernatant solution was decanted and dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) and stored at -20°C. The cell pellet was washed three times with the Tris-HCl buffer and the washed cell suspension thus obtained was subjected to ultrasonication. The cell debris was removed by centrifugation (10,000 rpm for 10 minutes). This supernatant solution (cell extract) and the dialyzed growth medium were tested for the protease activity.

12. Molecular weight determination

A column (2 x 45 cms) packed with Sephadex G-200 was used for determination of molecular weight of T20 protease according to the method of Andrews (61). Proteins used for calibration of column were, ferritin (440,000), catalase (210,000), aldolase (158,000), alcohol dehydrogenase (141,000), bovine serum albumin (64,000), ovalbumin (43,000), chymotrypsinogen (25,000) and lysozyme (11,000). A standard plot was obtained by plotting logarithm of molecular weight against Kav of various proteins. The calibrated column was used for estimation of molecular weight of T20 protease. Lyophilized extract redissolved in 0.02 M Tris-HCl buffer (pH 7.5), with a specific activity of 0.161 was applied on the column. The proteins were eluted in the same buffer and fractions (3.1 ml/ tube) were collected at a flow rate of 15 ml/hour. The absorption at 280 nm was measured to determine the concentration of the proteins in the fractions and protease assay was performed by Hull's method (52).

13. Antigenic relatedness of the proteases

In order to determine the antigenic relatedness of proteases from different bacterial isolates various immunochemical tests were performed.

a) Inhibition studies

Into the tubes containing the T20 protease

(approximately 0.5 EU/ml) and the Tris-HCl buffer, increasing amounts of the antibody previously raised in rabbit against purified T25 protease (62,.63) were added and incubated for 15 minutes at 25°C. The protease activity was then initiated by addition of 0.5 ml of 1% soluble casein. A control tube was also included with each test treated similarly except that the soluble casein was added after the addition of TCA.

b) Ouchterlony's double diffusion

The antigenic relatedness of protease from several isolates was determined by immunodiffusion as described by Stollar and Levine (64).

To prepare the gel frames, 6 clear glass slides (2.5 x 7 cms) were placed on each frame. These slides were then coated with a thin layer of 0.1% (w/v) Noble agar solution containing 0.05% (v/v) glycerine. The slides were dried in a 37°C incubator for 30 minutes. About 24 ml of running agar, (0.5% Noble agar in 0.025 M barbital buffer, pH 8.6) containing 0.2% sodium azide (w/v) were poured per frame. Care was taken to obtain a uniform layer. The agar was allowed to solidify for about 30 minutes and stored in a humidity chamber at 2-4°C until use. The gels were punched to make wells (diameter, 5 mm) and 15 ul antibody solution (about 4 mg protein/ml) were poured in central wells and 10 ul of enzyme (about 5 mg protein/ml) in the surrounding wells. These frames were then placed in the humidity

chamber and stored at 2-4°C for 18-24 hours to obtain precipitin bands.

c) Immunoelectrophoresis

The immunoelectrophoresis was carried out by the method of Scheidegger (65). The agar frames were prepared as described earlier. The gel puncher (Gelman) was used to obtain a trough down the center of slides with equally placed holes on either side. The gel from the holes was removed and 10 ul of enzyme solution (about 5 mg protein/ml) was poured on each side. In one hole 5 ul of a 0.1% bromophenol blue was poured as a marker dye. The frames were then aligned in electrophoresis chamber and filled with approximately 900 ml of 0.1 M barbital buffer (pH 8.6). For each frame 4 strips soaked in the buffer were attached to the ends connecting frames to the buffer. The cover was closed and the system was electrophoresed for about 2 hours at 3-5 m Amperes per frame. After the electrophoresis, the frames were removed from the chamber. The gel troughs previously cut were now removed and 30-40 ul of antiserum or purified antibody raised against protease from T25 or T16 were deposited along the length of the troughs. Each frame was then placed in a humidity chamber and incubated at 2-4°C to form precipitin arcs.

RESULTS

1. Bacterial Isolates

Twenty eight bacterial cultures were isolated from raw milk samples, of which 19 were found to belong to the genus Pseudomonas and were tentatively assigned to the species, fluorescens (49).. Out of the original 28 cultures, 8 were selected for this study with more emphasis centered on those showing high proteolytic activity.

2. Enzyme source

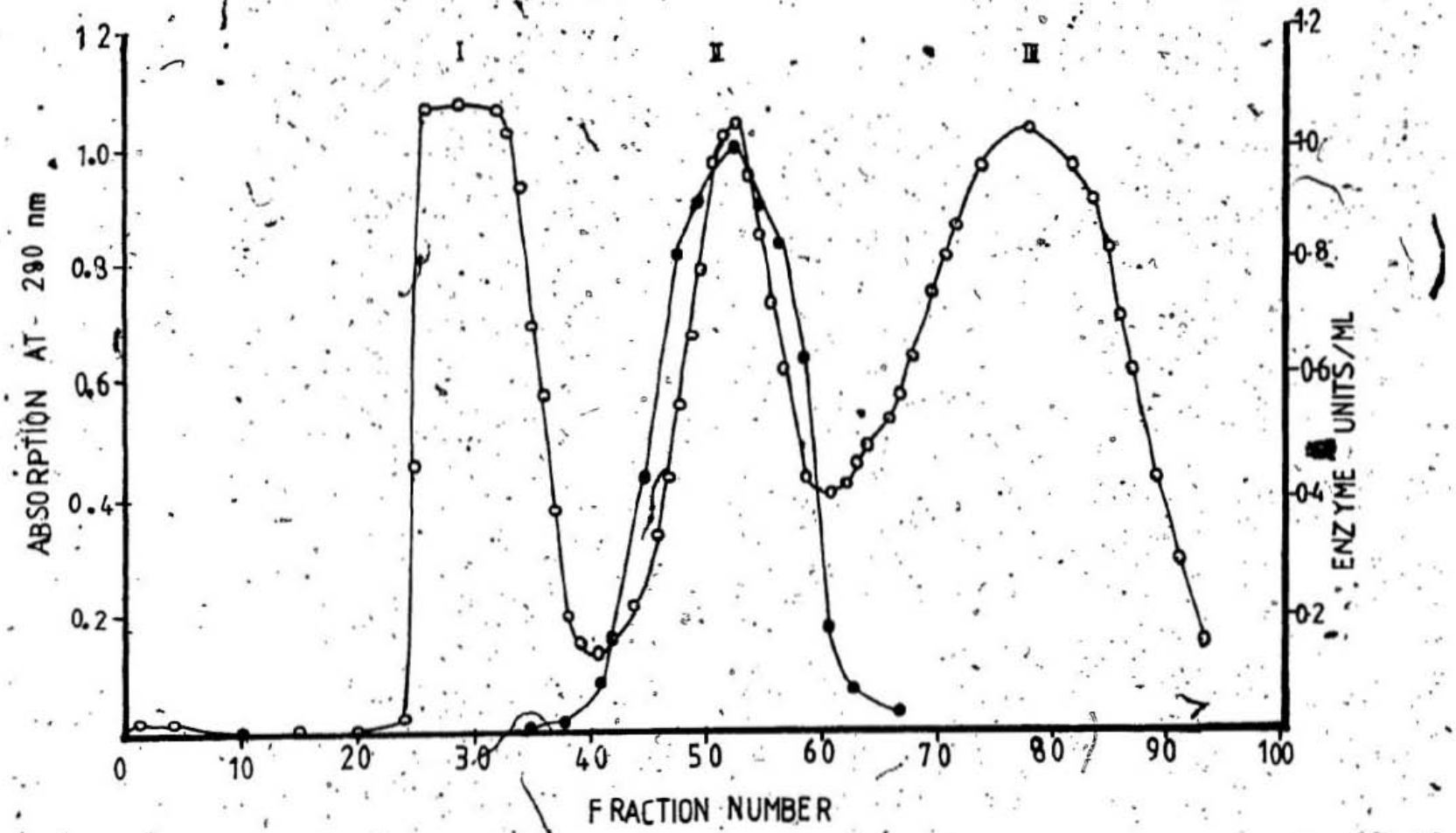
A crude enzyme preparation was obtained as outlined in Materials and Methods (Fig. 1). Dialysis of crude enzyme preparation was found to be necessary to reduce the high background absorption at 280 nm. Loss of protease activity did not occur on dialysis of enzyme preparations against 0.02 M Tris-HCl buffer (pH 7.5) for 48 hours. Similarly lyophilization and ultrafiltration did not cause considerable loss of protease activity and about 85-95% activity was recovered after these treatments. All proteases were found to be stable when stored frozen at $-20^{0}C$ for periods extending over many months.

3. Purification of the T20 protease

The protease from T20 was purified by a combination of ammonium sulfate precipitation, gel filtration and affinity column chromatography. Fig. 2 shows the elution profile of

Fig. 2 Gel filtration of T20 protease on Sephadex G-200 column

A concentrated sample having a specific activity of 1.610 (20 ml) was applied to column (2.5 x 81 Cm) containing Sephadex G-200 (398 ml bed volume). The column was washed with 0.02 M Tris-HCl buffer (pH 7.5). The protein (o—o) concentration in the fractions was determined spectrophotometrically at 280 nm. The protease activity (●—●) in the fractions was determined as described in the Materials and Methods.


I
II
III
ABSORPTION AT- 280 nm
ENZYME - UNITS/ML
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
100
0.2
0.4
0.6
0.8
1.0
1.2

proteins on a gel filtration column of Sephadex G-200. The proteins were separated into three peaks (I, II, III) and the protease activity was associated with peak II. The most active fractions (47-57) were pooled and concentrated by ultrafiltration to a volume of about 21 ml. This concentrated sample was then applied on a Z-L-phe-T-Sepharose column (Fig. 3). The bound protein was eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.01 M $CaCl_2$. The protein eluted into two distinct peaks. The initial peak (a) contained no protease activity and the second peak (b) contained most of the activity. The fractions (37-80) were pooled together and concentrated as before. Table 1 summarizes the steps in the purification of the protease. The purified protease showed 33 fold increase in activity with a recovery of 14 percent. Fig. 4 shows the results of gel electrophoresis of the purified protease.

4. Properties of protease

a) Effect of enzyme concentration on protease activity

Fig. 5 depicts the effect of increasing volume of T20 lyophilized extract (redissolved in the Tris-HCl buffer) on the protease activity. The protease activity increased with increasing volume of T20 extract up to 0.1 ml per tube of assay mixture; further increase in the amount of extract did not increase the T20 protease activity. This experiment demonstrated clearly that the T20 protease activity

Fig. 3 Purification of T20 protease by affinity column chromatography

Partially purified enzyme obtained by gel filtration was further purified on an affinity column (1 x 8 Cm) packed with Carbobenzoxy-L-Phenylahlanyl-triethylene tetraminyl-Sepharose 4B (Z-L-phe-T-Sepharose). The column was equilibrated with 0.025 M sodium acetate buffer (pH 5.7) containing 0.1 M NaCl and 0.01 M $CaCl_2$. After the sample application the unbound proteins were eluted with the above mentioned buffer. The arrow in the figure indicates the change of buffer to 0.1 M Tris-HCl (pH8) containing 0.5 M NaCl and 0.01 M $CaCl_2$. The proteins (o—o) in fractions were determined by reading the absorption at 280 nm and proteolytic activity (●—●) was assayed by Hull's method (52).

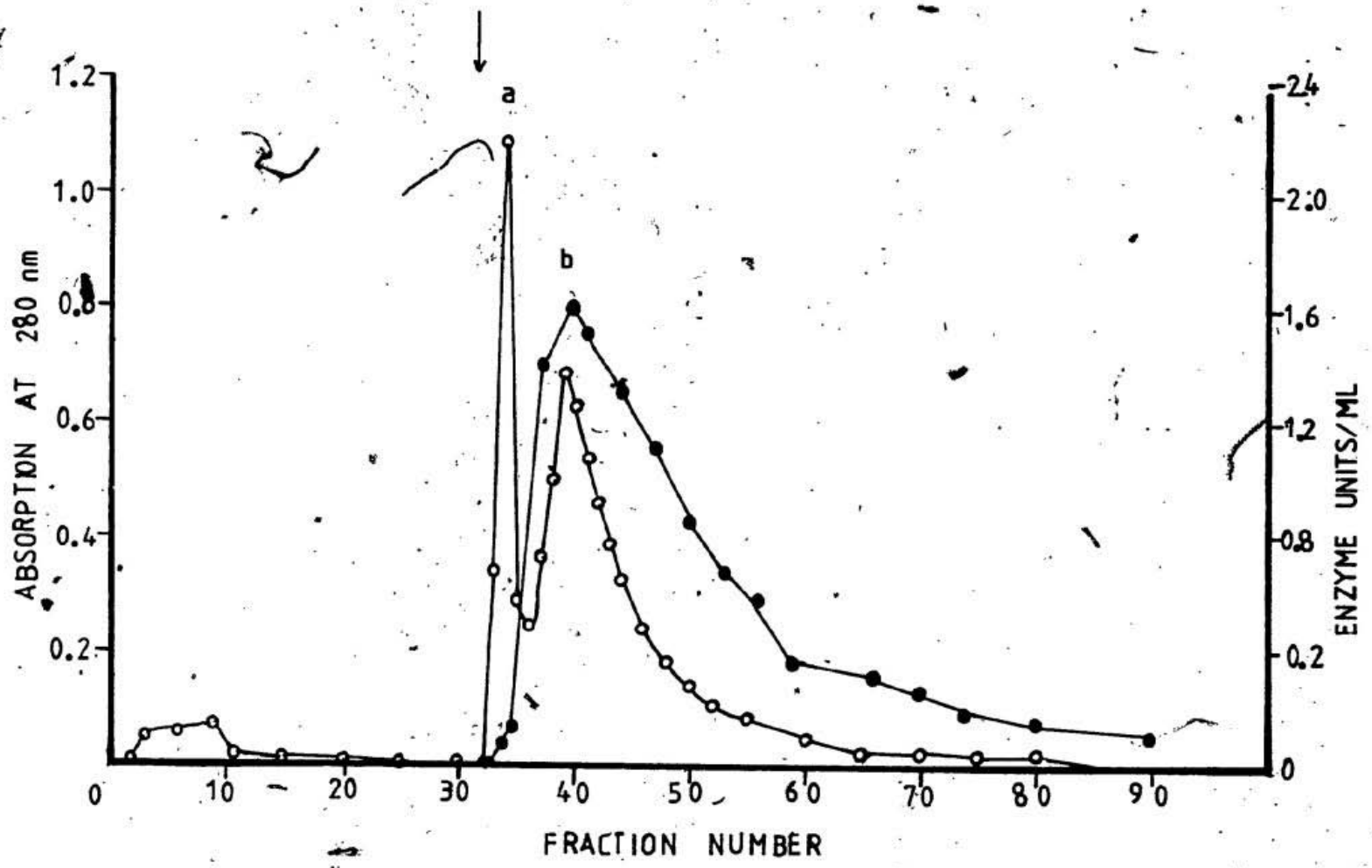

a
b
ABSORPTION AT 280 nm
ENZYME UNITS/ML
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
0.2
0.4
0.6
0.8
1.0
1.2
1.6
2.0
2.4

Fig. 4 Polyacrylamide gel electrophoresis of partially purified T20 protease

A partially purified sample (50 ug protein/gel) of T20 protease was electrophoresed on 7.5% polyacrylamide gels in the presence or absence of SDS (0.1%). The electrophoresis was completed in about 5 hours at a current of 2-4 mA per tube. The gels were stained in Coomassie Blue R 250.

