AN EXTRACELLULAR PROTEASE FROM AN EXTREME THERMOPHILE

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ABSTRACT

The major extracellular protease of the *Thermus*-like organism, *Thermus* T-351, has been isolated by Sephadex SP-C25 ion exchange chromatography, affinity chromatography on CBZ-D-phe-TETA-Sepharose, and Sephadex G75 gel filtration chromatography. The purified enzyme, assigned the title "Caldolysin", was shown to be homogeneous by a variety of electrophoretic techniques. Two other minor extracellular proteolytic enzymes (totalling 12% to 25% of initial enzyme activity) were separated during ion exchange chromatography, but have not been studied. The molecular weight and isoelectric point of purified Caldolysin were estimated to be approximately 20 000 Daltons and 8.5, respectively. The presence of 6 disulphide bonds was indicated by the reaction with dithionitro-benzoate. No free sulphydryl groups were detected.

When tested against a wide range of inhibitors, significant responses to EDTA, EGTA, and iodoacetic acid, but not o-phenanthroline or p-chloromercuri benzoate, were noted. Together with the presence of lytic activity (Caldolysin lysed a broad range of Gram-negative bacteria but only few Gram-positive organisms), this permitted the classification of Caldolysin as a metal-chelator-sensitive lytic protease. A number of similarities with *Myxobacter* and *Sorangium* lytic proteases have been noted.

It was shown that calcium was essential for the stability of Caldolysin, but not for its activity. No evidence was found for the presence of an active site-bound metal ion. This is a significant dissimilarity to Thermolysin, which contains a single catalytic zinc atom.

Casein, albumin, haemoglobin, collagen, elastin, and fibrin were all hydrolysed to varying degrees by Caldolysin. An apparent minimum size requirement for activity (a minimum of 3 or 4 amino acid residues) precluded the use of amino acid and dipeptide substrate analogues for the measurement of proteolytic activity. A preliminary determination of specificity indicated a preference for small neutral aliphatic amino acids on either side of the point of hydrolysis. No esterase activity was detected. Optimal hydrolysis of both casein and albumin occurred at approximately pH 8.

Temperature-activity relationships indicated that Caldolysin underwent conformational changes at 50°C and 92°C. Loss of enzyme activity below the latter temperature was thought to be primarily the result of autolysis while above this temperature denaturation occurred. Arrhenius plots showed a break at about 50°C, above which a reduction in activation energy occurred. The K_m at 40°C was considerably higher than that at either 65°C or 85°C.

Caldolysin is one of the most stable enzymes known. Thermostability was greater than that of most other documented proteases ($t_{\frac{1}{2}}$ (80° C) = 30 hours; $t_{\frac{1}{2}}$ (85°C) = 5-6 hours; $t_{\frac{1}{2}}$ (90°C) = 1 hour; $t_{\frac{1}{2}}$ (95°C) = 30 minutes; $t_{\frac{1}{2}}$ (100°C) = 4 minutes). In the absence of calcium, thermostability was reduced considerably ($t_{\frac{1}{2}}$ 80°C) = 8 minutes). Other metal ions provided only a slight thermostabilising effect compared with Ca [Ca≫Zn>Sr>Mg>Co>Ba>Cu]. Neither the presence of high ionic concentrations nor the presence of non-ionic solutes had any major effect on thermostability. However, high and low pH values significantly reduced thermostability at 75°C and 90°C. At room temperature in solution, little loss of enzyme activity was noted at pH's from 7-11 during a 95 day period. Activity half-lives at pH 3.6, 5, and 11.8 were 28 days, more than 60 days, and 4 days, respectively. Little activity loss resulted from storage of the enzyme in the frozen or lyophilised state for a period of six months. Caldolysin was shown to be very stable in the presence of denaturing agents (8M urea, 6M guanidine.HCl, 1% sodium dodecylsulphate) at ambient temperatures. At 75°C, a 50% loss of activity occurred within 59 minutes, 53 minutes, and 320 minutes, respectively.

A feature of the Michaelis-Menten kinetics of Caldolysin is marked substrate inhibition (K_m (azoalbumin) = 0.028; K_S' (azoalbumin) = 3) which, however, does not occur with small substrates or when the enzyme is immobilised. The enzyme also exhibits an initial activation during incubation at temperatures above 65°C. This effect has been correlated with the ionic strength, the incubation temperature, and the concentration of non-enzyme protein present. The latter causes

initial inhibition which is reversed on heating. It is postulated that activation/inhibition and substrate inhibition are related, and are caused by the presence of non-catalytic protein-binding sites in the molecule which are discrete from the active site.

Caldolysin was successfully immobilised to glass beads, CM-cellulose, and Sepharose 4B, with enzyme activity retentions of 1%, 31% and 73%, respectively. The K_m of Caldolysin was unchanged in the immobilised state, but the pH optimum of Sepharose-caldolysin was reduced by approximately one pH unit. Thermal stability of both apo-and holo-caldolysin-Sepharose was increased by 3-4-fold.

PREFACE

At the outset of this research project, it was intended to isolate, characterise, and compare proteolytic enzymes from selected thermophilic, mesophilic, and psychrophilic microorganisms. However, as the isolation and characterisation of the major extracellular protease from the extreme thermophile, *Thermus* T-351, progressed (this enzyme was subsequently assigned the trivial name of CALDOLYSIN, analogous to Thermolysin and because of its lytic properties), it became apparent that the unusual properties of this enzyme warranted extensive investigation. Accordingly, the project became centred on a detailed study of the activity, stability, and structure of Caldolysin, the results of which are presented in this thesis.

It had originally been intended to also seek (and possibly isolate) extracellular proteases from strains of *Saccharomyces cerevisiae* commonly used in oenology. This project stemmed from the observation by scientists at the Te Kauwhata Oenological and Viticultural Research Station, Hamilton, New Zealand, that fermentation of high-protein juices occasionally resulted in the production of gaseous H_2S. It was considered that this undesirable characteristic might be derived from the production of extracellular protease by the wine yeast, and subsequent proteolytic release of sulphur-containing amino acids. However, the negative results of the screening tests for extracellular yeast proteases precluded further work in this field.

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CHAPTER 1

INTRODUCTION

1-1 Proteolytic Enzymes: Historical background and enzyme classification.

Prior to the early 1900's, the existance of proteases from many animal and some plant sources had been either suspected or confirmed. However, it was not until 1917 that bacterial proteases were first observed, when Weinberg & Seguin (1917) detected proteolytic activity in cell-free extracts of *Clostridium histolyticum*. Detailed studies of microbial proteases were initiated in the early 1950's when proteases were isolated from *Aspergillus oryzae*, *Bacillus subtilis amyloliquifi* and *B. subtilis* (Crewther & Lennox 1950; Fukumoto & Negoro, 1951; and Güntelberg & Ottesen, 1952). Subsequently, both intracellular and extracellular proteases have been isolated from a variety of microorganisms (Matsubara & Feder, 1971). Interest in thermophilic proteases was first generated when, in 1962, Endo isolated a particularly heat-stable protease from *Bacillus thermoproteolyticus*. This enzyme, now known by the commercial name of Thermolysin, has been subject to detailed biochemical and structural analysis. Since 1962, a surge of interest in such enzymes, heightened by their apparent industrial potential, has resulted in the isolation and characterisation of thermostable proteases from numerous thermophilic microorganisms (see Table 1-1).

Early in the twentieth century, it was discovered that proteases could be grouped into broad categories specified by their physiochemical properties. A classification system for microbial proteases based on pH optimum, inhibitor response, and substrate specificity has been outlined by Morihara (1974), and is reproduced, with some alterations and omissions, in Table 1-2. Common protease nomenclature is not always consistent.

TABLE 1-2 Classification of microbial proteases (from Morihara, 1974)

GROUP and Sub-Group	pH Optimum	Common Inhibitors	Primary Specificity
SERINE PROTEASES	8	DFP, tosyl-L-lysine chloromethyl ketone	Basic amino acid residues at the carboxyl side of the splitting point.
Trypsinlike			
Alkaline	10	DFP, chloromethyl ketones	Aromatic or hydrophobic residues at the carboxyl side of the splitting point.
Myxobacter α-lytic	9	DFP	Small aliphatic residues at the carboxyl side of the splitting point.
Staphylococcal	4, 7.8	DFP	Aspartic or glutamic acid at the carboxyl side of the splitting point.
THIOL PROTEASES			
Clostripain	7	PCMB, tosyl-L-lysine chloromethyl ketone	Basic amino acids at the carboxyl side of the splitting point.
Streptococcal	7.5	PCMB	Broad
METAL-CHELATOR-SENSITIVE PROTEASES			
Neutral	7	EDTA, o-phenanthroline	Hydrophobic or bulky residues at the amino side of the splitting point.
Alkaline	7-9	EDTA ($>10^{-2}$M) o-phenanthroline	Broad
Myxobacter AL-1 protease 1	9	EDTA ($>10^{-2}$M)	Small amino acids at either or both sides of the splitting point.
Myxobacter AL-1 protease II	8.5 - 9	EDTA ($>10^{-2}$M)	Lysine residue at the amino side of the splitting point.
ACID PROTEASES	3-4	Diazoacetyl-DL-norleucine methyl ester	Aromatic or hydrophobic amino acid residues at both sides of the splitting point.

For instance, reference is made to active-site-specific components (e.g. metalloendopeptidase), pH optimum (e.g. *B. subtilis* neutral protease) or to their biological activities; (collagenase-like enzymes, elastases, keritinases, lytic proteases, and microbial rennins.)

TABLE 1-1. Proteases from some thermophilic bacteria and fungi.

Protease (common name)	Microorganism	Reference
Thermolysin	*B. thermoproteolyticus*	Endo (1962)
Aminopeptidase 1	*B. stearothermophilus*	Roncari & Zuber (1969)
Neutral protease	*B. stearothermophilus*	O'Brien & Campbell (1957)
-	*B. brevis*	Gibson & Gordon (1974)
-	*B. caldolyticus*	Heinen & Heinen (1972)
Thermomycolin	*Malbranchea pulchella*	Ong & Gaucher (1976)
Thermitase	*Thermoactinomyces vulgaris*	Rutloff *et al.* (1978)
-	*Thermomonospora viridis*	Upton & Fogarty (1977)
-	*Streptomyces rectus*	Mizusawa, Ichishima, & Yoshida (1969)

In addition to the categories outlined in Table 1-2, both carboxy- and aminopeptidases have been isolated from bacteria (Matsubara & Feder, 1971). These enzymes cleave single amino acid residues from the C and N termini of polypeptide chains.

1-2 The role of intracellular proteases

The multiple roles of intracellular proteases in mammalian systems have been well characterised (e.g. Barrett, 1977). Functions include activation of precursors (e.g. cleavage of plasminogen by the proteases kallikrein or urokinase to yield plasmin in the fibrinolysis sequence), metabolic control by specific hydrolysis of other enzymes, and non-specific proteolysis in protein degradation pathways.

The specificities of proteases involved in these processes varies according to the nature of the metabolic requirements. Urokinase, a serine protease, has been shown to catalyse the hydrolysis of a single arginyl-valine bond in plasminogen (Robbins *et al.*, 1967). Conversely, Cathepsin D, isolated from bovine uterus by Woessner & Shamberger (1971), was shown to cleave seven different peptide bonds in oxidised β-insulin. This acid protease, a major lysosomal enzyme in many vertebrates, is considered to be adapted for a role in intracellular digestion of proteins (Barrett, 1977).

The physiological functions of intracellular proteases in micro-organisms have been little studied by comparison. The limited number of bacterial intracellular proteases so far isolated includes those from *B. subtilis* (Stephanov *et al.*, 1977), *B. thuringiensis* var. *berliner* (Lecadet *et al.*, 1977), *B. licheniformis* (Strongin *et al.*, 1979), *Escherichia coli* (Strongin, *et al.*, 1979), and *B. cereus* (Cheng & Aronson, 1977). Various intracellular proteases have been implicated in general protein turnover (Cheng & Aronson, 1977) and in specific metabolic control mechanisms (Lecadet *et al.*, 1977). Furthermore, there is accumulating evidence that the precursors of some extracellular proteins are subjected to limited proteolysis prior to release from the periplasm (e.g. Drapeau, 1978).

Little is yet known of the roles of intracellular proteases in thermophiles. Roncari & Zuber (1969), in one of the few publications on thermophilic microbial intracellular proteases, reported the production of three types of aminopeptidase by strains of *B. stearothermophilus*. Each enzyme was found to possess different specificity and thermostability. More significantly, the relative proportions of Aminopeptidase 1, 11, and 111 varied in relation to the optimum growth temperature of the strain, with the most stable enzyme, Ap.1, being present in greater proportions in the more thermophilic strains (Amelunxen & Murdock, 1978a). It has been suggested that the synthesis of the three enzymes is under strict genetic control, although the relative rates of degradation may be a significant factor. However, the specific roles of these enzymes in cellular metabolism is, as yet, unclear. It is speculated, that since normal molecular controls such as allosterism are found operating in even extreme thermophiles, the role of proteolysis in these organisms is not likely to differ substantially from that found in mesophiles.

1-3 The role of extracellular proteases

Unlike the multifunctional intracellular microbial proteases, those excreted by the cell are apparently limited in their functional diversity. However, these enzymes have attracted considerably more research interest than their intracellular analogues, possibly due to the technical simplicity of isolating proteins from culture fluids.

It cannot be doubted that the primary function of microbial extracellular proteases is nutritional. This is substantiated by the broad specificity exhibited by most extracellular proteases. However, some evidence suggests that selected enzymes may serve more than one function. Drapeau (1978) isolated an extracellular metalloprotease which, by limited proteolysis, was able to activate the precursor of a second

protease excreted by the same organism.

The properties of microbial extracellular lytic proteases suggests that these enzymes may act in the prevention or suppression of inter-specific competition. However, no lytic protease yet isolated has been found to act against all microorganisms and most show marked specificity against Gram positive or Gram negative bacteria, but not both.

1-4 Specificity of Microbial Proteases

Current data on microbial protease specificity has all been accumulated over the past 25 years, and has been reviewed recently by Morihara (1974).

Proteases can be broadly divided into four basic groups: serine, thiol, metal-chelator sensitive, and acid, (see Table 1-2). These can be further subdivided according to their side-chain specificity.

The four categories of serine proteases (trypsin-like, alkaline, *Myxobacter* α-lytic, and staphlococcal) show distinct specificities. The first group exhibit specificity for basic amino acid residues, the second for aromatic and hydrophobic residues, the third for small aliphatic residues such as alanine, and the last for acidic residues at the carboxyl side of the cleavage point in either synthetic substrates (e.g. N-CBZ-L-glutamyl-α-phenyl ester (Drapeau, 1978)), or oxidised β-insulin. Serine proteases also exhibit esterase and amidase activity on N-acylated amino acid derivatives such as alanyl-glycine ethyl ester and benzoyl-alanine methyl ester.

Thiol proteases are subdivided into the categories of streptococcal and Clostripain-like. The former exhibit broad specificity with both β-insulin and synthetic substrate, while the latter are specific in nature, hydrolysing linkages with a basic amino acid residue (particularly arginine) at the carboxyl side of the splitting point.

Clostripain also possesses esterase and substantial amidase activity.

Metal-chelator-sensitive proteases are classified according to the sub-groupings of neutral, alkaline, and *Myxobacter* AL-1 proteases 1 and 2. *Myxobacter* AL-1 protease 1 is often referred to as *Myxobacter* β-lytic protease. Thermolysin, isolated from *B. thermoproteolyticus*, is a representative example of the neutral metalloprotease class. The substrate specificities of Thermolysin and other neutral proteases have been extensively studied (Feder & Schuck, 1970; Morihara, Tsuzuki, & Oka, 1968; Matsubara *et al.*, 1966; and Matsubara & Sasaki, 1968). From these reports, it has been well established that neutral metalloproteases are specific for hydrophobic or bulky amino acid residues (e.g. leucine or phenyl-alanine) at the amino side of the splitting point.