a) gel electrophoresis in presence of SDS

b) gel electrophoresis in absence of SDS

P protease

d dye

\+ positive electrode

\- negative electrode

a

b

Table 1

Purification steps for T20 protease

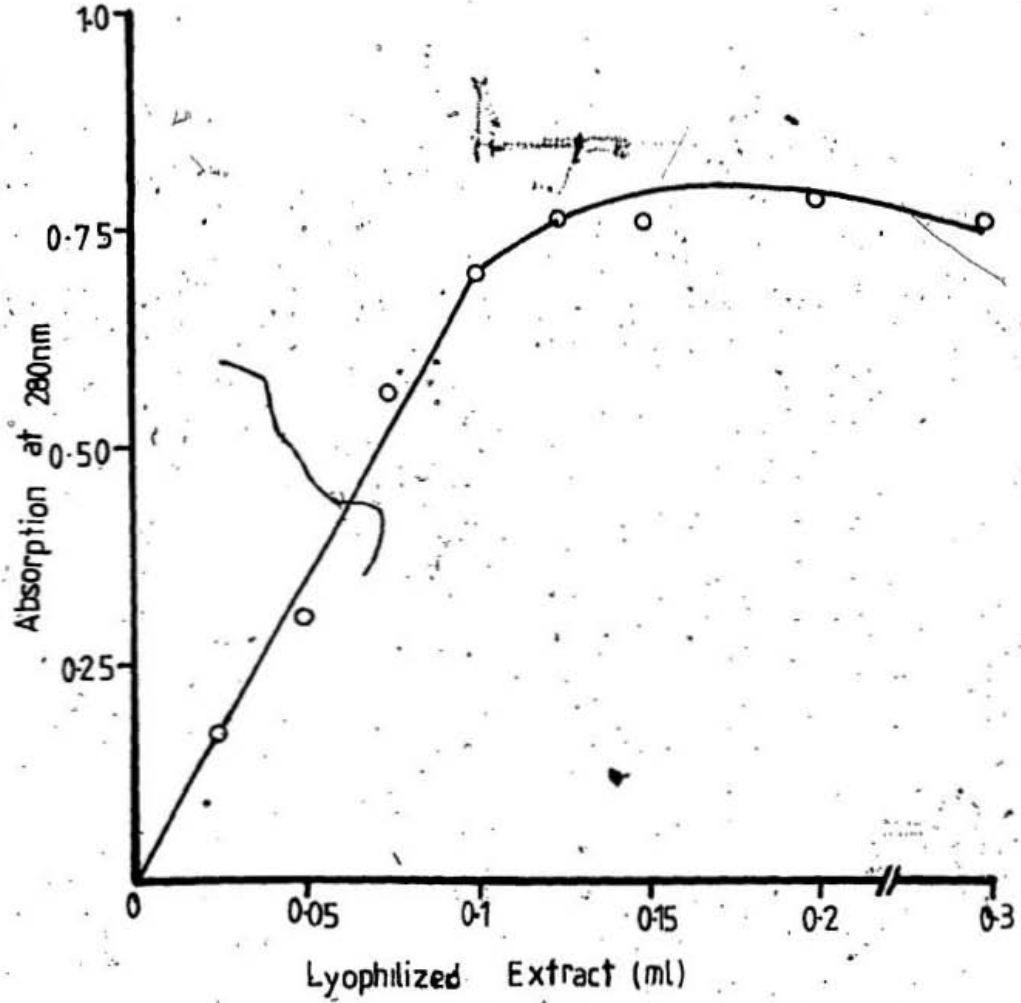
SNO	Steps	Volume	Total Units (EU)	Total Protein (mg)	Specific Activity*	% Recovery	Purification (fold)
1	Crude Extract	900	586.8	4050	0.144	100	1
2	Lyophilization	90	586.0	3420	0.171	100	1.2
3	Ammonium Sulfate Precipitation	21	471.2	294	1.610	80	11.2
4	Sephadex G-200	50	145.4	70	2.07	25	14.4
5	Affinity Column	30	80.4	16.8	4.786	14	33.2

* Specific activity: enzyme units per mg of protein

** The amount of enzyme required to release 1 u mole of tyrosine equivalence per ml per minute at 25°C

Fig. 5 Effect of enzyme concentration on the T20 protease activity

The protease activity was assayed by Hull's method (52) as described in Material and Methods except that the volume of lyophilized extract (redissolved in the Tris-HCl buffer, pH 7.5) varied. It ranged between 25 ul to 300 ul per tube in a total volume of 2 ml. Protein concentration in the extract was 6 mg per ml.


1·0
0·75
0·50
0·25
Absorption at 280nm
0
0·05
0·1
0·15
0·2
0·3
Lyophilized Extract (ml)

increases with increasing concentration of T20 protease in the assay mixture; also that the amount of the protease present in 0.1 ml extract was sufficient to produce a measurable level of protease activity. However, this experiment was performed using a crude enzyme preparation, and protease activity (specific activity) varied from one preparation to the other. Hence, nothing can be said with respect to the mg of T20 protease required for protease assay. In subsequent experiments the volume of extract was adjusted so as to give similar levels of protease activity.

b) Effect of pH on protease activity

Figure 6 demonstrates the effect of pH on the proteases from the isolate number T20, T18, T10 and T6. All the proteases were found to be maximally active in the neutral region. The T20 protease had a pH optimum of 7.2 when soluble casein was used as the substrate. All the proteases had very low activity at pH 5.5 ranging from about 14 to 28 percent of maximum activity in case of T6 and T18 protease respectively. When assayed at a pH of 10.5, the T10 and T6 proteases retained about 40 percent of the maximum activities. In contrast the T20 protease had no activity at or above the pH 10. Similarly the T18 protease had a very low activity (20%) at pH 10.5.

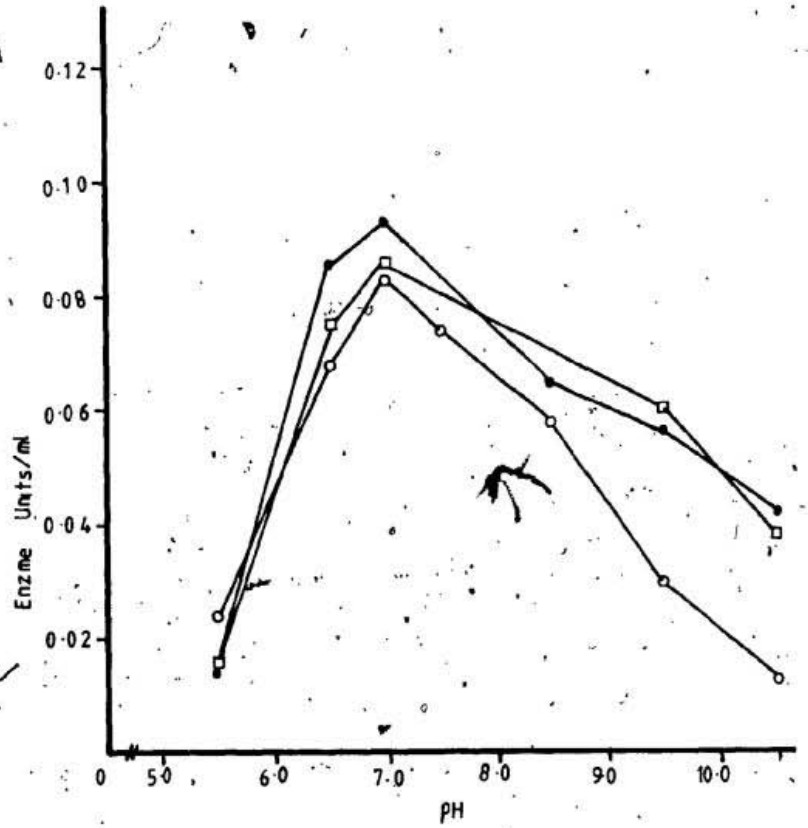
c) Effect of temperature on protease activity

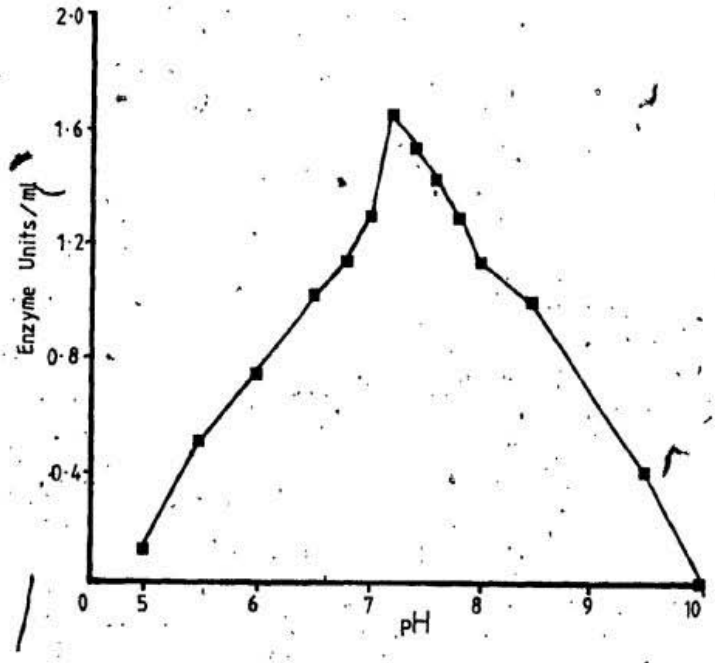
In order to assess the temperature at which the proteases from various isolates were maximally active, the

Fig. 6 Effect of pH on protease activity

The protease activity in the enzyme samples was determined using different buffers. For a pH range of 5.5 and 6.5, a 0.1 M Citrate Phosphate buffer was used. Similarly, for pH range between 7 and 8.5 and 9.5 and 11.5 Tris-HCl (0.1 M) buffer and Glycine-Sodium Hydroxide (0.1 M) buffer were used respectively.

o—o T18 protease. □—□ T10 protease.
■—■ T20 protease. ●—● T6 protease.


0·12
0·10
0·08
0·06
0·04
0·02
0
Enzme Units/ml
5·0
6·0
7·0
8·0
9·0
10·0
pH


2·0
1·6
1·2
0·8
0·4
0
Enzyme Units/ml
5
6
7
8
9
10
pH

proteases were incubated at various temperatures as described in Materials and Methods. As is depicted in Fig. 7, proteases appear to retain their activity in a wide temperature range. The optimum activity was observed at 35°C and a rapid decline of the protease activity was observed above this temperature. As is indicated in the figure at a temperature as low as 5°C, all of the proteases retained some activity and it ranged from about 0.04 to 0.06 enzyme units per ml in case of T20 and T18 respectively.

d) Time course of protease activity

The rate of proteolysis by T20, T18, T10 and T6 was found to be linear for up to more than 20 minutes (Fig. 8). The protease activity of isolate numbers T10 and T20 was linear up to 30 minutes while that of T6 and T18 was linear up to 25 and 40 minutes, respectively (Fig. 8). In subsequent experiments a 20 minute incubation period was selected to maintain linear rates during the protease assay.

e) Effect of substrate concentration on the protease activity

The effect of variation in casein concentration is shown in Fig. 9. The proteolytic activity of T18 and T6 increases with increasing concentrations of casein until it reaches a concentration of 3.5 mg/ml. Similarly, the upper limit of substrate concentration of T20 and T10 proteases was found to be 4.5 mg/ml. With substrate concentration

Fig. 7 Effect of temperature on the protease activity

To the temperature regulated tubes containing substrate and buffer, 0.1 ml enzyme was added. The protease activity was then determined as described in Materials and Methods.

○—○ T20 protease; □—□ T18 protease

×—× T10 protease; ◇—◇ T6 protease

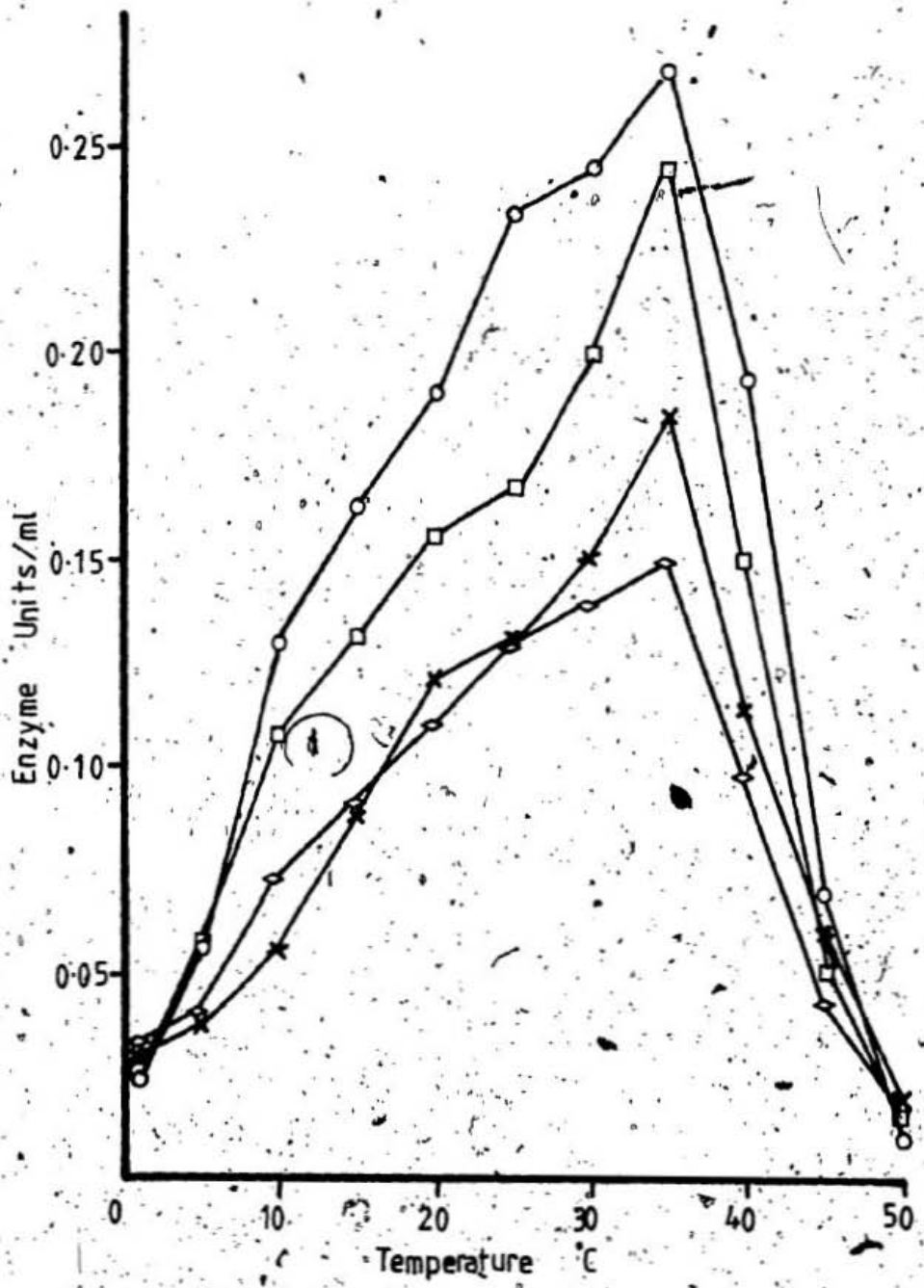
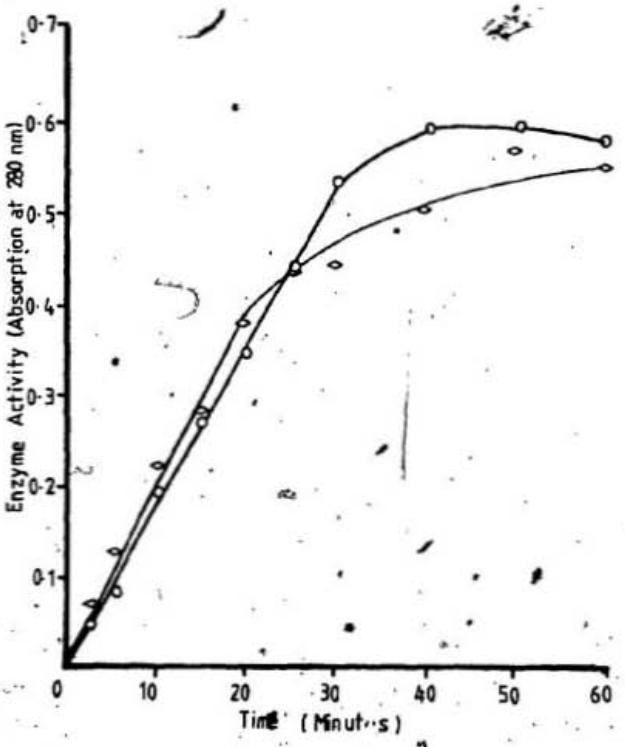

0·25
0·20
0·15
0·10
0·05
Enzyme Units/ml
0
10
20
30
40
50
Temperature °C

Fig. 8 Time course of protease activity

Protease assay was performed in separate tubes as described in Materials and Methods. Reaction was stopped at different time intervals by addition of TCA. The protease activity was then measured by Hull's method (52).