Specificity of the alkaline proteases is broad. Collagenase-like activity (hydrolysis of Gly-pro-gly-pro-ala) by the extracellular proteases of *Pseudomonas aeruginosa* and *Serratia marcescens* has been observed (Morihara, 1974). Nearly 40% of the peptide linkages in β-insulin are cleaved by these enzymes.

Myxobacter AL-1 proteases 1 and 2 are specific for small amino acid residues at either side of the splitting point, and lysine residues at the amino side of the splitting point, respectively. Protease 1 from *Myxobacter* AL-1 (Jackson & Matsueda, 1970) and from *Sorangium* (Whitaker *et al.*, 1965) are known to hydrolyse pentaglycine and tetraglycine, but not di- or triglycine.

The metal-chelator-sensitive proteases generally do not demonstrate esterase or amidase activity against small synthetic substrates. Some oligopeptides, and substrates with α-amino or α-carboxyl groups blocked, are hydrolysed.

The acid proteases show a broad specificity, with a tendency to hydrolyse linkages with aromatic or bulky residues on both sides. The

division of acid proteases into pepsin-like and rennin-like sub-groups is not firmly based on substrate specificity which, as shown by the hydrolysis of β-insulin (Morihara, 1974), is very similar.

CHAPTER 2. PART A.

MECHANISMS OF THERMOPHILY

2-1 Introduction

Over the past 30 years, most of the research on thermophiles has centred around the mechanisms responsible for their ability to survive at temperatures lethal to most other organisms. While initially being centred on the general mechanisms of microbial survival, emphasis has now shifted to a study of thermostability at the molecular level. Numerous reviews on aspects of thermophily have been published (see, for example: Koffler, 1957, Amelunxen & Murdock, 1978a, 1978b).

Prior to the discovery of extreme thermophiles, thermophilic bacteria were divided into three categories (Farrell & Campbell, 1969), as outlined in Table 2-1.

TABLE 2-1 Classification of thermophilic bacteria.

Category	Growth temperatures (°C)	
	Maximum temperature	Minimum temperature
1. Strict or obligate thermophiles	65 - 70	40 - 42
2. Facultative thermophiles	50 - 65	30
3. Thermotolerant bacteria	45 - 50	30

To include those microorganisms which grow readily at temperatures of 70° - 100°C (reviewed by Brock, 1978), the category of 'caldoactive' (*caldus* - hot) bacteria was introduced by Heinen & Heinen (1972). This category specifies a maximum growth temperature above 70°C, an optimum

above 65°C, and a minimum above 40°C (Williams, 1975). The term 'thermoduric' has been suggested to describe microorganisms with a growth optimum between 20°C and 37°C, but which survive the pasteurisation of milk (Robertson, 1927). It is assumed that this capability is a product of spore resistance and is not an intrinsic property of the organism.

The demarcation of the categories outlined above is, however, not absolute. Recent work on thermoadaptation (e.g. Haberstich & Zuber, 1974) has demonstrated that both thermophilic and mesophilic cultures of *B. stearothermophilus* and *B. caldotenax* can be successfully obtained by cultivation at intermediate temperatures.

Two dissimilar schools of thought developed from the early studies of thermophily. It was suggested (Gaughran, 1947; Allan, 1953) that rapid resynthesis of damaged or denatured cell constituents provided a mechanism for microbial survival. Supporting evidence produced by Bubela & Holdsworth, 1966a and 1966b, demonstrates that rates of protein turnover are greater in *B. stearothermophilus* than in *E. coli*. However, Amelunxen & Murdock (1978b), suggested that problems of interpretation of data can arise from the measurement of protein turnover in sporeformers, where a high rate is associated with sporulation. Koffler (1957) pointed out that if thermophilic growth was due to a simple kinetic function of resynthesis, then thermophiles should exhibit at least sixteen times more growth activity at 70°C than at 30°C (assuming the reaction rate doubles for each ten degree rise). Novitsky *et al.* (1974) and Crabb *et al.*, (1975) have shown that the growth rate of *B. coagulans* KU increases only by a factor of about 1.3 when the growth temperature is raised from 37°C to 55°C.

It is currently considered that rapid resynthesis is an unlikely general mechanism for thermal survival, especially in view of the amassed

evidence for the inherent thermostability of the molecular components of thermophiles.

The major line of research has been based on the assumption that the essential cell components of thermophiles are more stable than those of mesophiles, either as an inherent property, or brought about through the influence of stabilising factors. Ljunger (1970, 1973), in support of the latter alternative, attributes the heat stability of thermophilic bacteria to the active transport of calcium ions into the cells. The conclusions of Ljunger probably represent an over-simplification of the mechanisms of thermophily. Accepting that calcium uptake of cells is essential for survival, this might merely indicate the stabilising factor requirements of one or more key metabolic enzymes. The involvement of metal ions in the stabilisation of many thermophilic hydrolases is well established (Amelunxen & Murdock, 1978a). Furthermore, it was proposed by Amelunxen & Lins (1968) that the maximum microbial growth temperature may be related to the thermostability of a few critical cellular components such as enzymes.

Alternatively, the requirement for calcium noted by Ljunger might indicate the mechanism by which the cell maintains a highly charged molecular environment. Amelunxen & Murdock (1978a) present evidence to show that glyceraldehyde-3-phosphate dehydrogenase , a key glycolytic enzyme in *B. coagulans*, is stabilised by high ionic concentrations. Furthermore, the intracellular charge is calculated by Damadian (1973) to be very high, (equivalent to about 1 mole kg^{-1}), a composite of electrolytes, protein, phospholipid, and nucleic acid charges. Stahl (1978) suggests that the divalent calcium ion may influence the molecular charge environment in *B. stearothermophilus*, in which an active transport system has been inferred.

In 1924, Heilbrunn reported that thermophilic microorganisms had lipids with higher melting points than mesophiles. It is now well established that the lipid composition of thermophiles responds to growth temperature (Welker, 1976; Oshima, 1978), and it is tempting to suggest that increased membrane stability (the result of a modified lipid composition) is a factor controlling the maximum growth temperature. However, in view of the incomplete understanding of the mechanisms of membrane stability, it is more realistic to assume that the modification of lipid composition is merely one of the many factors contributing to the stability of cellular systems in thermophiles.

Current evidence suggests that not one, but maybe all of the proposed stabilising factors may operate concurrently, in varying proportions to produce the observed degree of cellular survival. However, whatever mechanisms operate at the organism level, the basis of these mechanisms can only be elucidated by investigations at the molecular or atomic level. Thus, the interactions within and between molecules, particularly proteins, have developed as the primary loci of investigations in the past ten years. Those factors thought to stabilise thermostable proteins will now be discussed.

2-2 Transferable Protective Factors

In preliminary studies on cell-free extracts of thermophilic microorganisms, a variety of enzymes were shown to be significantly more thermostable than homologous mesophilic enzymes (see review by Amelunxen & Murdock, 1978a). Koffler (1957) and Koffler & Gale (1957) suggested that transferable protective factors within the cell might impart thermostability. Their experiments were based on the extent of coagulation after heat treatment as an index of thermostability, and the interpretation of this data is questionable (Amelunxen & Murdock, 1978a).

The present extent of knowledge of molecular thermophily precludes the involvement of transferable protective factors as a likely mechanism, for the following reasons (Amelunxen & Murdock, 1978a):

1. Lack of effect from mixture experiments of cell-free extracts;
2. Unchanged thermostability during purification to homogeneity; and
3. The known ability to account quantitatively for the molecular weight based on amino acid content, for homologous enzymes from mesophiles and thermophiles.

It is accepted that factors might exist which, by virtue of their low molecular weight and non-dissociable character, have not been detected.

Stabilising factors such as metal ions will be considered below in section 2-8. While metal ions could be classified as transferable protective factors, they are not of the type envisaged by Koffler.

2-3 Molecular size, flexibility, and allosterism

One of the first thermophilic enzymes isolated, α-amylase from *B. stearothermophilus* (Manning & Campbell, 1961; Manning *et al.*, 1961; Campbell & Manning, 1961; Campbell & Cleaveland, 1961) was shown to be a low molecular weight protein with a high proline content, implying an unusual tertiary structure. It was suggested that all thermostable proteins might show these characteristics. Many thermophilic enzymes have been characterised, and their molecular weights and structures are essentially the same as their mesophilic counterparts (Ljungdahl & Sherod, 1976).

During the earlier studies on thermophiles, it was suggested (Brock, 1967) that thermostable proteins would be relatively rigid and inflexible, having sacrificed efficiency and functional control for stability. Such structures would be incompatible with allosterism, with which conformational flexibility is a primary requirement.

However, there are now numerous examples of enzymes isolated from thermophiles and extreme thermophiles which are allosterically functional, in some cases in identical fashion to their mesophilic counterparts. Ljungdahl & Sherod (1976) divide thermophilic allosteric enzymes into two classes:

1. Those enzymes which demonstrate conformational flexibility only at high temperatures.
2. Enzymes subject to allosteric control at both high and low temperatures.

The first category appear to provide partial support for the assertions of Brock (1967), since proteins of this class apparently possess insufficient conformational entropy at lower temperatures to overcome the rigidity of the tertiary structure. Tsuboi *et al.* (1978) have demonstrated, by means of analysis of the kinetics of H/D exchange between protein and ^2H_2O, that polypeptide chain elongation factor (EF-Tu) from *Thermus thermophilus* remains rigid up to 50°C, whereas the equivalent protein from *E. coli* is 'flexible' at 38°C. Enzymes of this type may be responsible for the inability of thermophiles to grow at mesophilic temperatures.

2-4 Secondary Structure

The α-amylase from *B. stearothermophilus* has been shown to exist in a semi-denatured random coil structure. This discovery promoted the contention that thermophilic proteins might exhibit differences in secondary structure.

Stelwagen & Barnes (1976) were able to find no difference in the helical content of two mesophilic and two thermophilic enolases, although they established an inverse corelation between the amount of β-structure

and the thermal transition temperature. This result was confirmed by Singleton *et al.*, (1977) in calculations of helical and pleated-sheet content of proteins on the basis of amino acid compositions. They concluded that the consequence of a reduction in β-structure content would be a less rigid molecule. However, glutamine synthetase from *B. stearothermophilus* was shown to have 42% less α-helix and 21% more β-sheet than the same enzyme from *E. coli* (Hunt & Ginsburg, 1972). This result, derived from circular dichroism measurements, was not confirmed by Wedler & Hofman (1974) using the mathematical calculation of Chou & Fasman (1974).

Davidson & Fasman (1967) noted that β-structure formation in poly-lysine was facilitated at higher temperatures through the formation of hydrophobic bonding, and speculated that increased protein rigidity induced at high temperatures by this mechanism might contribute to activity loss in enzymes. Singleton *et al.* (1977) have concurred with this theory, in their contention that reduced β-sheet content in thermophilic proteins is an adaptation to maintain flexible, conformationally active structures.

Despite these assertions, a number of other investigations of homologous thermophilic and non-thermophilic proteins have detected no difference in helical or β-structure content (Yoshida *et al.*, 1975; Susuki & Imahori, 1974; Hibino *et al.*, 1974; Ogasahara *et al.*, 1970; Fontana *et al.*, 1976; Hasegawa *et al.*, 1976; and Wedler & Hofman, 1974).

A cautionary note on the use of amino acid composition data to predict secondary structure has been provided by Amelunxen & Murdock (1978b). For example, Singleton *et al.* (1977) calculated helical contents of 42% and 30% for thermophilic and non-thermophilic ferredoxins respectively. However, Devananthan *et al.* (1969) and Adman *et al.* (1973) have shown experimentally that Clostridial and *Micrococcus* ferredoxins have little defined secondary structure.

It has now been conclusively established that there is little or no gross difference between the secondary structure of thermophilic proteins and their mesophilic counterparts.

2-5 Aggregation

Wedler & Hofman (1974) have proposed that concentration-dependent aggregation of dodecamers of glutamine synthetase from *B. stearothermophilus* is a contributing factor in the thermostability of the enzyme. The association occurs without change in the enzymic activity. The driving force for the aggregation of glutamine synthetase is probably the association of apolar regions on the upper and lower faces of "the stacked hexagons of the dodecameric native enzymes". The stability of hydrophobic interactions increases up to approximately 70°C (Brandts, 1967), and further stabilisation is provided by removal of the apolar regions from solvent contact, and the increased entropy resulting from release of water of solvation.

However, little other evidence is available to suggest that aggregation is a significant mechanism of thermophily. The existance of *T. aquaticus* and *Thermus* X-1 enolases in octameric rather than dimeric form, as in mesophiles, is considered to be unrelated to their enhanced thermostability (Stelwagen *et al.*, 1973).

Aggregation observed in heated solutions of avidin (Donovan & Ross, 1973), enolases (Stelwagen *et al.*, 1973) and a variety of other proteins (Koffler, 1957) is presumed to be the product of heat induced protein melting and precipitation. This is in no way associated with enhancement of thermostability, since, as shown by Koffler & Gale (1957), proteins from thermophiles are more resistant to such aggregation than those from mesophiles.

2-6 Cysteine

There are a number of reported examples of thermophilic enzymes which possess fewer free sulphydryl groups than mesophilic analogues (Hocking & Harris, 1976; Hengartner *et al.*,1976; Sukuki & Imahori, 1974; Barnes & Stelwagen, 1973; Yoshida *et al.*, 1975; Cass & Stelwagen, 1975; and Saiki *et al.*, 1978). Data presented in the last reference shows that malate dehydrogenases from chicken heart mitochondria, chicken heart supernatant, *Neurospora crassa*, *E. coli*, *T. aquaticus*, and *T. flavus* possess 20, 11.4, 7.9, 8.3, 6.8, and 4.0 cysteine residues respectively, demonstrating an excellent inverse corelation between cysteine content and thermostability.

It has been suggested that a decrease in cysteine content in proteins from more thermophilic environments is a molecular adaptation to reduce the number of surface sulphydryl groups which are inevitably subject to oxidation (Hocking & Harris, 1976). However, adaptive pressure to delete internal -SH groups should be significantly lower on this basis, since the protected intramolecular regions are generally considered to be reductive. Furthermore, such selection should be less significant for intracellular proteins (such as malate dehydrogenase, above), the environment of which is also reductive. Amelunxen & Murdock (1978a) suggest that changes in cysteine content may be related to inactivation rather than denaturation.

It may be significant that thermostable extracellular proteases and amylases from a number of microorganisms contain no cysteine (e.g. Pangburn *et al.*, 1976; Barach & Adams, 1977; and Laxer *et al.*, 1976). The functional conditions of these enzymes would be strongly oxidising, a product of aerobism and high temperature. Exceptions in this group are the extracellular proteases of several thermophilic fungi (*Streptomyces*, Mizusawa & Yoshida, 1973; *Thermoactinomyces* and *Thermomonospora*,

Desai & Dhala, 1969; and *Micropolyspora*, Mizusawa & Yoshida, 1976). However, these enzymes contain only a single free -SH group in the active site. It is assumed that the hydrophobic and reductive nature of the active site 'cleft' protects this residue from oxidation.

2-7 Disulfide bonding

It was suggested by Anfinson (1972) that most extracellular proteins, which require greater stability to environmental change, contain disulfide bridges. However, contrary to this suggestion, many extracellular thermostable bacterial hydrolases lack cystine (Amelunxen & Murdock, 1978a). The important factor regulating the presence of S-S bonds may not be the requirement for stability, but conditions in which they are synthesised. The synthesis of proteins in thermophiles may be subject to strong selective pressure to delete cysteine, since this highly reactive residue is likely to be subject to a variety of undesirable side-reactions prior to disulfide formation, at thermophilic temperatures.

It is concluded that cystine, while being important in some proteins, is not an essential prerequisite for thermostability.