T18 protease; T10 protease

T20 protease; T6 protease


Enzyme Activity (Absorption at 280 nm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
Time (Minutes)

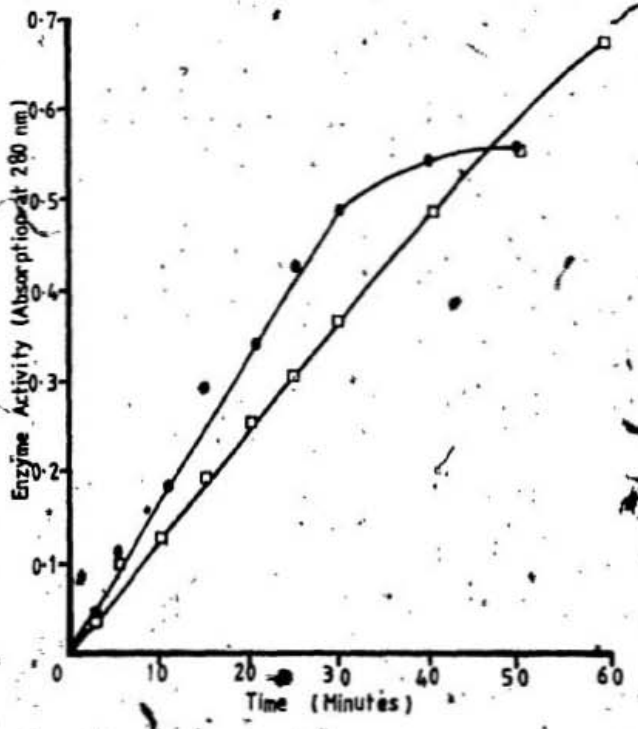
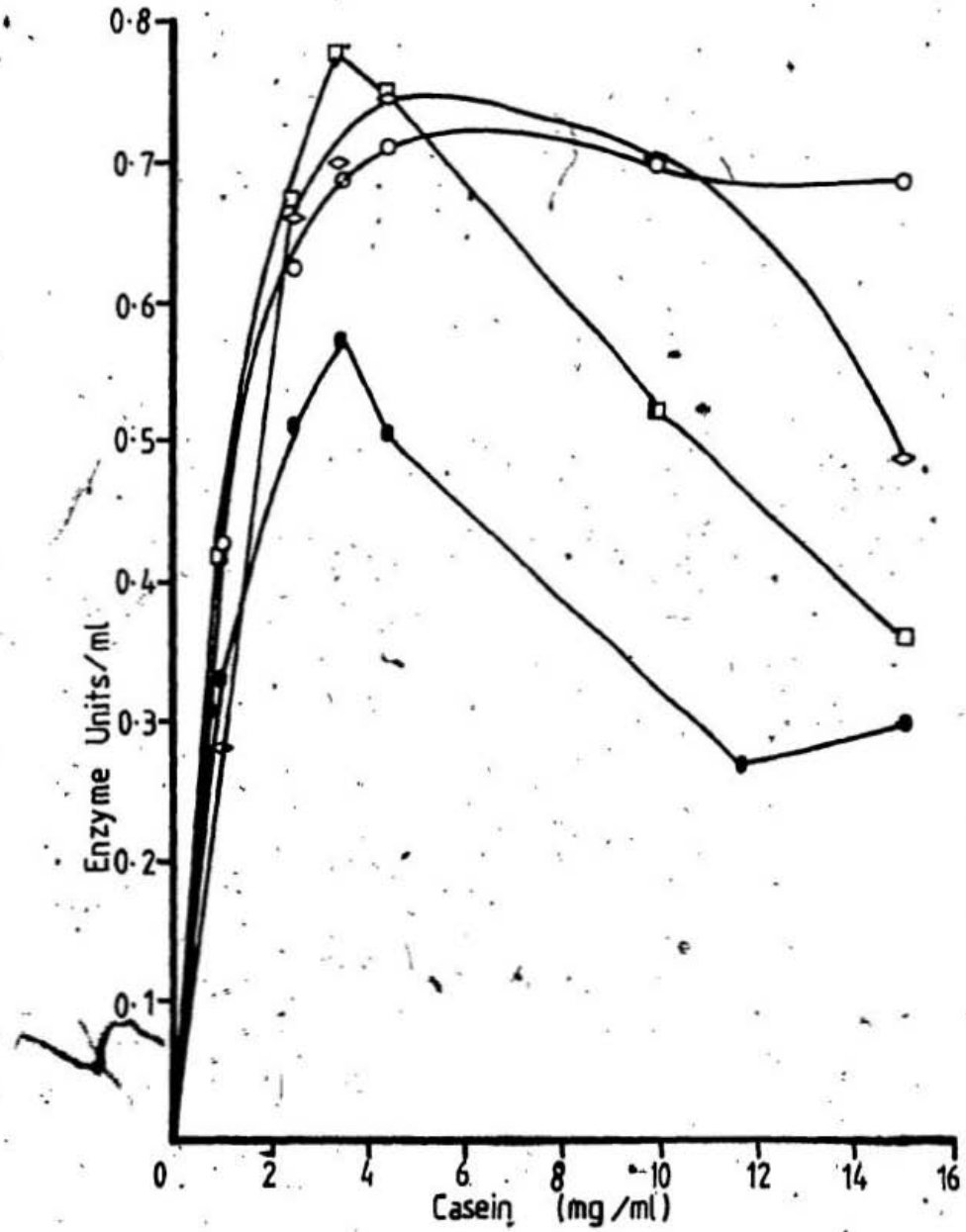

Enzyme Activity (Absorption at 280 nm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
Time (Minutes)

Fig. 9 Effect of soluble casein concentration on the protease activity

Protease activity was determined as described in Materials and Methods in the presence of increasing concentrations of soluble casein, ranging from 1-15 mg/ml.

○—○ T20 protease; □—□ T18 protease

●—● T10 protease; ◇—◇ T6 protease


0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Enzyme Units/ml
0
2
4
6
8
10
12
14
16
Casein (mg/ml)

above the optimum concentration, the proteases from T18, T10 and T6 showed inhibition of activities.

The effects of varying the concentrations of α and β-casein on various protease activities are illustrated in Figs. 10 and 11. When α-casein was used as the substrate, the protease activity of T10 and T6 increased linearly with increasing concentration up to about 3.6 M x 10^{-7}. The T20 protease activity increased linearly up to the concentration of 5.5 M x 10^{-7}. At higher concentrations the phenomenon of inhibition was observed in all cases.

Like α-casein, increases in protease activities of all isolates were observed when increasing concentrations of β-casein were used as the substrate. Similarly, inhibition of protease activity was also observed when β-casein concentration was increased after a certain limit. For example, the T10 protease activity increased linearly with an increasing β-casein concentration up to 2.2 M x 10^{-5} before showing any sign of inhibition.

The apparent Km of α and β-casein for various proteases are presented in Table 2. The value of apparent Km of α-casein is at least 100 fold less than that of β-casein. Hence, the proteases investigated in this study have higher affinity for α-casein as compared to that of β-casein.

f) Substrate specificity of the proteases

The protease activity obtained in the presence of various substrates is presented as a percentage of the

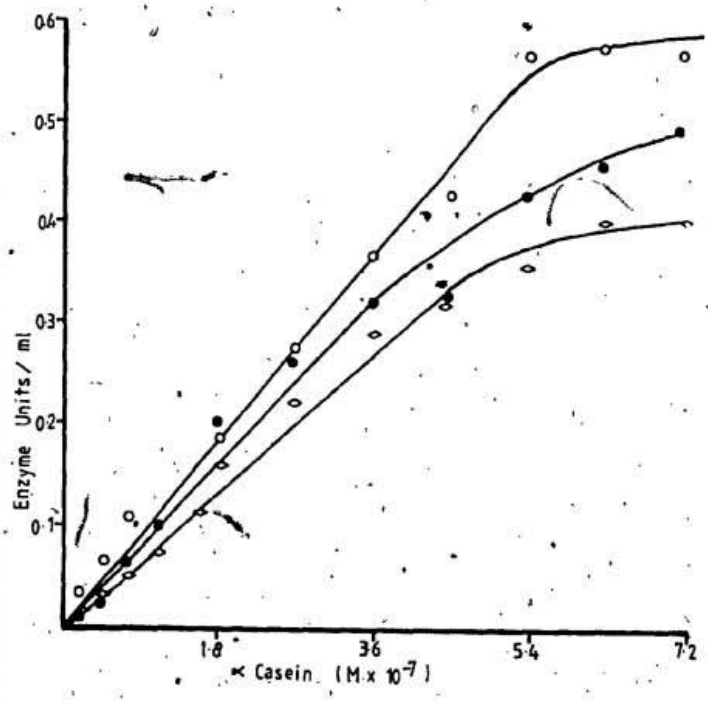
Fig. 10 Effect of α-casein concentration on protease activity

Various volumes of α-casein were added to the buffer to give final concentrations ranging from 0.1 to 7 $Mx10^{-7}$. The proteolytic activity was measured as described in Materials and Methods. The kinetic constants were determined by Lineweaver Burke Plot (60).

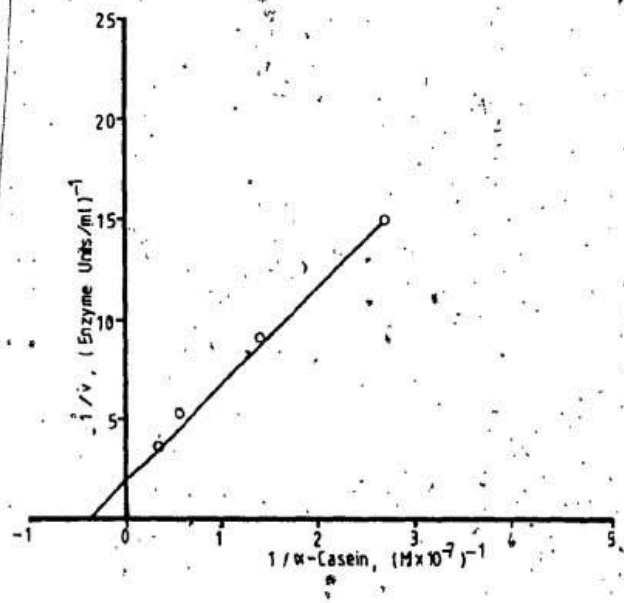
I, o—o T20

II, ●—● T10

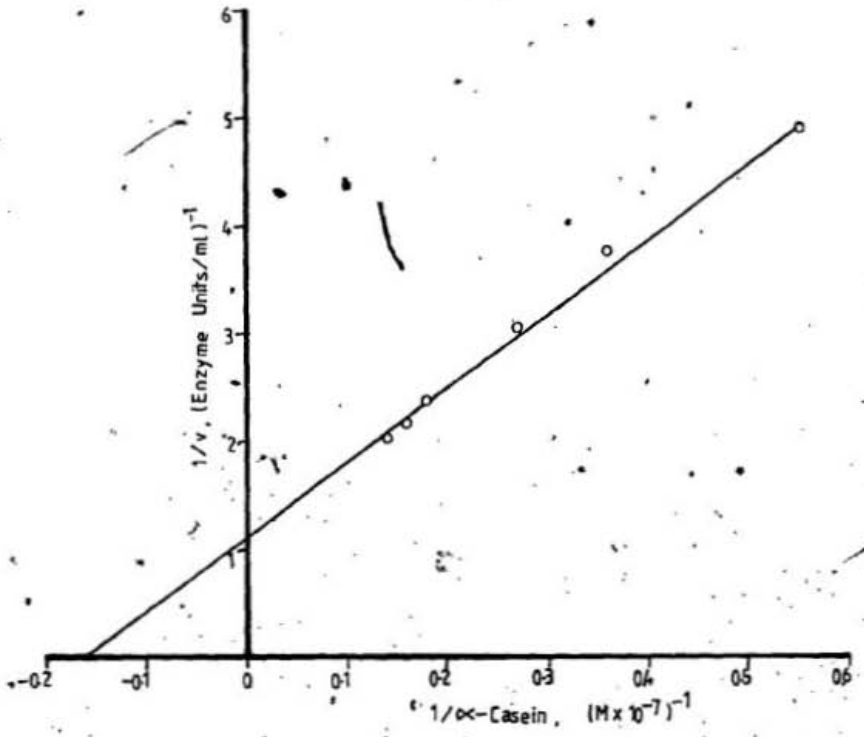
III, ◇—◇ T6


0·6
0·5
0·4
0·3
0·2
0·1
Enzyme Units/ ml
1·8
3·6
5·4
7·2
∝ Casein (M x 10⁻⁷)

(I)


25
20
15
10
5
1/v, (Enzyme Units/ml)⁻¹
-1
0
1
2
3
4
5
1/∝-Casein, (M x 10⁻⁷)⁻¹

(II)


6
5
4
3
2
1
1/v, (Enzyme Units/ml)$^{-1}$
-0·2
-0·1
0
0·1
0·2
0·3
0·4
0·5
0·6
1/∝-Casein, (M x 10^{-7})$^{-1}$

(III)

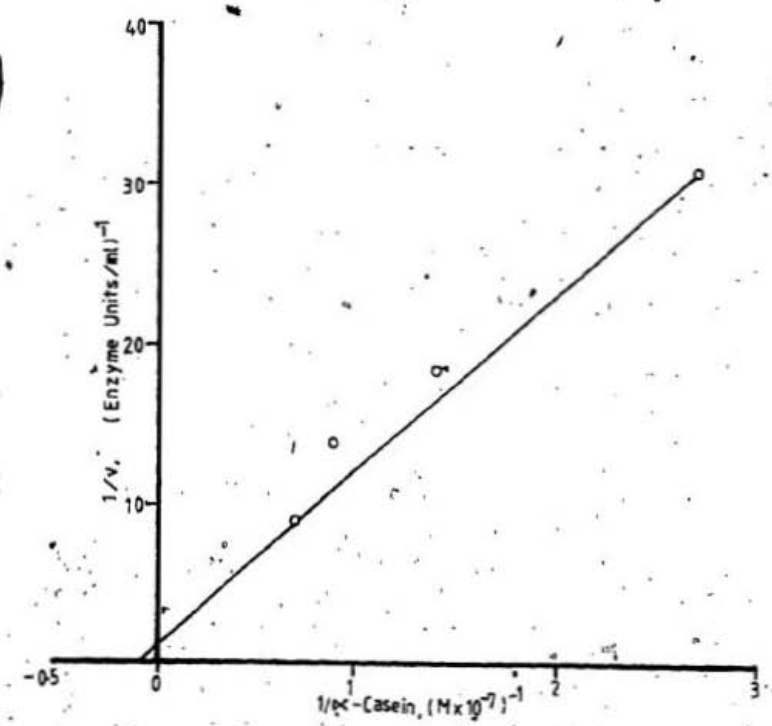

40
30
20
10
1/v, (Enzyme Units/ml)$^{-1}$
-0·5
0
1
2
3
1/∝-Casein, (M x 10^{-7})$^{-1}$

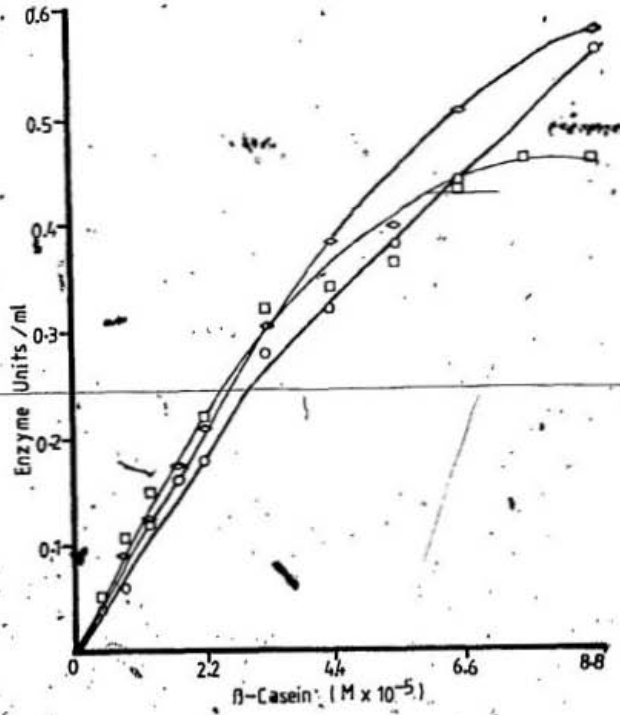
Fig. 11 Effect of β-casein concentration of T20 protease activity.

Various volumes of β-casein solution were added to the buffer to give final concentrations ranging from 0.1 to 8.8 $Mx10^{-5}$. The proteolytic activity was measured by Hull's method (52) as described in Materials and Methods. The kinetic constants were determined by Lineweaver Burke Plot (60).

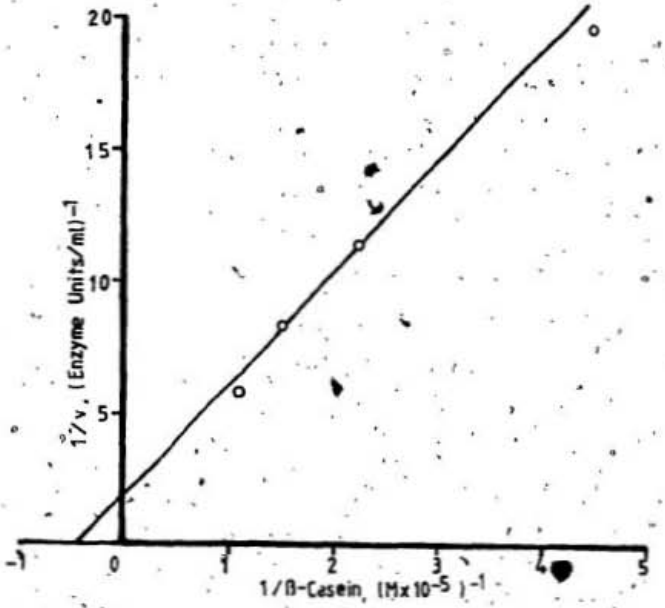
I, ◇—◇ T20

II, □—□ T10

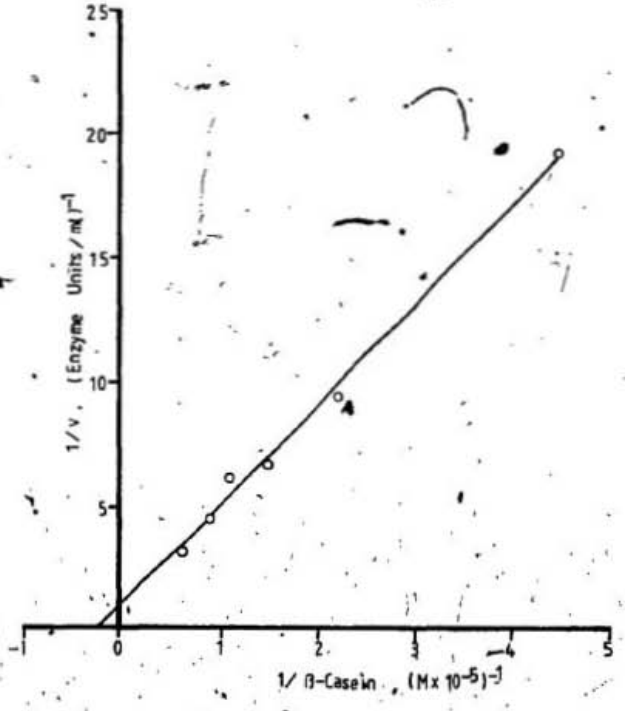
III, ○—○ T6


0.6
0.5
0.4
0.3
0.2
0.1
Enzyme Units/ml
0
2.2
4.4
6.6
8.8
β-Casein (M x 10⁻⁵)

(I)


20
15
10
5
1/v, (Enzyme Units/ml)⁻¹
-1
0
1
2
3
4
5
1/β-Casein, (M x 10⁻⁵)⁻¹

(II)


25
20
15
10
5
1/v , (Enzyme Units / ml)⁻¹
-1
0
1
2
3
4
5
1/ β-Casein , (M x 10⁻⁵)⁻¹

(III)

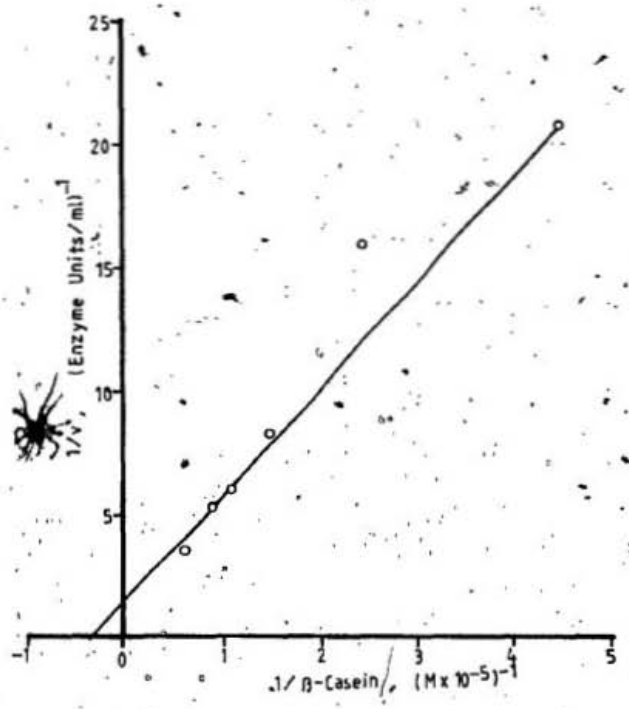

25
20
15
10
5
1/v , (Enzyme Units/ml)⁻¹
-1
0
1
2
3
4
5
1/ β-Casein , (M x 10⁻⁵)⁻¹

Table 2

Apparent Km of various proteases for α and β-casein

Substrate	Km*			
	Source of protease			
	T20	T18	T10	T6
α-Casein	2.6	5.8	6.2	9.5
β-Casein	2.0	3.4	4.2	3.3

* α-Casein M x 10^{-7}

β-Casein M x 10^{-5}

activity obtained with soluble casein (Table 3). Most of the proteases had maximum activity on soluble casein which is a mixture of various caseins. Although the T20 protease hydrolyzed α, β and γ-caseins quite efficiently, it had very little activity on k - casein (only 14%). The protease T18 had more proteolytic activity on α and k casein, 89 and 90 percent, respectively. However, in the presence of β and γ-casein it exhibited only 59 and 54 percent activity, respectively. The T10 protease also hydrolyzed α, β and γ-caseins efficiently and its activity in the presence of α-casein was slightly greater than that obtained with soluble casein. The T6 protease hydrolyzed β and k-caseins with almost equal efficiency, 93 and 98 percent, respectively. However, in the presence of γ-casein it showed a reduced activity of 57 percent. Out of the four proteases tested, T10 and T6 were found to possess very low activity on α-lactalbumin, a whey protein, while T18 protease did not hydrolyze α-lactalbumin. In contrast, the T20 protease hydrolyzed it to some extent with about 22 percent of the activity. Ovalbumin was not hydrolyzed by any of the proteases except by T6 protease which showed only 6 percent activity on this protein. Proteases T6, T10 and T18 showed 32, 50 and 12 percent activity, respectively, in the presence of bovine serum albumin. In contrast T20 exhibited no activity in the presence of bovine serum albumin. When hemoglobin was used as a substrate, T20, T10 and T6

Table 3

Substrate specificity of the proteases

Substrates	% Relative Activity*			
	T20	T18	T10	T6
Soluble Casein	100	100	100	100
α-Casein	57	89	104	ND
β-Casein	57	59	84	93
γ-Casein	50	54	59	57
κ-Casein	14	90	ND**	98
α-lactalbumin	22	0	2	3
Ovalbumin	0	0	0	7
Bovine Serum Albumin	0	12	50	32
Hemoglobin	36	0	21	18

The protease assays were performed as described in materials and methods. The concentration of the substrates in the reaction mixture was 5 mg.

* % Relative activity is calculated with respect to the activity obtained in the presence of soluble casein.

** Not determined

proteases were found to attack it with 36, 10 and 18 percent activity, respectively. Surprisingly T18 failed to attack hemoglobin.

g) Protease activity in the presence of synthetic substrates

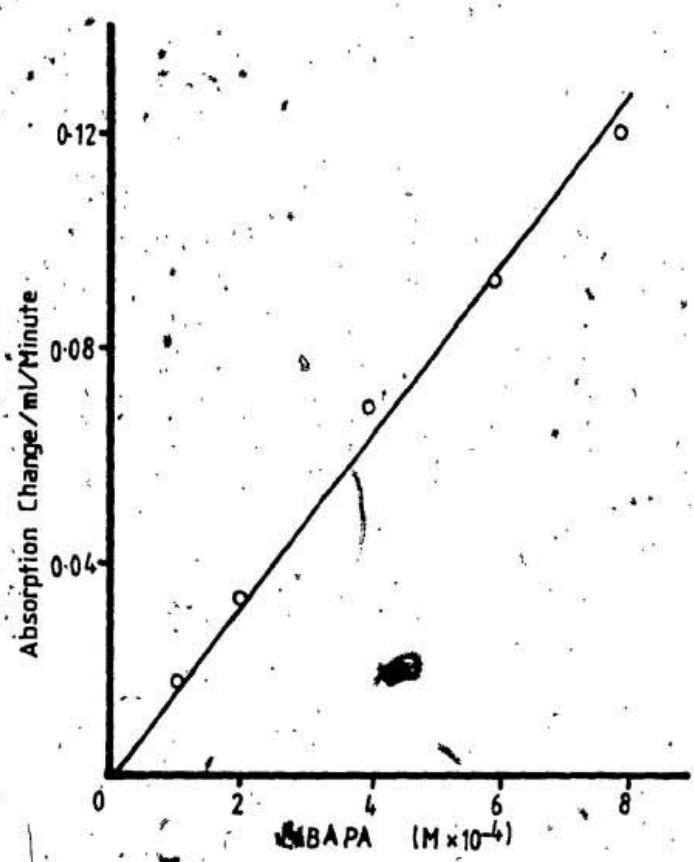
Various synthetic substrates commercially available were tested using T20 protease. It hydrolyzed N-benzoyl-L-arginine-p-nitroanilide but had no activity on N-benzoyl-L-tyrosine-p-nitroanilide and Glutaryl-L-phenylalanine-p-nitroanilide. Similarly, the T20 protease did not hydrolyze the trypsin substrate, paratosyl-L-arginine methyl ester. A synthetic peptide hydrolyzed by T20 protease was glycyl-phenylalanine-β-naphthylamide. In order to determine the affinity of the enzyme for synthetic substrates, the effect of varying the concentration of the substrates on the enzyme activity was determined and from the Lineweaver Burke plots (Figs. 12 and 13) Km's were calculated (Table 4).

h) The stability of the T20 protease at different pH

In order to determine the effect of pH on the stability of T20 protease the lyophilized extract dissolved in the Tris-HCl buffer having high protease activity (2.810 EU/ml) was incubated at various pH for 102 hours at 10°C. This protease was found to be stable over a wide range of pH (Table 5); the loss of protease activity ranged from only 2 percent at pH 5.5 to about 16 percent of original activity

Fig. 12 Protease activity on L-BAPA(T20)

Proteolytic activity on L-BAPA was measured according to the method of Erlanger et al. (55). The reaction mixture contained various amounts of substrate ranging in concentration from 1-8 $Mx10^{-4}$. The kinetic constants were determined by Lineweaver Burke Plot (60).


0·12
0·08
0·04
Absorption Change/ml/Minute
0
2
4
6
8
L-BAPA (M×10⁻⁴)

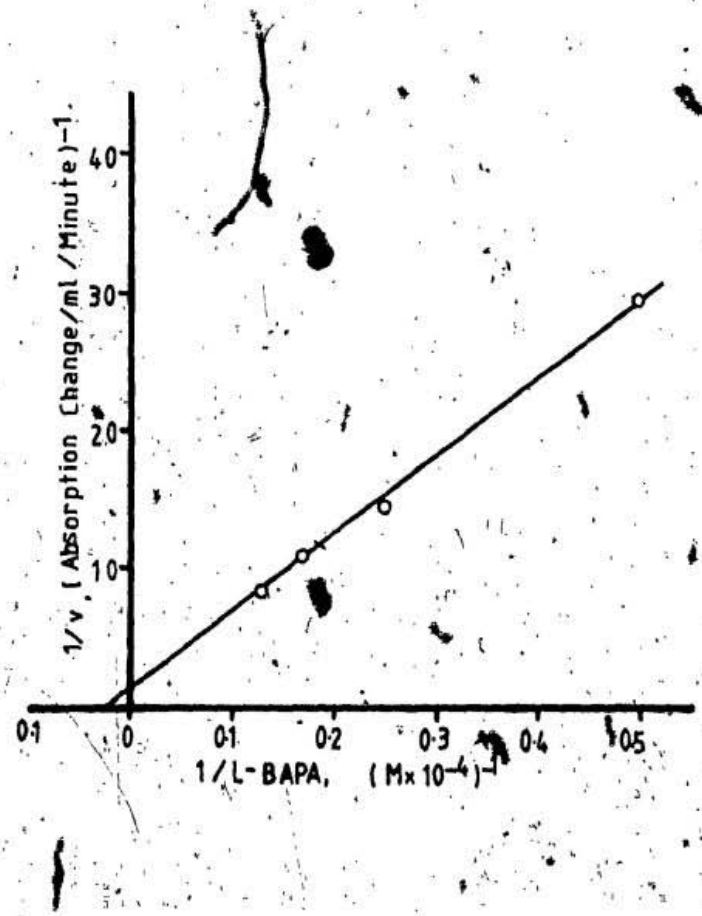
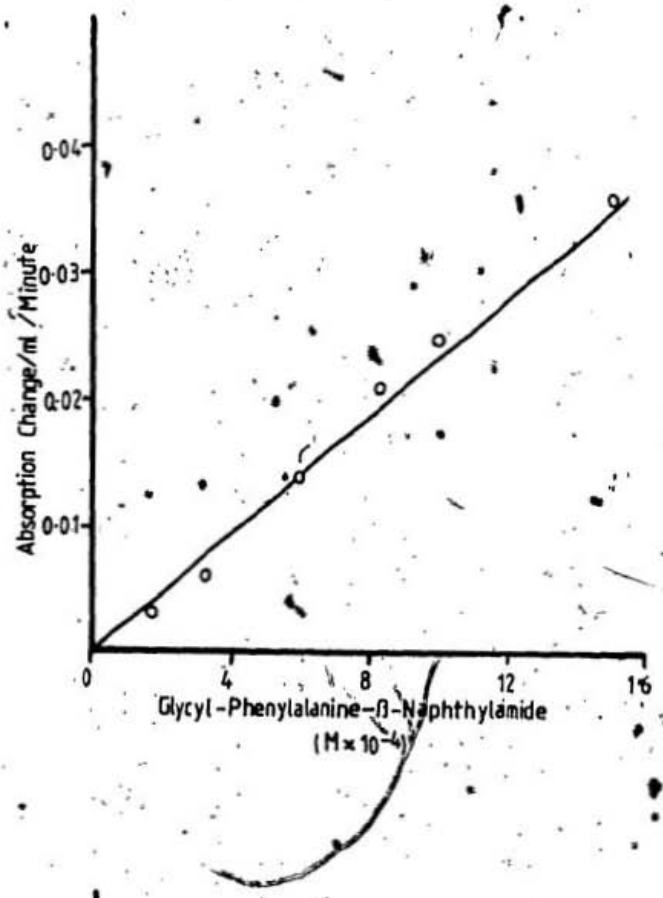

40
30
20
10
1/v, (Absorption Change/ml/Minute)⁻¹.
0·1
0
0·1
0·2
0·3
0·4
0·5
1/L-BAPA, (M×10⁻⁴)⁻¹

Fig. 13 Protease activity on Glycyl-Phenylalanine-β-Naphthylamide(TZO)

The proteolytic activity was assayed by measuring the absorption change at 340 nm per ml per minute. The Km was then determined by Lineweaver Burke Plot (60).