2-8 Non-protein stabilising factors

It has been found that not all thermophilic proteins are intrinsically thermostable at the optimum or maximum growth temperatures of the organisms from which they are isolated. Amelunxen & Murdock (1978a) list a selection of thermophilic enzymes with intrinsic and extrinsic stabilities. Of those proteins listed, nearly 30% are normally found with a stabilising cofactor. These are listed in Table 2-2 below.

It is seen from Table 2-2 that metal ions form the majority of stabilising cofactors, and yet the mechanisms involved are still poorly understood. In those examples studied, metal ion stabilisation appears

TABLE 2-2 Thermophilic enzymes and associated stabilisers

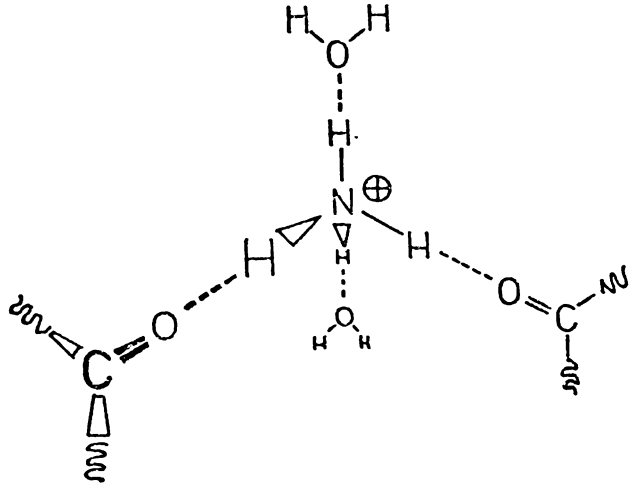
Enzyme	Source [1]	Thermostability (°C)		Reference
		Intrinsic	Stabilisers [2]	
Glucokinase	B.s.	65	0.2M NaCl	Hengartner & Zuber, 1973
Glucose-6-P isomerase	B.s.	55	substrate (<65)	Muramatsu & Nosoh, 1971
Enolase	B.s.	40	Mg^{2+} (60)	Boccu *et al.*, 1976
Enolase	T. X-1	74	Mg^{2+} (88)	Stelwagen & Barnes, 1976
Enolase	T.a.	88	Mg^{2+} (100)	Stelwagen & Barnes, 1976
NADP isocitrate dehydrogenase	B.s.	55	substrate (<70)	Pearse & Harris, 1973
Formyltetrahydrofolate synthetase	C.ta.	<60	NH_4^+, K^+ (60)	Ljungdahl *et al.*, 1970
Glutamine synthetase	B.s.	<60	cofactors + substrate (70)	Wedler & Hofman, 1974
α-amylase	B.s.	50	Ca^{2+} (<70)	Ogasahara *et al.*, 1970
α-amylase	B.cald.	45	Ca^{2+} (70)	Heinen & Lauwers, 1976
Thermolysin	B.th.	<50	Ca^{2+} (80)	Dahlquist *et al.*, 1976

1. B.s. - *B. stearothermophilus;* T. X-1 - *Thermus* X-1; T.a. - *T. aquaticus;* C.ta. - *Clostridium thermoaceticum;* B.cald. - *B. caldolyticus;* B.th. - *B. thermoproteolyticus*

2. Figures in brackets refer to thermostability in the presence of stabilisers.

to occur by intervention at acidic binding sites such as the carboxylate groups of aspartate and glutamate residues (Roche & Voorduow, 1978). The binding of calcium and zinc to thermolysin, which has been studied in detail, will be discussed in Part B of this chapter.

Hsui *et al.*, (1964) have proposed that metal ions might form intramolecular cross-links similar in function to disulfide bridges. It is assumed that other metal ions such as K^+ and Mg^{2+} (or even NH_4^+) could function in a similar manner, by acting as ionic intermediaries between charged side-groups of spatially proximal but sequentially distant residues in the peptide chain. The protons of the ammonium ion would provide, in addition, the facility for cross-linked H-bonding. e.g.



Of those enzymes stabilised by substrates or modifiers, glutamine synthetase is an interesting example (Wedler & Hofman, 1974). The mechanism of substrate thermostabilisation apparently operates by active site interaction, implying that the active site cleft is the primary locus of protein unfolding. In a number of other enzymes, the active site region is more strongly stabilised than the remainder of the polypeptide chain, and substantial unfolding can occur without influencing the active-site conformation and enzymatic activity. It is considered that substrate stabilisation in thermophilic enzymes may act as a further metabolic control, in addition to established mechanisms such as allosterism.

A number of other non-protein stabilising influences have been reported. *B. stearothermophilus* alkaline phosphatase is found to be loosely bound to the cytoplasmic membrane, in which state thermostability is significantly greater than when dissociated (Amelunxen & Murdock, 1978b). Other examples of enzymes stabilised by membrane association have been reported (Bubela & Holdsworth, 1966a). Research in this laboratory has suggested that the membrane-bound NADH dehydrogenase from *Thermus* T-351 is more stable than the soluble form of the same enzyme (Walsh & Daniel, unpublished results).

It has been observed that proteins isolated from 37°C-grown *B. coagulans* cells contain three times as much carbohydrate as those extracted from cells grown at 55°C (Amelunxen & Murdock, 1978a). Furthermore, there is a 20°C difference in the melting temperatures of these proteins. Whether these two factors are casually related has yet to be established.

2-9 The influence of Water on intramolecular interactions

It is known that proteins and other macromolecules orient watermolecules to a considerable extent, and it therefore follows that cell water must possess a high degree of organisation, (Ling, 1967). With increasing temperature, the rotational freedom of ordered water molecules increases with a very large increase in molecular entropy, as the hydration shells surrounding proteins and other molecules are reduced. However, at the point of protein unfolding, total water entropy will decrease as intramolecular residues are exposed to the solvent and acquire solvation shells. Thus the energetics of denaturation are integrally involved with the thermodynamic changes in the solvent, water. Changes in protein structure and solvent interactions contributing to the difference in free energy in the native and denatured states are outlined in Table 2-3 (from Brandts, 1967).

TABLE 2-3 Thermodynamic changes in the denaturation of proteins

	Native		Denatured
1.	Rigid structure with little rotational freedom	⟸⟹	Flexible structure
2.	Peptide-peptide hydrogen bonds	⟸⟹	Peptide-solvent hydrogen bonds
3.	Non-polar side-chains in protein interior	⟸⟹	Non-polar side-chains exposed to solvent
4.	High charge density due to compactness	⟸⟹	Low charge density in extended conformation
5.	Ionisable side-chains with local interactions (e.g. salt bridging)	⟸⟹	Ionisable side-chains fully solvated.

Changes in temperature will affect the major intramolecular secondary interactions (salt-bridging, hydrogen-bonding, and hydrophobic bonding) both directly and through the influences of the solvent. With increasing temperature, ionic interactions will be significantly destabilised via shifts in ionisation, an effect compensated by increases in the dielectric properties of water. Brandts (1967) has demonstrated in a model calculation for chymotrypsin that the intramolecular stabilisation resulting from ionic bonding would be unchanged between 20°C and 70°C.

The influences of both heat and solvent on hydrophobic interactions (also known as apolar or Van der Waals forces) are particularly important in the maintainance of protein integrity. The associations of non-polar amino acid side-groups and solvent molecules are known to be complex. Localisation of large amounts of water around side-groups of denatured protein at low temperature is associated with a large negative entropy. With increasing temperature, the 'clathrate' solvent structures around such groups are disrupted, resulting in a substantial increase in entropy

(Brandts, 1967). The overall effect of temperature on hydrogen bonding in chymotrypsin has been calculated by Brandts, (1964). It was concluded that the stabilising effects of hydrophobic bonds should increase up to approximately 72°C. The importance of apolar interactions in thermophily will be discussed further in section 2-10.

It is concluded that the integral involvement of water in inter- and intramolecular interactions makes it difficult to separate molecular and solvent influences. However, any change in protein structure which alters the distribution of, or capacity for bound water molecules (e.g. changes in the exterior charge density, or the number of external apolar residues) will effect the thermodynamics of protein denaturation, and hence the stability of the native molecule.