Absorption Change/ml /Minute
0·04
0·03
0·02
0·01
0
4
8
12
16
Glycyl-Phenylalanine-β-Naphthylamide
(M x 10⁻⁴)

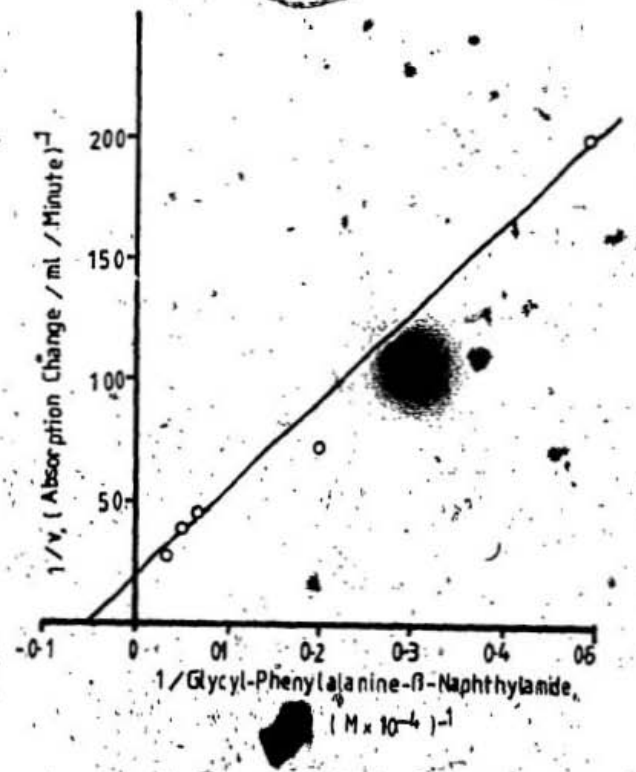

1/v, (Absorption Change / ml / Minute)⁻¹
200
150
100
50
-0·1
0
0·1
0·2
0·3
0·4
0·5
1/Glycyl-Phenylalanine-β-Naphthylamide,
(M x 10⁻⁴)⁻¹

Table 4

Km of T20 protease on synthetic substrates

Substrate	Km $M \times 10^{-3}$
L-BAPA	3.5
Glycyl-Phenyl alanin-β-Naphthylamide	16.9

Table 5

The stability of T20 protease at different pH

pH	% Residual * Activity
2.5	87
3.0	84
3.5	92
4.0	96
4.5	94
5.0	100
5.5	98
6.0	95
6.5	91
7.0	96
7.5	100
8.0	94
8.5	88
9.0	84
9.5	81
10.0	72
10.5	58
11.0	60
11.5	24

A highly concentrated enzyme (1.91EU/ml) preparation was added to various buffers ranging in pH from 2.5-11.5. The following buffers in 0.1 M concentration were used: Citrate-Phosphate buffer, Tris-HCl buffer and Glycine-Sodium Hydroxide buffer. These buffer-enzyme solutions were stored under refrigeration (10^{0}C) and proteolytic activity was measured as described in materials and methods.

* Calculated with respect to the original activity present in the extract.

at pH 9. However, the loss was much more at pH 11.5 and only 24 percent of original activity was retained after storing the protease at this pH under similar conditions.

i) Heat stability of the proteases

Table 6 demonstrates the effect of 10 minute heat exposure on the protease activity. The T18 protease retained 28 percent of its activity after 10 minutes exposure at 100°C while T20, T10 and T6 proteases retained about 20 percent of their original activity. However, the loss of protease activity was much more at lower temperatures; for example, only 3 percent of the activity of T20, T18 and T10 were retained after heat exposure of 10 minutes at 50°C and only 11 percent in the case of T6 protease.

Figure 14 depicts the heat stability of protease T20 at 100°C. The inactivation of this protease takes place in 2 stages. There is a rapid loss of protease activity in which more than 50% of the activity is lost in the first 10 minutes. In the second phase, however, the inactivation rate is very slow and appears to follow a first order kinetics.

Figure 15 shows the effect of heat exposure on T20 protease in the presence of varying concentrations of $CaCl_2$. The protease activity decreased with increasing concentration of calcium chloride up to 10 umoles. With further increases in concentration, no increase in inactivation was observed.

Table 6

Protease activity after exposure for 10 minutes at different temperatures

Temperature °C	% Activity			
	Source of Protease			
	T20	T18	T10	T6
Control	100	100	100	100
100	19	28	20	20
90	17	33	24	27
80	13	27	27	22
70	12	15	23	16
60	7	9	14	14
50	3	3	3	11

Aliquots of enzyme solution (2 ml) from various isolates dialyzed in 0.02 M Tris-HCl (pH 7.5) were heated in water baths maintained at the required temperatures. After exposure to heat for 10 minutes, the residual activity was measured as described in materials and methods. The residual activity is presented as a percentage of protease activity originally present in unheated samples (control).

Fig. 14 Time course of heat treatment at 100°C

The T20 protease solution in the buffer was heated for various time intervals. The protease activity was measured as described in Materials and Methods. The residual activity after heat exposure is presented as the percentage of activity originally present in the unheated sample (0 time).

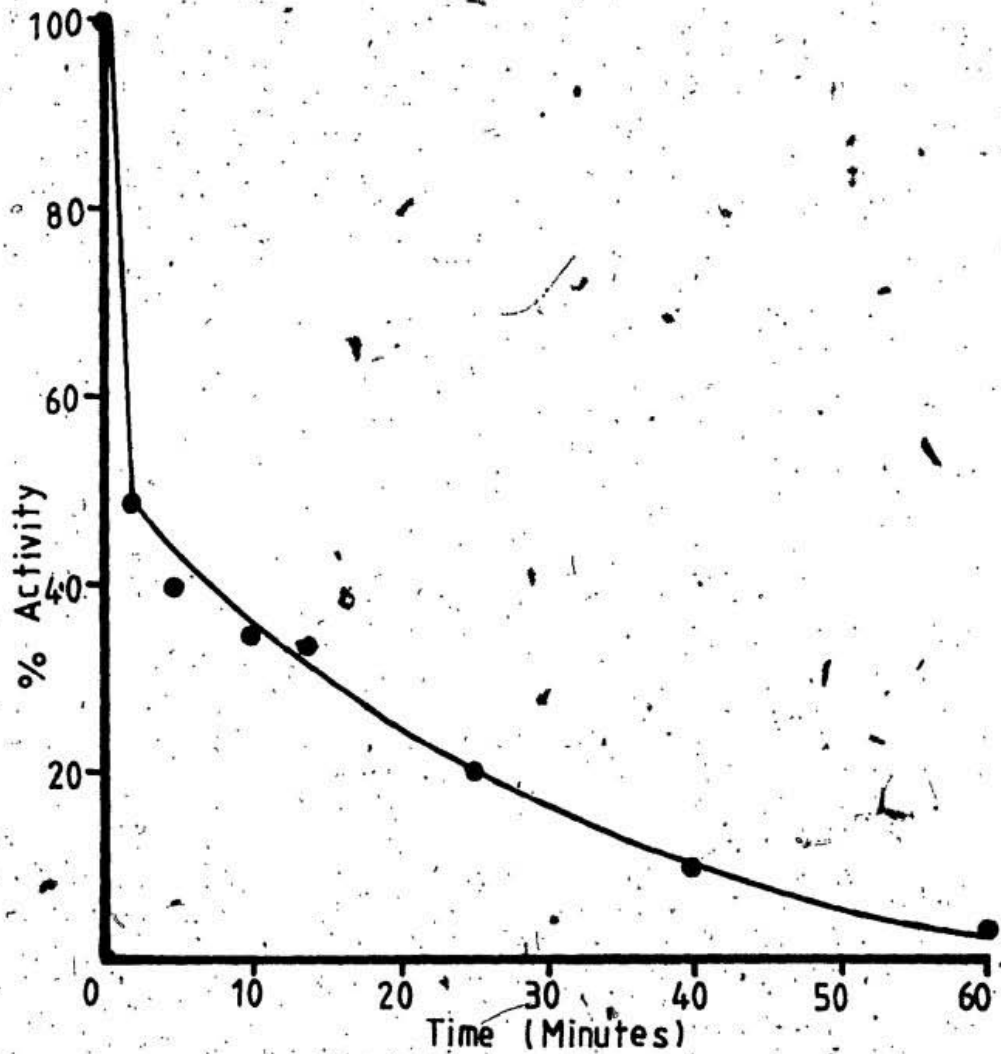
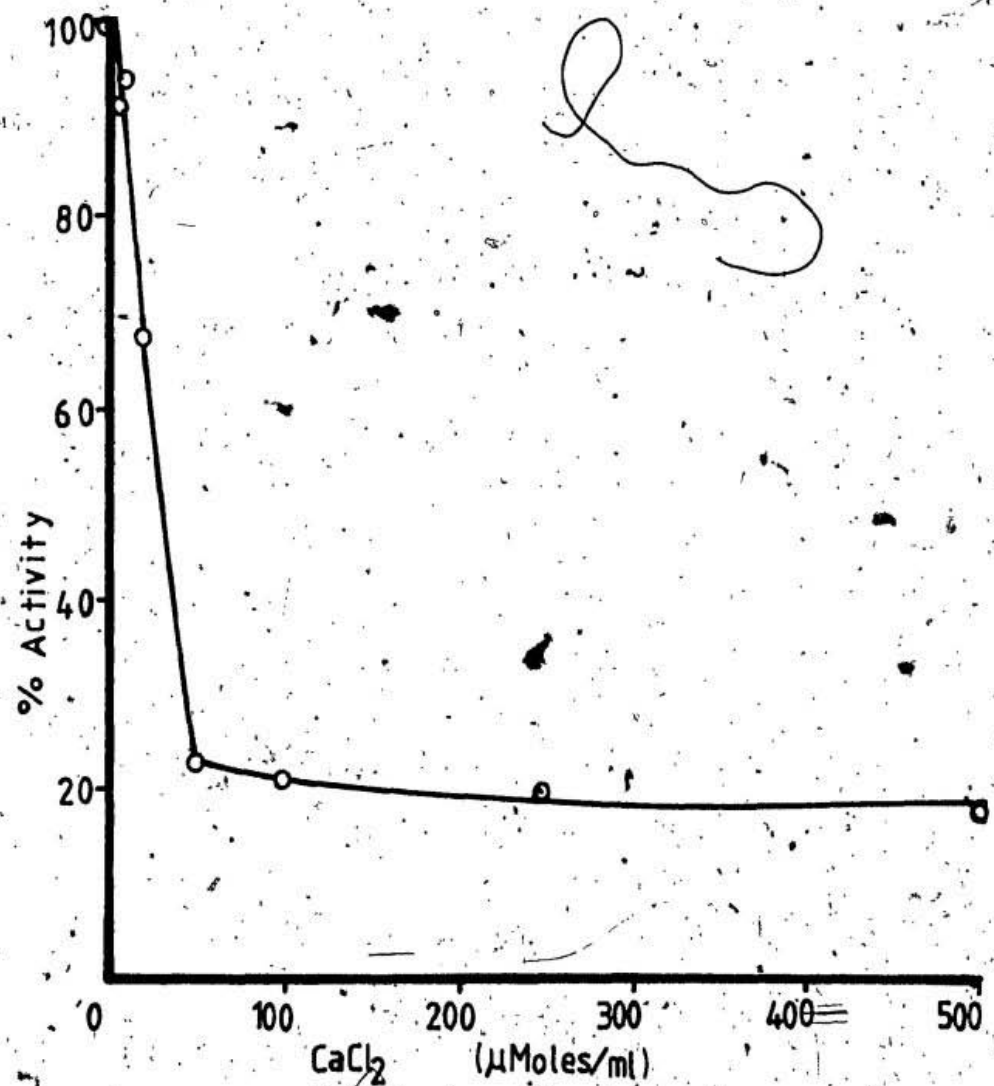

100
80
60
40
20
% Activity
0
10
20
30
40
50
60
Time (Minutes)

Fig. 15 . Heat exposure of T20 protease in presence of calcium chloride

The T20 protease (1 ml) was heated in the presence of various concentrations of $CaCl_2$ ranging from 5 - 1000 umoles. After the heating at 100°C for 10 minutes, the protease activity was measured after dialysis as described in Materials and Methods. The residual activity is presented as percentage of protease activity obtained in absence of calcium chloride.


100
80
60
40
20
% Activity
0
100
200
300
400
500
CaCl2 (µMoles/ml)

5. Enzyme modulation studies

Table 7 demonstrates the effect of various enzyme modulators on protease activity. The sulfhydryl reagents, such as N-ethylmaleiamide and p-chloromercuribenzoate had an inhibitory effect on proteases from various isolates. The inactivation by N-ethylmaleiamide at a concentration of 2.5 mM was as much as 51 percent in case of T20 protease and 33 percent in case of T6. However, at a concentration of 1.25 mM only 27 percent of T20 protease activity was inhibited. Similarly, an increase in inhibition was also observed when para-chloromercuribenzoate concentration was increased from 1.25 to 2.5 mM. Cysteine seemed to stimulate all of the three proteases tested, however, the stimulation was very minimal. For example, the highest stimulation obtained by cysteine was only 11 percent in case of T20 protease, and the higher concentration did not change it. EDTA, a chelating agent for divalent cations, had a marked effect on protease activity; and at a concentration of 2.5 mM, it inhibited about 76% of T20 protease activity. Similarly 8-hydroxy-quinoline, a metal chelating agent, also inhibited various proteases quite efficiently (Table 7). The trypsin inhibitors from chick embryo, lima and soya beans and tosyl-L-lysine-chloromethyl ketone did not have an effect on any of the protease activities. Similarly pepstatin and antipain also did not inhibit any of the proteolytic activity.

Table 7

Effect of [illegible]e modulators on the protease activity

Modulators	% Relative Activity							
	T20		T18		T10		T6	
	1.25 mM	2.5 mM	1.25 mM	2.5 mM	1.25 mM.	2.5 mM	1.25 mM	2.5 mM
Control	100	100	100	100	100	100	100	100
N-Ethylmaleiamide	73	49	77	52	82	55	87	67
P-Chloromercuribenzoate	98	97	85	50	92	84	83	49
Cysteine	111	109	ND*	ND	104	108	111	104
EDTA	84	24	83	72	59	60	67	61
8-Hydroxyguinoline	85	68	78	43	85	70	86	47

Various modulators at two different concentrations were allowed to react with enzymes (roughly 0.5 EU/ml) from several isolates in the Tris-HCl buffer for 10 minutes. The protease activity is presented as the percentage of activity observed in untreated control.

* Not determined

6. Effect of metal ions

The effect of various metal ions on protease activities from different isolates is shown in Table 8. Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited all of the proteases considerably, and the inhibition was more than 70% in case of T18, T10 and T6 protease at a concentration of 2 mM. Only T20 protease retained slightly higher activity in most of the cases. Similarly, Ni^{2+} had a slightly lower inhibitory effect on most of the protease activities. However, it showed an increased inhibition with an increase in concentration of the metal ion from 1 to 2 mM. Ca^{2+}, Co^{2+} and Mg^{2+} showed variable results; and effect of these metal salts varied from isolate to isolate, and from one concentration to another.

Table 9 shows the effect of various metals in the restoration of protease activity after complete inactivation by dialysis against the Tris-HCl buffer containing 0.1 M EDTA. The metal ions Li^{+}, Hg^{2+}, Ni^{2+}, Sn^{2+}, Cd^{2+}, K^{+} and Na^{+} did not restore the activity of any of the proteases at a concentration of 2mM. Similarly Zn^{2+} and Cu^{2+} did not restore the protease activity to any considerable extent, except in one case when Cu^{2+} restored about 22% of the original activity of T20 protease. Metal ions which appear to show stimulation of the protease activity included Ca^{2+}, Mn^{2+}, Mg^{2+} and Sr^{2+}. However, the reactivation of the protease activity in the presence of these metal ions was less than that originally present in the extract. Only in

Table 8

Effect of metal ions on protease activity

Metals	% Relative Activity							
	T20		T18		T10		T6	
	1mM	2mM	1mM	2mM	1mM	2mM	1mM	2mM
None	100	100	100	100	100	100	100	100
Calcium Chloride	88	88	93	96	92	98	102	110
Cobaltous Chloride	84	85	72	68	78	77	78	72
Cupric Sulfate	39	39	20	0	27	23	24	19
Manganese Chloride	102	94	87	100	93	104	87	102
Magnesium Sulfate	96	94	ND*	94	82	91	106	92
Zinc Sulfate	57	24	23	4	23	26	25	14
Mercuric Chloride	57	54	30	4	46	26	60	32
Nickel Sulfate	79	68	65	45	71	52	67	64

The enzyme and metal ions were allowed to react with each other in cold (at 10^{0}C) for 15 minutes, and then assayed for proteolytic activity as described in Materials and Methods. The protease activity is represented as the percentage of the activity observed with untreated control. The concentration of enzyme was same for any protease and roughly 0.5 to 0.8 EU/ml of all proteases was used for assay.

* Not determined

Table 9

Restoration of protease activity by metal ions

Salt	% Relative Activity *			
	T20	T18	T10	T6
None	0	0	0	0
Untreated** Control	100	100	100	100
Calcium Chloride	87	97	47	55
Cobaltous Chloride	58	63	25	25
Cupric Sulfate	22	7	3	0
Manganese Chloride	103	99	38	57
Magnesium Sulfate	61	61	15	36
Zinc Sulfate	0	5	3	0
Mercuric Chloride	4	0	0	0
Nickel Sulfate	0	0	0	0
Lithium Chloride	0	0	0	0
Strontium Chloride	61	35	40	51
Stannous Chloride	0	0	0	0
Aluminium Sulfate	64	78	80	20

Table 9
(Continued)

	% Relative Activity*			
	T20	T18	T10	T6
Ammonium Molybdate	19	96	10	8
Barium Chloride	34	9	8	40
Cadmium Chloride	6	0	0	0
Lead Nitrate	34	40	10	40
Potassium Chloride	0	0	0	0
Sodium Phosfate (Mono basic)	0	0	0	0

Enzyme extracts from various isolates were extensively dialyzed in the Tris-HCl buffer containing 0.1 M EDTA. This resulted in complete inactivation of the protease in these samples. The inactivated samples were redialyzed against Tris-HCl buffer to remove EDTA. The redialyzed samples were then tested for the protease activity, in the presence of various metal ions shown above.

* % Relative activity is calculated with respect to original activity present in the extract before EDTA treatment.

** Protease activity originally present in the extract prior to dialysis against buffer containing EDTA.

the case of T18 and T20 did Mn^{2+} reactivate the protease to almost 100% activity. Similarly, Ca^{2+} in the case of T18 reactivated the enzyme to its original level.

7. Enzyme synthesis

a) Growth curve protease production

Figure 16 demonstrates the protease production in various growth phases of T20. The protease production begins early in the logarithmic phase and continues into the stationary phase. After reaching a peak in the stationary phase there is a gradual decline in the protease activity.

b) Induction of proteases

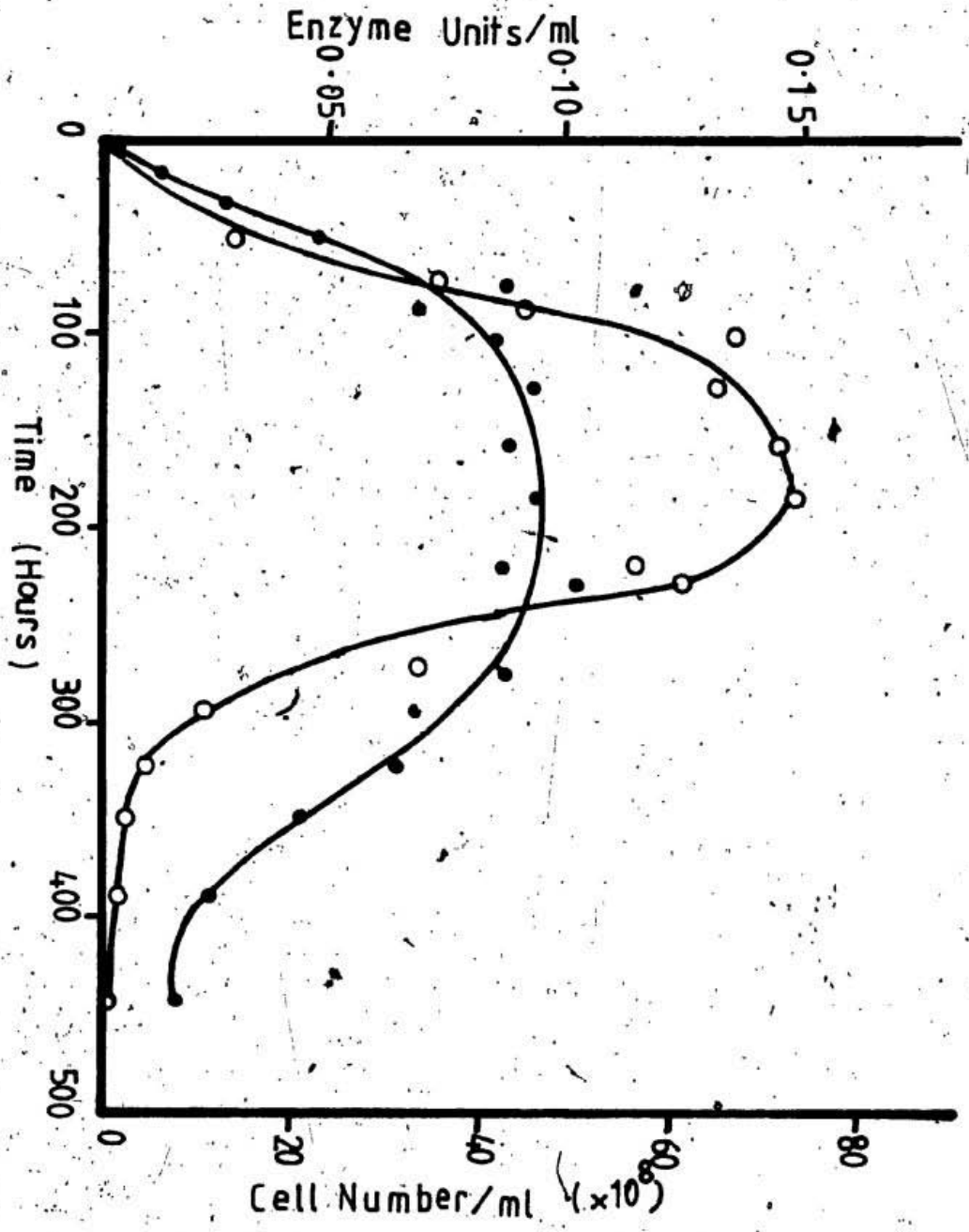
The proteolytic activity of each isolate was found to be elicited when grown in the presence of various carbon compounds. Table 10 (a) shows the growth and protease production by various isolates in the presence of various carbon compounds at a final concentration of 10 mM. All of the bacterial cultures grow in the presence of casein and produce protease activity except in one experiment when T20 did not produce protease in presence of casein plus citrate or succinate (Table 10, b). Comparatively higher levels of protease activity were detected when T20 was grown in presence of one percent milk powder. The amount of protease produced was also found to vary depending upon the carbon compound added into the growth medium in addition to the

Fig. 16 Growth curve and protease production

A thick suspension of bacteria was inoculated into TSB with 2% milk powder (w/v). At different time intervals samples (4 ml) were withdrawn and assayed for proteolytic activity. Viable counts were also performed on these samples at appropriate dilutions and plating.

●—● Cell number

○—○ Protease activity


Enzyme Units/ml
0·05
0·10
0·15
0
100
200
300
400
500
Time (Hours)
0
20
40
60
80
Cell Number/ml (×10⁶)

Footnote to Table 10:

Washed cell suspensions (1 ml) were innoculated into mineral salt media containing different carbon sources (10 mM except in 'C', where the final concentration was 100 mM) with or without 0.5% (w/v) soluble casein or milk powder (1%, w/v). The flasks were then incubated at 25°C for 5 days on a shaker. The growth was measured by reading absorption at 600 nm and protease activity was assayed by Hull's method (52).

Table 10 (a)

Protease production in the presence of various carbon sources

Carbon Source	T20		T18		T10		T6	
	P[a]	G[b]	P	G	P	G	P	G
Casein	18	+	68	+	9	+	13	+
Casein + Pyruvate	12	+	52	+	10	+	19	+
Casein + Citrate	0	+	28	+	8	+	8	+
Casein + Succinate	0	+	29	+	18	+	28	+
Casein + Glutamine	8	+	24	+	14	+	28	+
Casein + Glucose	6	+	66	+	18	+	26	+
Casein + Lactose	0	+	54	+	8	+	29	+
Pyruvate	6	+	0	+	0	-	4	+
Citrate	0	+	0	+	0	+	0	+
Succinate	0	+	0	+	0	+	0	+
Glutamine	0	+	0	+	0	+	0	+
Glucose	0	+	0	+	0	+	0	+
Lactose	0	-	0	-	0	-	0	-
None	0	-	0	-	0	-	0	-

[a] P, protease activity, enzyme units/ml x 10^{-3}

b, Growth

Table 10 (b)*

Protease production by T20 in the presence of various carbon sources

Carbon Source	Growth	Protease Activity EU/ml x 10^{-3}
Casein	+	10
Casein + Pyruvate	+	12
Casein + Citrate	+	0
Casein + Succinate	+	0
Casein + Glutamine	+	11
Casein + Glucose	+	2
Casein + Lactose	+	0
MP*	+	20
MP + Pyruvate	+	30
MP + Citrate	+	2
MP + Succinate	+	62
MP + Glutamine	+	62
MP + Glucose	+	42
MP + Lactose	+	18
None	-	0

* Skim milk powder (1% w/v).

Table 10 (c)

Carbon Source*	Growth	Protease Activity
Lactose	-	-
Phenylalanine	+	-
Glutamine	+	-
Glycine	+	-
Tyrosine	+	-
Glucose	+	-
Pyruvate	+	-
TSB	+	+
TSB + Milk	+	++

* Concentration 100 mM

** The final concentration of milk was 1% (w/v) and TSB was prepared according to the instruction of manufacturer.

milk powder. For example in presence of lactose or citrate with milk powder, lower amounts of protease were produced, while glutamine with milk powder seemed to elicit higher levels of protease activity under similar conditions. Table 10c shows the absence of proteolytic activity in the growth medium of T20, when grown in the presence of various carbon compounds at a final concentration of 100 mM. In this experiment no protease activity was detected in the presence of any carbon source alone except in TSB with or without milk powder (1% w/v) some protease activity was detected. Since no protease activity was detected in the cell extract from various bacterial cultures after cell disruption (table 11), the protease activity in the medium was probably a result of synthesis 'de novo' of the enzyme protein as discussed later.

8. Immunological relatedness between proteases

Various immunological reactions demonstrated that proteases from T20, T18, T10 T6, T25 and T16 are immunologically related to each other: Fig. 17 shows the inhibition of T20 protease by antiserum prepared against purified T25 protease. More than 50 percent inhibition of T20 protease was obtained at 4 mg per ml concentration of IgG.

Single precipitin bands were obtained when T20, T18, T10 and T6 proteases were reacted with antiserum produced against T25 protease and T16 protease (Fig. 18). All of the

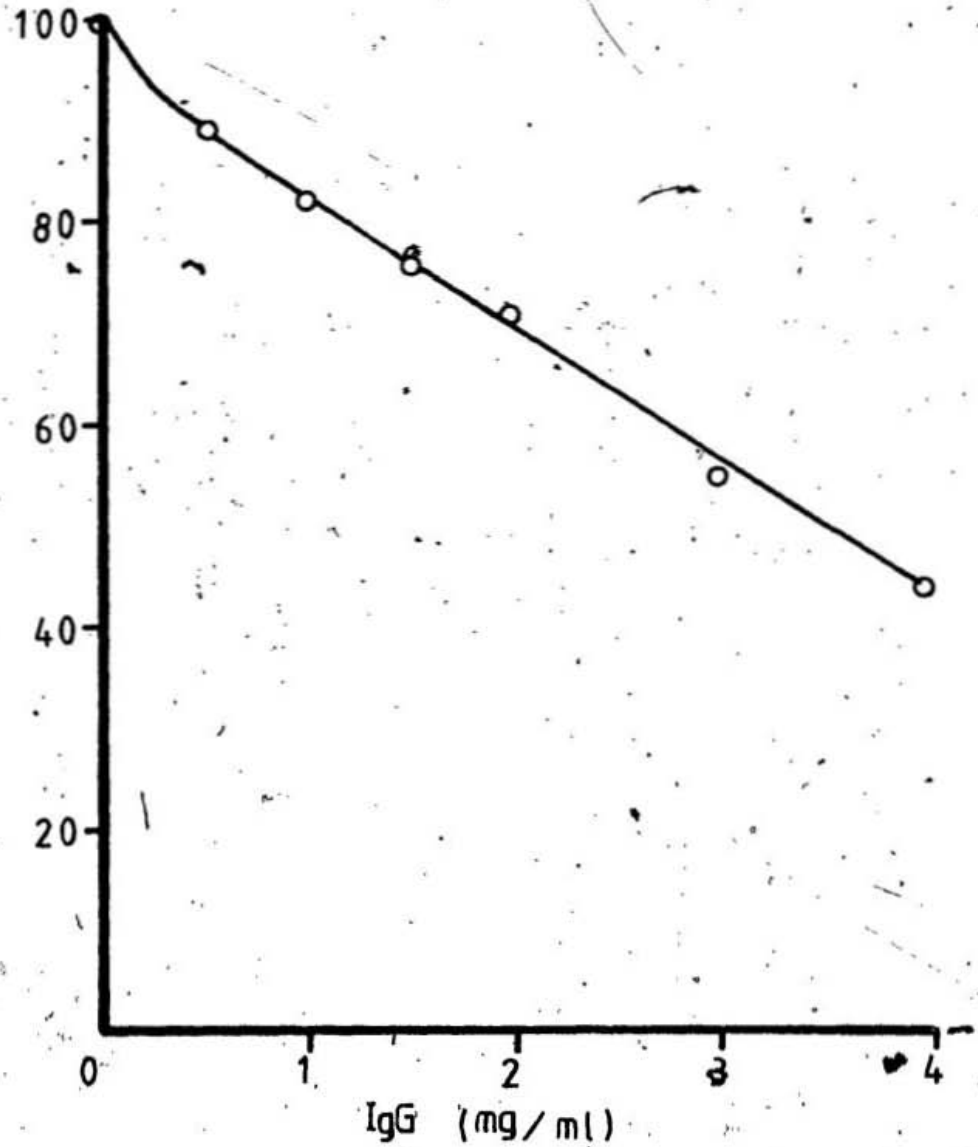
Table 11

Protease activity in growth medium and cell extracts of various isolates

Protease Activity (EU/ml)	T20	T18	T10	T6
Growth Medium	0.318	0.287	0.281	0.267
Cell Extract	0.000	0.000	0.000	0.000

Fig. 17 Inhibition of T20 protease activity by IgG raised against T25 protease

The antigen (T20 proteins) containing about 0.5 enzyme unit/ml was allowed to react with the antibody in buffer for 15 minutes at 25°C. The reaction was started by addition of the substrate. The residual activity is presented as the percentage of activity observed in the absence of antibody.


100
80
60
40
20
0
1
2
3
4
IgG (mg/ml)

precipitin bands formed by antigen-antibody reaction in Ouchterlony's double diffusion technique fused together. The lines of identity indicated that each of the four proteases carried antigens with similar antigenic determinants.

The precipitin bands formed by immunoelectrophoresis are presented in Fig. 19. All of the crude enzyme preparations tested formed a single precipitin arc. Similarly, a single precipitin arc was also obtained when purified T20 protease and proteases produced at two different temperatures were used.

9. Determination of molecular weight

Molecular weight of the T20 protease was determined by gel filtration on a Sephadex G-200 column as described in Materials and Methods. Fig. 20 shows a standard plot obtained from the experimental data. The molecular weight of T20 was found to be 43,000 by this method (Fig. 21).

Fig. 18 Ouchterlony's double diffusion technique

The enzyme solutions (10 ul) from various isolates containing about 5 mg protein/ml were placed into the wells surrounding a central well in which 15 ul antiserum was placed. The frames were then placed in a humidity chamber and incubated for 24 hours at 10°C. The immunodiffusion gels show cross reactions among (A) T20, (B) T18, (C) T6 (D) T10. In (I) IgG raised against T25 protease and in (II) IgG raised against T16 protease was poured.