2-10 Hydrophobicity

Since hydrophobic bonds are known to become stronger with increases in temperature up to about 70°C (Brandts, 1967), considerable research has centred on the possibility that greater numbers of hydrophobic residues or an increase in the average hydrophobicity may be found in thermophilic proteins, when compared to similar proteins from mesophiles.

Hydrophobicity may be calculated by three different methods: average hydrophobicity ($H\phi_{av.}$) (Bigelow, 1967); the frequency of non-polar side chains (NPS) (Waugh, 1954); and the ratio (p) of the volume occupied by non-polar residues (Fisher, 1964). It was noted by Bigelow (1967) that $H\phi_{av.}$ correlated well with NPS but not with p, a difference he attributed to the choice of which side-chains were apolar, and which not.

Bull & Breese (1973) found a good correlation between hydrophobicity and experimentally determined melting temperatures of 14 non-thermophilic proteins. However, when the hydrophobicity parameter was applied to two thermophilic and two mesophilic enolases, the correlation between predicted

and actual stabilities was reversed. Other extensive analyses of thermostability and hydrophobicity (Singleton & Amelunxen, 1973; Singleton, 1976; Singleton *et al.*, 1977) have failed to provide a positive correlation.

Failure to show a general relationship between hydrophobicity and thermostability does not exclude the possibility that minor increases in the hydrophobic nature of selected regions within a protein may cause a significant increase in thermostability (Ljungdahl *et al.*, 1970). In a comparison of *Drosophila melanogaster* alcohol dehydrogenase allozymes of different thermostability, Sampsell & Milkman (1978) calculated that the difference in free energy of activation between each of the four allozymes could be attributed to the addition of one extra hydrophobic bond.

It is suggested that if increased hydrophobic bonding is a general mechanism for the enhancement of thermostability, then it will be the product of the substitution of relatively few apolar residues, each acting in a highly specific manner.

2-11 Hydrogen bonding

Hydrogen bonds are either unaffected or marginally destabilised by increases in temperature, but are substantially weakened by solvent access (Brandts, 1967). However, many of the hydrogen bonds involved in the tertiary structure of proteins are submerged within the molecular interior, and hence shielded from solvent interference (Amelunxen & Murdock, 1978b), where they are able to provide a positive contribution to thermostability.

Attempts to establish a clear corelation between increases in the number of H-bond-forming amino acids and enhanced thermostability have generally been unsuccessful. Barnes & Stelwagen (1973) detected a

positive relationship between H-bond forming amino acid content and stability in enolases, but their choice of amino acids is disputed by Amelunxen & Mardock (1978a). Cass & Stelwagen (1975), Hagesawa *et al.* (1976), and Suzuki & Imahori (1974) were unable to detect any significant differences in H-bond formation between enzymes from mesophilic and thermophilic sources.

The method used for the determination of H-bonding potential, like that for hydrophobicity, gives an average estimate. It is possible that single amino acid substitutions, providing limited numbers of additional hydrogen bonds or modifying the environment in the vicinity of critical H-bonds, might provide sufficient stabilisation to account for the difference in thermostability between mesophilic and thermophilic proteins. It is noteworthy that substitutions of the latter type would probably not be recognised, as they would not necessarily be H-bond forming amino acids.

Hocking & Harris (1976) calculated that a 30-fold difference in the thermal stabilities of triose phosphate isomerases from rabbit muscle and *B. stearothermophilus* at 60°C was the result of a difference in stabilisation energies of only 0.2 kJ $mole^{-1}$. Hydrogen bond energies range from 4 to 42 kJ $mole^{-1}$ (McKay & McKay, 1969) whereas apolar interactions are approximately 4 kJ $mole^{-1}$ (Sampsell & Milkman, 1978).

It is clear that in energetic terms, the difference between mesophilic and thermophilic proteins can be accounted for by the addition of very few additional intramolecular bonds, as has been demonstrated by Grütter *et al.*, (1979).

2-12 Salt Bridging

Salt-bridging, the non-mediated interaction of oppositely charged groups, is considered to be one of the mechanisms responsible for

enhanced thermostability in a limited number of proteins. Ionic associations are known to be destabilised by increases in both temperature (Brandts, 1967) and salt concentration. Therefore, salt-linkages on the surface of intracellular proteins (where ionic concentrations of about 1 mole kg^{-1} may be experienced (Damadian, 1973)), should be considerably weakened (Amelunxen & Murdock, 1978a). However, internal salt-bridges, especially those protected by a hydrophobic environment, could play a significant role in the stability of thermophilic proteins. For example, the energetic difference between ferrodoxins from *C. tartarivorum* and *C. thermosaccharolyticum* is calculated to be less than 5 kJ $mole^{-1}$ (Perutz & Raidt, 1975). The molecular changes responsible for the enhanced thermostability were thought to be the replacement of two hydrogen bonds (stabilisation energy = 20 kJ $mole^{-1}$) by stronger salt-linkages. This conclusion has been subject to some criticism (Amelunxen & Murdock, 1978a) on the basis that the salt-bridges are almost certainly on the surface of the globular ferredoxin structure.

Chell & Sundaram (1978) and Sundaram *et al.*, (1976) conclude that the enhanced thermolability of proteins with increasing concentrations of KCl is an indication of the presence of external salt-bridging. This conclusion is disputed by Amelunxen & Murdock (1978b), who suggest that the data implies preliminary cleavage of some other intramolecular bond, followed by the dissociation of *internal* salt-bridges.

The presence of intramolecular salt-bridging has been demonstrated in *B. stearothermophilus* glucose phosphate dehydrogenase (Biesecker *et al.*, 1977). In comparison with the lobster muscle enzyme, the bacterial tetramer derives added stabilisation from three additional salt-bridges per sub-unit. Positioned between sub-units, these linkages are shielded from solvent interaction.

It is evident from the inconsistencies in the literature that the distribution and mechanisms of ionic interactions are yet not well understood.

2-13 Acidic residues

An increase in the ratios of acidic to basic residues, consistent with a decrease in isoelectric point, has been reported by Shing *et al.*, (1975) for triosephosphate isomerases from a thermophile, a mesophile, and a psychrophile. That this is more than an isolated example is shown in data presented by Singleton (1976), Singleton *et al.*, (1969), Tanaka *et al.*, (1971), and reviewed by Amelunxen & Murdock (1978a). Unfortunately, owing to incomplete data in most of these examples, glutamine and glutamate (and asparagine and aspartate) have been grouped as Glx (and Asx). The instability of glutamine at elevated temperatures (Ratcliffe *et al.*, 1978) may have acted as a strong selective pressure for the replacement of glutamine by glutamic acid or asparagine. Thus it was considered to be an interesting exercise to determine whether the ratios, glu/gln and gln/asn would demonstrate any corelation with the T_m values of proteins from different sources (Table 2-4). It is concluded that insufficient data is available to permit a definite statement on the correlation between glutamine and glutamic acid residues in mesophiles and thermophiles. However, in the data available, the Glu/Gln ratio is generally higher in the thermophiles than in the mesophiles. There is apparently no correlation between the asparagine/glutamine ratios and the temperature preference of the organism.

Whether or not an increase in acidic residues results from the adaptive pressure mentioned above, the carboxyl group is well suited for the formation of intramolecular interactions of the type which might enhance thermostability, for example: (from Amelunxen & Murdock, 1978a)

a. The formation of two H-bonds with another carboxyl group when both are protonated.

b. The formation of an ion dipole interaction with the phenolic hydroxyl of tyrosine.