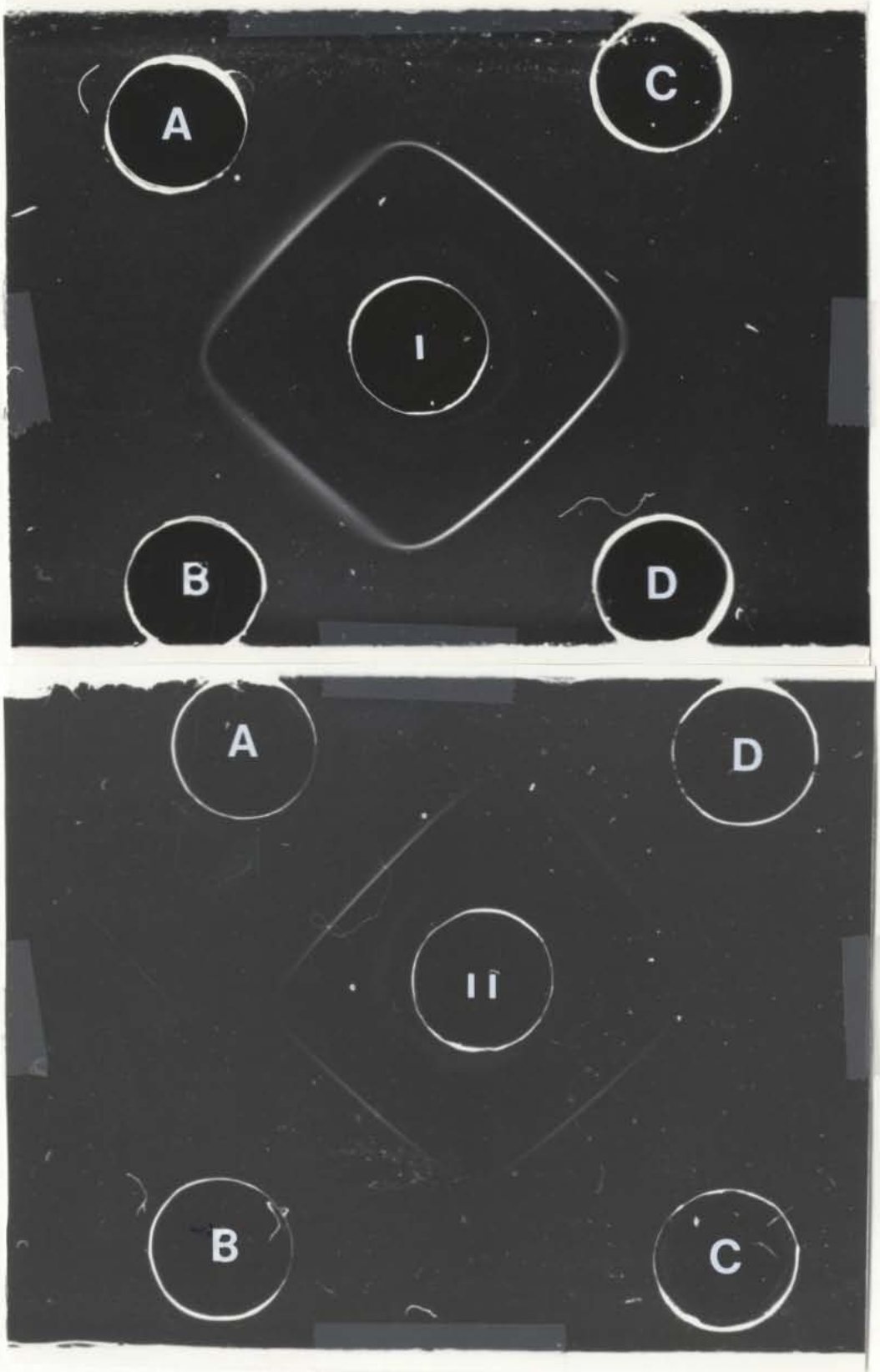

A
C
I
B
D
A
D
II
B
C

Fig. 19 Immunoelectrophoresis of proteases.

About 10 ul of protease solutions were poured in two wells on each side of a central trough. The gels were then electrophoresed for about 2 hours at a current of 3 mA per frame. After the electrophoresis the gel from the central trough was removed and antiserum or antibody was applied and stored in cold for 24 hours for development of the precipitin arcs.

a) T6 protease

b) partially purified T20 protease

c) T18 protease

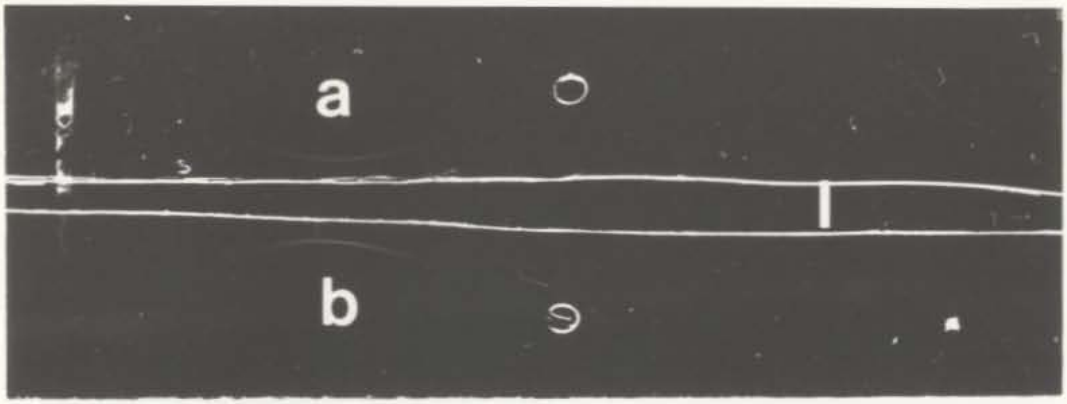
d) T10 protease

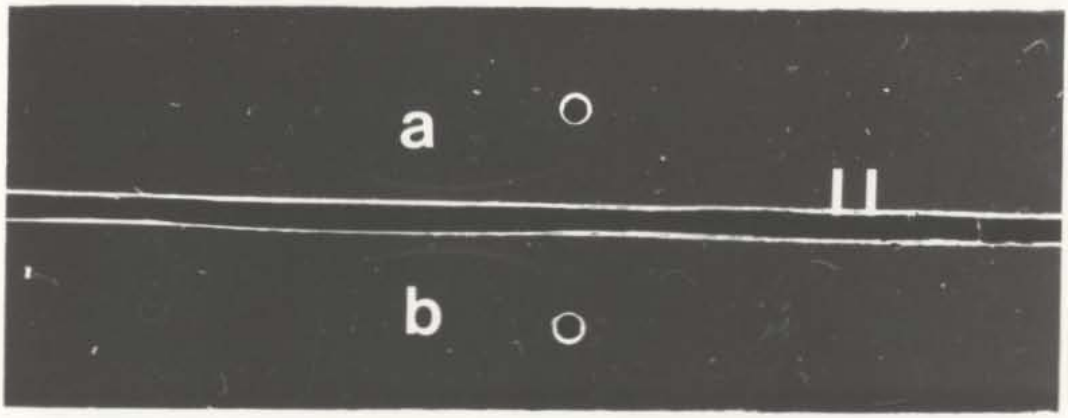
e) T20 protease produced at 25°C

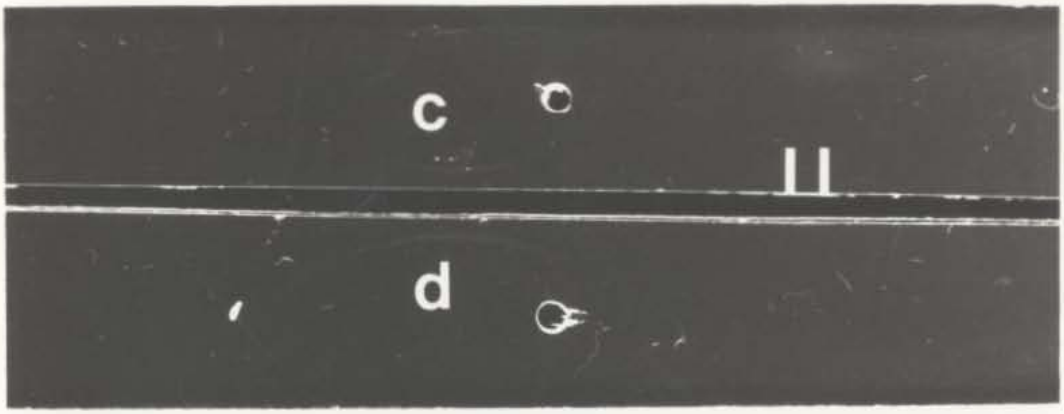
f) T20 protease produced at 5°C

i) IgG raised against T25 protease

ii) IgG raised against T16 protease


a
b


a
b


c
d

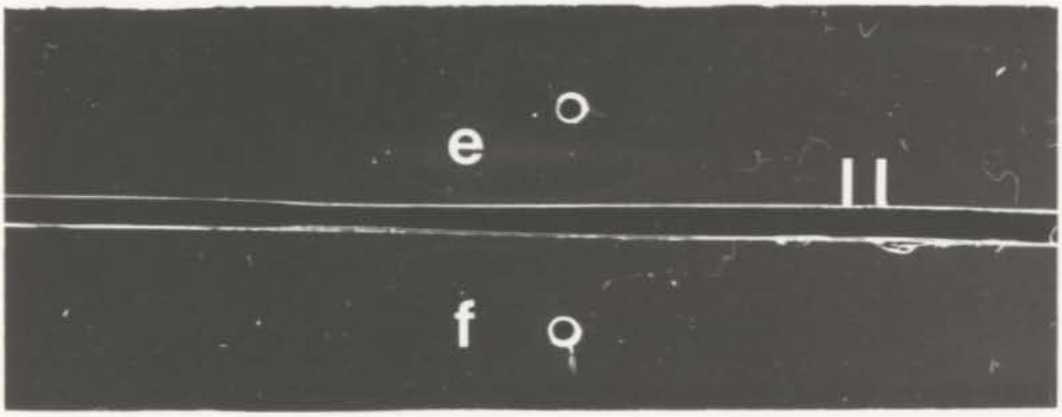
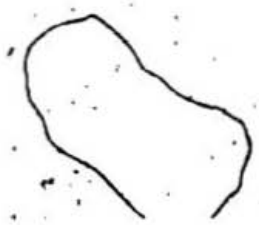

e
f

Fig. 20 Standard curve for molecular weight determination

A column (2 x 45 cms) packed with Sephadex G-200 was used for molecular weight determination of various proteases. The proteins used for the calibration of the column were, Ferritin (440,000), Catalase (240,000), Alcohol dehydrogenase (141,000) Aldolase (158,000), Bovine Serum Albumin (64,000), Ovalbumin (43,000), Chymotrypsinogen A (25,000) and Lysozyme (11,000). The column was calibrated with 0.02 M Tris-HCl buffer. Fractions (3.1 ml) were collected and the absorption at 280 nm was measured. The Kav of various proteins were determined using Vo of Blue Dextran (200,000) and the log of molecular weight was plotted against Kav.



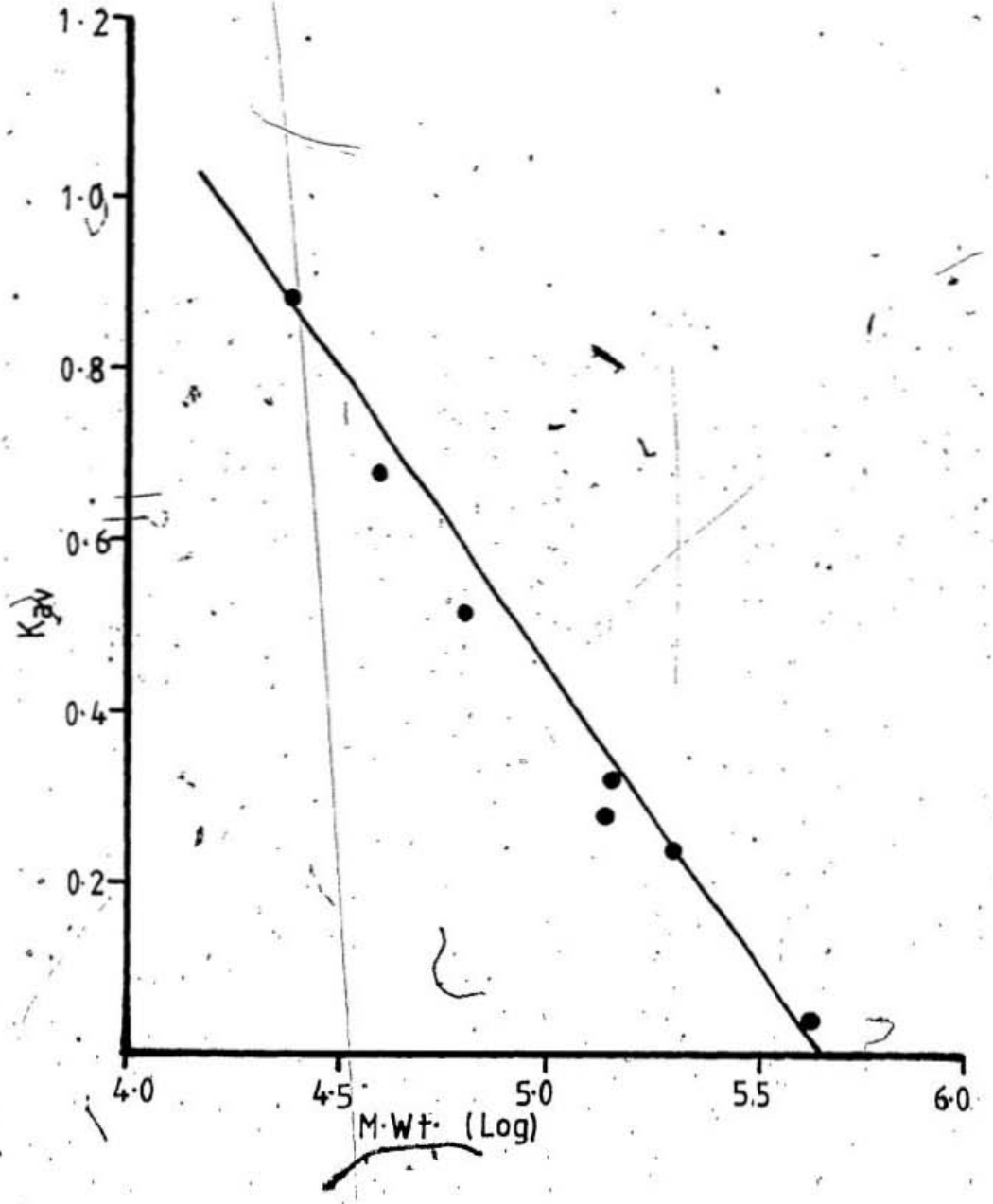
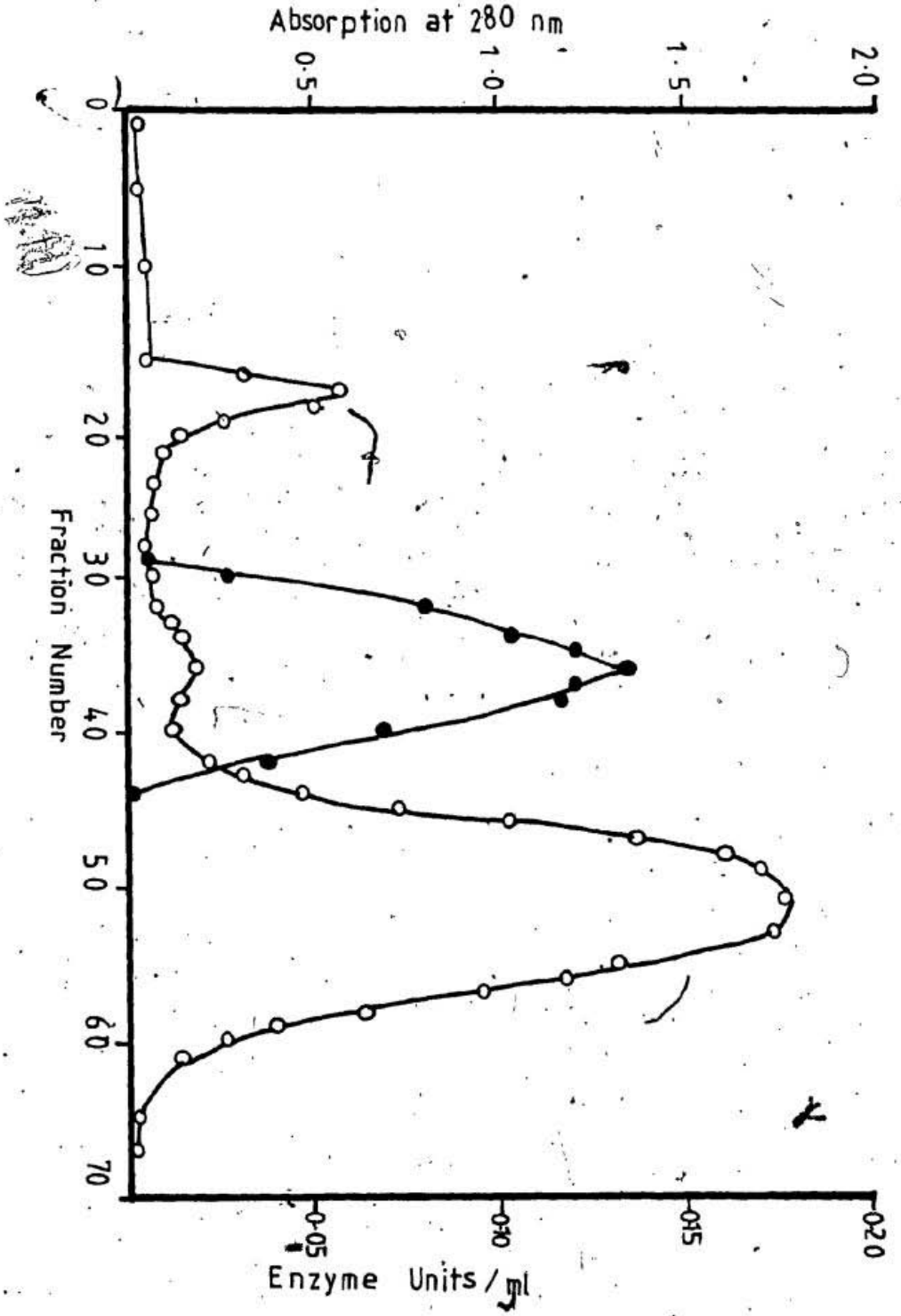

1·2
1·0
0·8
0·6
0·4
0·2
K_{av}
4·0
4·5
5·0
5·5
6·0
M·Wt· (Log)

Fig. 21 - Determination of molecular weight of T20 protease on Sephadex G-200 column

A concentrated sample (4 ml) was applied on a Sephadex column equilibrated with various proteins for molecular weight determination. The proteins were eluted with 0.02 M. Tris-HCl buffer (pH 7.5) at a flow rate of 18 ml per hour. Fractions (3.1 ml) were collected and proteins (O—O) were determined by measuring the absorbance at 280 nm; also assayed for proteolytic activity (●—●) as described in Materials and Methods using 0.1 ml of each fraction.


Absorption at 280 nm
0·5
1·0
1·5
2·0
0
10
20
30
40
50
60
70
Fraction Number
0·05
0·10
0·15
0·20
Enzyme Units / ml

DISCUSSION

1. Partial purification of protease

The protease from T20 was partially purified by a combination of ammonium sulfate precipitation, gel filtration and affinity chromatography. Other workers have also used a combination of various techniques to purify the extracellular proteolytic enzymes for various Pseudomonas species. These techniques include salting out, gel filtration and affinity chromatography (26, 29, 30, 32, 35). In present studies, the T20 protease was partially purified with a fourteen percent recovery of the proteolytic activity originally present in the crude extract (Table 1).

To precipitate the T20 protease from the crude extract, solid ammonium sulfate (at 60% saturation) was used. The precipitated proteins were then subjected to gel filtration (Fig. 2). The peak of proteolytic activity on gel filtration corresponding to the proteins in peak II was found to be symmetrical. This suggested that only one molecular form of the protease was present in the growth medium of the isolate number T20. However, electrophoretic homogeniety was not achieved at this stage. Hence this protease was further purified by affinity column chromatography. The protease bound to the affinity column quite efficiently under acidic conditions. For the elution of the bound proteins 0.1 M Tris-HCl buffer (pH 8) was used. The proteins eluted readily in this buffer. Howeyer,

proteins not showing any proteolytic activity were also eluted in this buffer, as indicated by two distinct peaks in the elution profile of the affinity column (Fig 3). The proteolytic activity was associated with the second peak (b), the peak 'a' not showing any protease activity.

The finding that the non-proteolytic protein bound to the column efficiently and eluted with the protease suggested that this protein might be the inactivated protease which retained its capacity to bind to the affinity column. Moreover, the partially purified protease showed multiple bands when electrophoresed with or without SDS. Only one protein band (corresponding to p in Figure 4) showed protease activity when unstained gel was placed on milk agar plate soon after polyacrylamide gel electrophoresis without SDS as described in Materials and Methods. Hence multiple bands in the gels were most probably due to the autodigestion of the protease. On the basis of above findings it is concluded that the most probable explanation for not achieving electrophoretic homogeniety and for low recovery is the autodigestion of the protease during its purification. Other proteases are also known to be autodigested on purification. Boethling (33) could not obtain a single protein band when he electrophoresed purified extracellular protease from P. maltophillia. He also found autodigestion to be the cause of electrophoretic heterogeniety of the purified protease from this source.

2. Properties of proteases

a) pH optimum

All of the proteases investigated were found to be maximally active in the neutral pH range. The pH optimum for the T20 protease was found to be 7.2 (Fig. 6). Other proteases were also found to be maximally active in the same region. Hence, these proteases can be classified as neutral proteases. Other Pseudomonas proteases are also reported to possess pH optima in the similar range (28, 29, 35, 36).

b) Temperature optimum

When proteases from various isolates were assayed at different temperatures, all of the proteases showed maximum activity at a temperature of 35°C. This is in agreement to the finding of Alichanidis and Andrews (29). Slight shoulders in temperature versus activity curves might be due to multiple forms of the protease (each having different temperature optima) or due to the experimental errors. As discussed earlier, only one protease is produced by T20 and probably by other isolates. Hence, these slight shoulders were most probably due to experimental error. Other Pseudomonas extracellular proteases had much higher temperature optima. For example, the temperature optimum for MC 60 and B52 proteases was as high as 45°C (34, 36).

All proteases investigated in this study showed very little activity at a temperature of 50°C., even though the

proteases were stable at higher temperatures. This is in agreement with the findings of other investigators (29, 35, 36). The findings in the pH and temperature profiles of various proteases are quite important with respect to their industrial application. As depicted in Figure 7, all of the proteases retained considerable activity over a wide range of temperatures. The T20 protease, for example, retained 9 percent and 49 percent of maximum activity at a temperature of 1 and 10°C respectively. This low activity at low temperatures and the maximum activity at or near neutral pH may contribute to flavor defects and spoilage of the milk and other dairy products, which are usually stored under refrigeration. White and Marshall (66) observed lower flavor scores in cheese made from milk containing protease from P. fluorescens P26 held at 4°C for 12 hours. Law et al. (67) observed gelation of milk by protease from another P. fluorescens strain.

c. Effect of substrate concentration on the protease activity

The effects of varying the soluble casein concentration are presented in Figure 9. The enzyme velocity increases upto a concentration of about 3.5 mg/ml in the case of T18 and T6 proteases and 4.5 mg/ml in the case of T20 and T10 proteases. No further increase in protease activity was observed with further increase in concentration of soluble casein. Rather there is a decrease in protease activity of

isolate number T18, T10, and T6 with increase in concentration of soluble casein over 4.5 mg/ml. This decrease in protease activity suggests that at a higher substrate concentration, inhibition of protease activity is taking place. However, the nature of the inhibition is not known. It is not known whether this is an indication of true substrate inhibition or if it is in fact inhibition caused by the increasing amount of product accumulating as a result of high protease activity at higher substrate concentration. Therefore, on the basis of these experiments, no comment can be made regarding the nature of inhibition encountered at higher substrate concentration.

Just as in the case of soluble casein, inhibition of protease activity was also observed in most of the cases when α and β-casein were used as substrates. However, the degree of inhibition observed was much lower than that observed when soluble casein (which is a mixture of various casein fractions) was used as the substrate.

Depending upon the source, various proteases showed a linear increase in activity with increasing concentration of α or β-casein before showing any sign of inhibition. Since for each protease investigated the activity increased linearly up to a certain concentration of the protein substrates (α or β-caseins) and inhibition of protease activity was detected at only higher concentrations, probably the phenomenon of substrate inhibition is involved. This is consistent with the inhibition caused by soluble

casein. However, a greater degree of inhibition was observed when soluble casein was used as the substrate. This is as expected since a comparatively higher soluble casein concentration is used for protease assay.

While interpreting the Km values, it should be taken into account that an end point assay method with a 20 minute incubation period was used for measurement of protease activity. Although linearity of protease activity with time was established at a soluble casein concentration of 2.5 mg per ml as described in Materials and Methods, the rate of protease activity might not be linear for 20 minutes at lower substrate concentration. Therefore, the apparent Km values presented in Table 2 could probably be slightly lower or higher than the actual values. Within these limitations the conclusion that can be drawn is that the proteases investigated here have a higher affinity for α-casein than for β-casein. This is in agreement with the observation of Kiru et al. (68) who observed changes in casein composition of milk when flavor defects first appeared in milk and part of α-casein was removed. Overcast (69) observed some distinct changes in elution pattern of casein when milk was incubated with P. fragi, P. putrifaciens and P. fluorescens. β-casein disappeared completely after 42 days at 3-5°C. The degree of hydrolysis of milk proteins and selectivity depends upon bacterial culture as well as the conditions employed (46, 47). The data on Km values of various extracellular proteolytic enzymes of psychrotrophic

pseudomonads are not available. The activity of 0.024 enzyme units per ml of crude culture supernatant reported for P. fluorescens B-52 (35) protease is much lower than that for T20 protease which was found to be 0.652 (Table 1). Hence the proteases described in this report are much more active than the P.fluorescens B-52 protease. Other proteases are also reported to be several fold less active than the proteases investigated here (30).

d) Substrate specificity of proteases

Proteins other than soluble casein were hydrolysed by T20, T18, T10 and T6 (Table 3). However, no single protein was hydrolysed with similar efficiency. Hence, on the basis of these observations the substrate of choice would be soluble casein, which is a mixture of various proteins (α, β, γ and k-caseins).

In this study very little work was done on synthetic peptides. Further work is needed to deduce the amino acid specificity of these proteases. The T20 protease hydrolyzed the synthetic substrates, L-BAPA and Glycyl-Phenylalanine-β-Naphthylamide. The Km for the latter was much higher (Table 4). This is as expected, since Glycyl-Phenylalanine-β-Naphthylamide is a specific substrate for cathepsin C (70). The Km for L-BAPA was also higher than the Km of trypsin, which was 0.939×10^{-3}M (55).

No activity was observed on N-Benzoyl-L-Tyrosine-p-Nitroanilide or Glutaryl-L-Phenylalanine-p-Nitroanilide.

This suggests that probably the T20 protease is specific for hydrolyzing a peptide bond of a basic amino acid near the carboxyl terminal. The inability of T20 protease to show any activity on TAME suggests that the T20 protease does not have any esterolytic activity like trypsin. Moreover, the protease activity on cathepsin C substrate (Glycyl-L-phenylalanyl-β-Naphthylamide) by T20 protease also suggests that this enzyme has dipeptidase activity.

3. Enzyme modulation studies

The proteases from T20, T18, T10 and T6 were found to be inhibited by metal chelating agents like, EDTA and 8-hydroxy quinoline. Hence these proteases are metalloproteases as are some proteases from other pseudomonads (28, 29, 32, 35). In addition to being inhibited by metal chelators the proteases in this study were also inhibited slightly with sulfhydryl reagents such as N-ethylmaleiamide and p-chloromercuribenzoate. The extracellular protease from P. fluorescens strain AR11 was also found to be inhibited by metal chelating agents as well as by sulfhydryl reagents (29). In contrast, some other pseudomonads produce extracellular proteases which are inhibited only by metal chelators (28, 35).

4. Effect of metal ions on protease activity

Once it was established the proteases from the four isolates (T20, T18, T10 and T6) were metalloproteases,

attempts were made to identify the metal ions involved. Out of the many metal ions tried Cu^{2+}, Zn^{2+}, Hg^{2+} and Ni^{2+}, inhibited the protease activity of T20, T18, T10 and T6. Metal ions like Ca^{2+}, Mn^{2+} and Mg^{2+}, had either no effect or a very slight inhibitory or stimulatory effect. Since the identity of the metals involved in protease activity could not be determined by these experiments, a new set of experiments was designed. In the second set of experiments the proteases of various isolates were completely inactivated by treatment with EDTA as described in Materials and Methods. The protease activity was then assayed in presence of various metal ions. With a few exceptions, Cu^{2+}, Zn^{2+}, Hg^{2+}, Ni^{2+}, Li^{+}, Sn^{2+}, K^{+} and Na^{+} were unable to restore any of the protease activity (Table 9). Cu^{2+}, Zn^{2+} and Hg^{2+} were previously shown to be inhibitory (Table 8). Metal ions such as Ca^{2+}, Co^{2+}, Mn^{2+}, Sn^{2+}, Al^{2+}, Ba^{2+}, Mg^{2+} and Pb^{2+} restored the protease activities of various isolates to some extent; the degree of restoration of protease activity differed from one metal ion to the other and also among the different isolates. Other proteases are known to require divalent cation for activity. For example the protease from *P. fluorescens* B52 requires Ca^{2+} for stabilization of enzyme while Zn^{2+} is involved in the protease activity (35). On the basis of present studies the identity of the metal involved in protease activity is not known. The only conclusion which can be drawn is that some

divalent cation might be involved either for maintenance of the enzyme structure or involved in protease activity.

5. Heat stability

Many psychrotrophic pseudomonads isolated from milk and other dairy products are known to produce extracellular heat resistant proteases (40, 41, 45, 47). The T20, T18, T10 and T6 proteases were also found to be heat resistant (Table 6). Although Ca^{2+} and/or Zn^{2+} are known to play important roles in providing heat stability to other proteases (35) it seems that both of these ions do not play an important role in the retention of proteolytic activity of any isolate investigated in this study. The presence of Ca^{2+} in reaction mixture during heat exposure of T20 protease did not provide extra heat stability to this enzyme (Fig. 15). Since the role of endogenous Ca^{2+} ion was not investigated, its participation in providing heat stability to T20 protease cannot be ruled out and hence no conclusion can be drawn with respect to the role of calcium ions in providing heat stability to these proteases. As far as Zn^{2+} is concerned it cannot be the metal ion involved in maintenance of protease activity during heat exposure as it has an inhibitory effect on all protease activities (Table 8). Therefore, on the basis of these experiments, the mechanism of heat stability cannot be deduced. Moreover, like many other proteases these proteases were also shown to be inactivated to a greater extent at lower temperatures than

at higher temperatures. This phenomenon of 'Low Temperature Inactivation', is observed for other proteases (42), and has already been discussed in detail (page 18).

6. Control of synthesis

The protease production by T20 begins in early logarithmic phase of growth and continues in stationary phase. Similar findings were reported for other psychrotrophic proteases (36, 47). The reason for the increasing amounts of protease at the beginning of logarithmic phase and then its decline in late stationary phase is not clear. However, it may be explained as follows: in the early logarithmic phase when the bacteria are growing exponentially, they require continuous energy source as well as amino acids for synthesis of cellular proteins. So the extracellular protease production begins, probably stimulated by milk proteins or any of their degradative product(s), produced by basal levels of protease synthesized at all times irrespective of composition of medium (71). The assumption that the secretion of protease involves synthesis 'de novo' is supported by the general observation that most extracellular enzymes are not stored inside the cells. This was also found to be true for extracellular proteases investigated in this report, as no protease activity was detected when assayed in the cells after disruption (Table 11).. As more and more proteins are degraded, increasing amounts of the stimulator are produced

which then elicit further protease production. In late stationary phase the supply of the 'inducer' is decreased and/or a catabolic inhibitor (probably a repressor) is produced which then decreases (represses) the protease production. No concrete evidence was provided by these studies and further work is needed to provide the evidence in support of these speculations. However on the basis of these experiments, it seems probable that the proteases reported here are inducible enzymes in which milk proteins play an important role and that catabolic repression may also be involved in control of synthesis.

7. Molecular weight determination

The molecular weight of T20 protease was determined by gel filtration on Sephadex G-200 column, and was found to be 43,000. A molecular weight of 44,000 was estimated for another isolate in this laboratory (72). Other investigators have determined the molecular weights of other pseudomonad proteases which range from as low as 23,000 (30) to 50,000 (32).

8. Antigenic relatedness of proteases

The IgG raised against T25 protease was effective in inhibiting T20 protease activity. This finding together with the observation that the precipitin bands in Ouchterlony's double diffusion tests fused together indicated that the proteases from various bacterial cultures

share similar antigenic determinants. Additional evidence in support of antigenic similarity of various proteases is the finding that similar precipitin bands were formed when IgG raised against purified T16 protease was used. Formation of single precipitin arcs in immunoelectrophoresis with either of the two IgG preparations provided further proof of antigenic similarity of various proteases. In addition to this, formation of single precipitin arcs with crude extract or partially purified T20 protease indicated that only one protease is produced by T20. Immunoelectrophoresis of T20 protease synthesized at two different temperatures (25^{o} and $5^{o}C$) also provided the evidence in support of the assumption that the enzyme synthesized at two different temperatures is also the same (Fig. 19).

CONCLUSIONS

The following conclusions are drawn from these investigations:

1. The proteases from bacterial isolates numbers, T20, T18, T10 and T6 are neutral proteases.
2. These proteases are active over a wide range of temperatures, having a temperature optimum of 35°C.
3. All of these proteases require metal ions for their activity, hence are classified as metalloproteases.
4. All reactions of casein (α, β, γ and k) are hydrolyzed by the proteases investigated here, although various proteases show different rates of hydrolysis of these proteins.
5. Proteins other than casein are not acted upon by these proteases efficiently; hence the substrate of choice is casein which is their natural substrate.
6. The synthetic substrates, L-BAPA and glycyl-phenylalanine-β-naphthylamide are hydrolyzed by T20 protease.
7. All bacterial cultures produce highest level of protease activity when grown in presence of milk.
8. These proteases are extracellular in nature and are not stored in the cells before secretion.
9. The proteases from several isolates of Pseudomonas fluorescens share common antigenic determinants.

10. All proteases investigated can survive heat treatments at high temperatures (100°C).

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