TABLE 2-4 Quantitative comparison of selected amino acid residues in proteins

Protein [1]	Source [2]	Class [3]	T_m	Glu/Gln	Asn/Gln	
Ferrodoxins						
	B. stearothermophilus [4]	T		5.0	1.0	Hase *et al.* (1976)
	C. tartivorum	T		2.5	0/2	Perutz & Raidt (1975)
	C. thermosaccharolyticum	T		7/0	0/0	"
	H. halobium	H		5.67	3.2	"
	M. aerogenes	M		1.0	1.5	"
	C. pasteurianum	M		1.0	1.5	"
	C. acidiurici	M		1.0	0.5	"
	P. elsdenii	M		7/0	0/0	"
	D. gigas	M		0.5	0.83	Hase *et al.* (1976)
	C. limicula 1	M		2.5	1.0	"
	C. limicula 11	M		8/0	1.0	"
	C. vinosum [5]	M		1.5	0.75	"
	Spinach [6]	M		3/0	1/0	Dayhoff (1976)
Proteases						
Thermolysin	*B. thermoproteolyticus* [7]	T	84	1.86	1.60	Pangburn *et al.* (1976)
Subtilisin BPN'	*B. subtilis*	M	65	0.36	1.55	Markland & Smith (1971)
Subtilisin Carlsberg	*B. subtilis*	M	65	0.71	2.71	Markland & Smith (1971)
Carboxypeptidase A	Porcine	M	63	0.71	1.60	Bradshaw *et al.* (1969)
Neutral protease	*B. subtilis* [8]	M	59	0.64	2.00	Pangburn *et al* (1976)
Chymotrypsin	Bovine	M	56	0.50	1.20	Blow (1971)
Papain	*Carica papaya*	M		0.67	1.08	Glazer & Smith (1971)

1. GPDH - glyceraldehyde-3-phosphate dehydrogenase.
2. *M. aerogenes - Micrococcus aerogenes; C. pasteurianum - Clostridium pasteurianum; C. acidiurici - Clostridium acidiurici; P. elsdenii - Peptostreptococcus elsdenii; C. tartarivorum - Clostridium tartarivorum; C. thermosaccharolyticum - Clostridium thermosaccharolyticum; H. halobium - Halobacterium halobium; D. gigas - Desulfovibrio gigas; C. limicula - Chlorobium limicula; C. vinosum - Chromatium vinosum.*
3. M - mesophile; T - thermophile; H - halophile.
4. 67 amino acid residues from N-terminus.
5. 63 amino acids from N-terminus.
6. Amino acid residues from postion 35 to 100.
7. 180 of the 316 residues.
8. 180 of the 326 residues.

c. Ionic associations with metal ions (Ca^{2+}, Zn^{2+}, Mg^{2+}, etc.)

d. Salt-bridging

The acidity constants (pK_a-COOH) of carboxyl groups are not significantly modified by temperature changes, but are increased markedly by decreases in environmental polarity, thus facilitating the internal location of carboxyl groups for participation in interactions such as c. or d. above. However, it is noted by Amelunxen & Murdock (1978b) that the majority of salt-bridges are formed by arginine, not by glutamate or aspartate, a fact which is supported by a documented tendency for some thermophilic proteins to have unusually high levels of arginine (Singleton *et al.*, 1977; Hocking & Harris, 1976). Both aspartate and glutamate do play a vital role in the coordination of metal ions in thermolysin (Roche & Voorduow, 1978; Dahlquist *et al.*, 1976).

2-14 Conclusions

The most striking conclusion derived from recent literature on thermophily is that thermophilic proteins are, in the majority of cases, very similar to their mesophilic analogues. No gross structural changes, marked sequence alterations, or strong trends for the presence or absence of a particular type of amino acid have yet been documented, or are likely to be. It is concluded by almost all workers in the field of molecular thermophily that the mechanisms involved in the enhancement of thermal stability of macromolecules are both subtle and various.

When the differences between mesophilic and thermophilic proteins are translated into energetic terms, this conclusion becomes obvious. Relatively small changes in the free energy of stabilisation of a molecule can be responsible for substantial differences in thermostability (e.g. Wilkinson & Knowles, 1974). Often, the energy separation between mesophilic and thermophilic proteins is of the same order of magnitude

as the stabilisation energies of the common intramolecular bonds. Thus it can be deduced, as found experimentally, that substitution of a few residues to provide a small number of additional linkages should be sufficient to account for the phenomonen of molecular thermophily.

The substitution of selected residues within proteins has escaped notice until recently for the following reasons:

1. Changes in small numbers of amino acids are usually not apparent in broad comparative analyses of amino acid composition.
2. The variety of potentially stabilising interactions (H-bonds, apolar bonds, salt-bridges, etc.) results in little consistency in minor sequence changes in dissimilar proteins.
3. Phylogenetic changes in protein sequence often confuse the analysis of thermally induced alterations.
4. There has been a paucity of protein sequence and X-ray crystallography data from which to derive accurate molecular structures.

It is now known that the replacement of a small number of residues in critical regions of a polypeptide chain can enhance or decrease thermostability, as shown dramatically by the work of Grütter *et al.* (1979) and Langridge (1968). A study of point mutations of the β-galactosidase molecule demonstrated clearly that the substitution of a serine in certain regions of the sequence could reduce thermostability, whereas insertion into other regions enhanced thermostability or produced no change. Grütter *et al.* (1979) showed that a decrease of approximately 14°C in the melting temperature of Bacteriophage T4 lysozyme resulted from the substitution of a single arginine residue by histidine. The important conclusions from this work can be summarised as:

1. Single amino acid substitutions can effect the thermostability (in either a positive or negative manner) without grossly affecting the tertiary structure.

2. Certain regions within a protein are critical for the maintenance of thermal stability.

Loss of thermal stability resulting from a single amino acid replacement could result from either the deletion of a residue involved in a critical intramolecular bond, or merely the modification of the environment of such a linkage. For example, an increase in the polarity of the local environment about a salt-bridge can reduce the pK_a of the carboxyl groups, thus weakening the ionic association.

Note added in Proof

In a recent publication, Argos *et al.* (1979) support the general conclusion that increases in the thermal stability of proteins can be achieved by minor amino acid changes, without significant alteration of the protein backbone conformation. The authors conclude that such changes increase internal and decrease external hydrophobicity, and favour helix stabilising residues in helices.

CHAPTER 2. PART B.

STABILISING INFLUENCES IN THERMOPHILIC PROTEOLYTIC ENZYMES

Although a considerable number of thermophilic proteases have been isolated and characterised, only thermolysin, the extracellular metalloendopeptidase isolated from *B. thermoproteolyticus* (Endo, 1962), has been studied in detail (see reviews by Amelunxen & Murdock (1978a, 1978b), Weaver *et al.*, 1976, and Roche & Voorduow, 1978). With the exception of metal ion binding sites, thermolysin has been shown to contain no unusual structural characteristic which could be responsible for it's thermal stability (Titani *et al.*, 1972). Some intramolecular stabilisation is known to result from the presence of 19 tyrosine residues, the hydroxyl moieties and aromatic regions of which interact in hydrogen and hydrophobic bonding respectively (Ohta *et al.*, 1966).

The electron density map of thermolysin indicates that the molecule consists of two hemispherical lobes separated by a deep cleft (the active site) within which a zinc atom is situated (Matthews *et al.*, 1972, 1974). The zinc ion is chelated by three residues, His-142, His-146, and Glu-166, a structure which is homologous with the active site of bovine carboxypeptidase A (Lipscombe *et al.*, 1969).

The major contribution to thermal stabilisation in thermolysin is derived from bound calcium ions, clearly shown by the response of the enzyme to EDTA (Weaver *et al.*, 1976). More detailed analysis has demonstrated that two of the four bound calcium ions are responsible for a large proportion of the protein stabilisation (Dahlquist *et al.*, 1976). These ions are located in exposed sites on the polypeptide surface, one interacting with asp-57, asp-59, and a peptide carbonyl group, while the ligand of the second site include asp-200, a threonyl hydroxyl, and two peptide carbonyl groups (Matthews *et al.*, 1974). These interactions

are essentially ionic and are presumed to be vital in the prevention of modification of the tertiary structure. Voorduow & Roche (1975b) have speculated that Ca(3), bound to asp-57 and 59, is the key to the unfolding of thermolysin. While both Ca(3) and Ca(4) are evidently situated in critical loci, Ca(3) is nearer to the N-terminus. In the case of Staphylococcal nuclease, the polypeptide chain unfolds from the amino terminus (Jardetzky *et al.*, 1971) and by analogy, Ca(3) of thermolysin could be the starting point for denaturation (Voorduow & Roche, 1975b).

Calciums (1) and (2) are situated in a double site at the centre of five acidic residues and act in a cooperative manner (Voorduow & Roche, 1974b). Since the double binding site is situated rather close to the active site cleft,it has been suggested that the two calciums may be involved in the stability of the molecule by linking the two lobes (Colman *et al.*, 1972). However, terbium, which binds strongly to the double calcium site, shows no stabilising effect (Dahlquist *et al.*, 1976).

Voorduow *et al.*, (1976a) have calculated intrinsic and ion stabilisation contributions to the total free energy of activation of five microbial proteases containing calcium ions (Table 2-5).

TABLE 2-5 Intrinsic (ΔF), total (ΔF_T), and calcium contributed (ΔF_{Ca}) free energies of activation for microbial proteases [a] (from Voorduow *et al.*, 1976).

Enzyme	ΔF	ΔF_T	ΔF_{Ca}	Number of Ca ions
Subtilisin Carlsberg	22.5 ± 5.5	25.5 ± 1.0	3.0	1
Subtilisin BPN'	22.4 ± 0.3	24.6 ± 0.2	2.2	1
Thermomycolin	24.1 ± 0.5	26.7 ± 0.1	2.6	1
Thermolysin	20.5 ± 0.2	28.6 ± 0.4	8.1	4
B. subtilis neutral protease A	22.0 ± 0.4	22.9 ± 3.0	0.9	>2

a. calculated at 70°C; units are kcal $mole^{-1}$.

This data indicates that while thermolysin has the highest total free energy (i.e. the highest thermostability), it has a lower intrinsic thermostability than even a mesophilic protease such as Neutral protease A. The intrinsic contribution refers to intramolecular forces such as H-bonding, hydrophobic bonding, etc. While it is known that tyrosines are important in tertiary structure stabilisation in thermolysin, factors responsible for the ΔF contributions in the other proteases listed are still obscure.

Voorduow *et al.*, (1976) have proposed a definition of molecular thermostability, on the basis that previous classifications have been purely arbitrary. They suggest (arbitrarily) that "thermostable enzymes are those which have total kinetic thermal stabilities of (ΔF_T) >25.0 kcal mole^{-1} at 70^{o}C under conditions (pH, pCa^{2+}, I, etc.) which result in maximisation".

In an independent calculation of free energies of activation, Tajima *et al.*, (1976) derive data for Thermolysin and *B. subtilis* neutral protease (Table 2-6) which is similar to that quoted above.

TABLE 2-6 Free energy of activation data for two proteases (from Tajima *et al.*, 1976)

Enzyme	ΔG [a]	ΔG_{Ca} contribution
Native thermolysin	27	7
0.3 g mole Ca^{2+} apo-enzyme [b]	20	
Native neutral protease	22	1
1.4 g mole Ca^{2+} apo-enzyme [b]	21	

a. at 80^{o}C; units are kcal mole^{-1}

b. Apo-enzymes completely metal-ion-free were not obtained by the gel filtration method used.

However, anomalous results are obtained by Tajima *et al.*, (1976) in their calculation of the calcium-binding free energies for the two proteases in Table 2-6. The total free energy for *B. subtilis* neutral protease is calculated on the basis of four calcium ions (Table 2-7).

TABLE 2-7 Free energy changes for the successive binding of calcium ions (from Tajima *et al.*, (1976)) [a]

Calcium ions	Thermolysin ΔG_i (kcal mole^{-1})	*B. subtilis* neutral protease ΔG_i (kcal mole^{-1})
(1)	6.3	7.1
(2)	5.7	6.4
(3)	5.3	5.8
(4)	4.7	4.1
$\Delta G_i T$	22.0	23.4

a. at 6°C.

On the basis of these calculations, the calcium contribution in neutral protease is greater than that in thermolysin, in contradiction to the conclusions of Voorduow *et al.*, (1976). However, sequence data for *B. subtilis* neutral protease indicates that the Ca (4) site does not exist (Pangburn *et al.*, (1976)). Thus ΔG_i total for neutral protease should lie between 16 and 19 kcal mole^{-1}, in which case thermolysin has the greater calcium stability contribution.

A feature of some extracellular proteases is the absence of cystine (Pangburn *et al.*, 1976; Mizusawa & Yoshida, 1973). However, this is noted in both thermophilic and mesophilic enzymes. It is concluded that, rather than being a positive influence on stability, the absence of

cystine prevents potential destabilisation. Thermomycolin, the extracellular serine protease from *Malbranchea pulchella*, has been found to contain a single disulfide bond (Gaucher & Stevenson, 1976) proving that such bonds are not completely detrimental to the synthesis and activity of all extracellular proteases.

The alkaline protease from *Streptomyces rectus* contains a single cysteine residue buried in a hydrophobic region at the base of the active site (Mizusawa & Yoshida, 1976). It is considered that a hydrogen bond from this residue is implicated in the thermostabilisation of the molecule.

CHAPTER 3

THE GENUS *THERMUS*

3-1 Classification, morphology, and species diversity

Since the isolation of *T. aquaticus* in 1969 (Brock & Freeze, 1969), a number of other Gram-negative, non-sporulating, thermophilic aerobes, all subsequently assigned to the same genus, have been isolated (Table 3-1).

TABLE 3-1 Species diversity in the genus *Thermus*

Organism	Reference	Growth temperatures [a]		
		T_{min}	T_{opt}	T_{max}
T. flavus	Rozanova & Khudyakova, 1974	-	70 - 75	80
T. thermophilus HB 8	Oshima & Imahori, 1971, 1974	45	65 - 72	85
T. ruber	Loginova & Egarova, 1975	-	60	80
Thermus X-1	Ramaley & Hixson, 1970	40	69 - 71	80
Thermus strains NH & DI	Pask-Hughes & Williams, 1975	45	70	80
16 strains resembling *Thermus*	Brock & Boylen, 1973	35-55	65 - 70	75 - 80
Icelandic Gram-negative strain	Williams & Pask-Hughes, 1973	45	70	80
T. aquaticus	Brock & Freeze, 1969	40	70	79

a. For laboratory grown cultures: data quoted in Brock (1978) and Williams (1975).

T. thermophilus was initially assigned a new genus title, *Flavobacterium thermophilum* HB8, but was reclassified after a closer morphological study.

The Thermus-like organisms listed above have been isolated from a variety of locations including thermal pools in Iceland, Japan, U.S.A., and New Zealand (Brock, 1978), and hot-water tanks in both U.S.A. (Brock & Boylen, 1973) and Britain (Pask-Hughes & Williams, 1975). The environmental characteristics favouring *Thermus* growth include temperatures of 70°C to 100°C and a neutral or alkaline pH. That growth actually occurs at 95°C and above has been conclusively demonstrated by Bott & Brock, (1969).

T. aquaticus and the other species and strains possess many similar characteristics. They are all aerobic, heterotrophic, gram-negative, non-motile, non-sporulating, rod-shaped organisms with a high sensitivity to penicillin (Brock, 1978). Initial isolates of *Thermus* will often possess a filamentous morphology, which shortens to rods with changes in temperature or with culture age or sub-culturing. Cells are generally yellow-pigmented (Williams, 1975) although strain X-1 is reported to be non-pigmented (Ramaley & Hixson, 1970). A recent paper reports that an orange-pigmented strain of the normally red *T. ruber* has been isolated (Loginova *et al.*, 1978).

Most *Thermus* isolates demonstrate an unusual morphological feature in the formation of 'rotund bodies' (Brock & Freeze, 1969). These are groups of cells surrounded by an independent membrane, the latter presumed to be a product of the association of the outer cell envelope layer from each cell.

The G/C ratios of *T. aquaticus* (4 strains), *Thermus* X-1, *T. thermophilus*, and strains NH and DI are quoted by Williams, (1975). These range from 61.5% to 68.4%, suggesting a marked similarity between these species. A recent comparative analysis of seven *Thermus* isolates by Degryse *et al.* (1978) has shown a high degree of homology. The authors

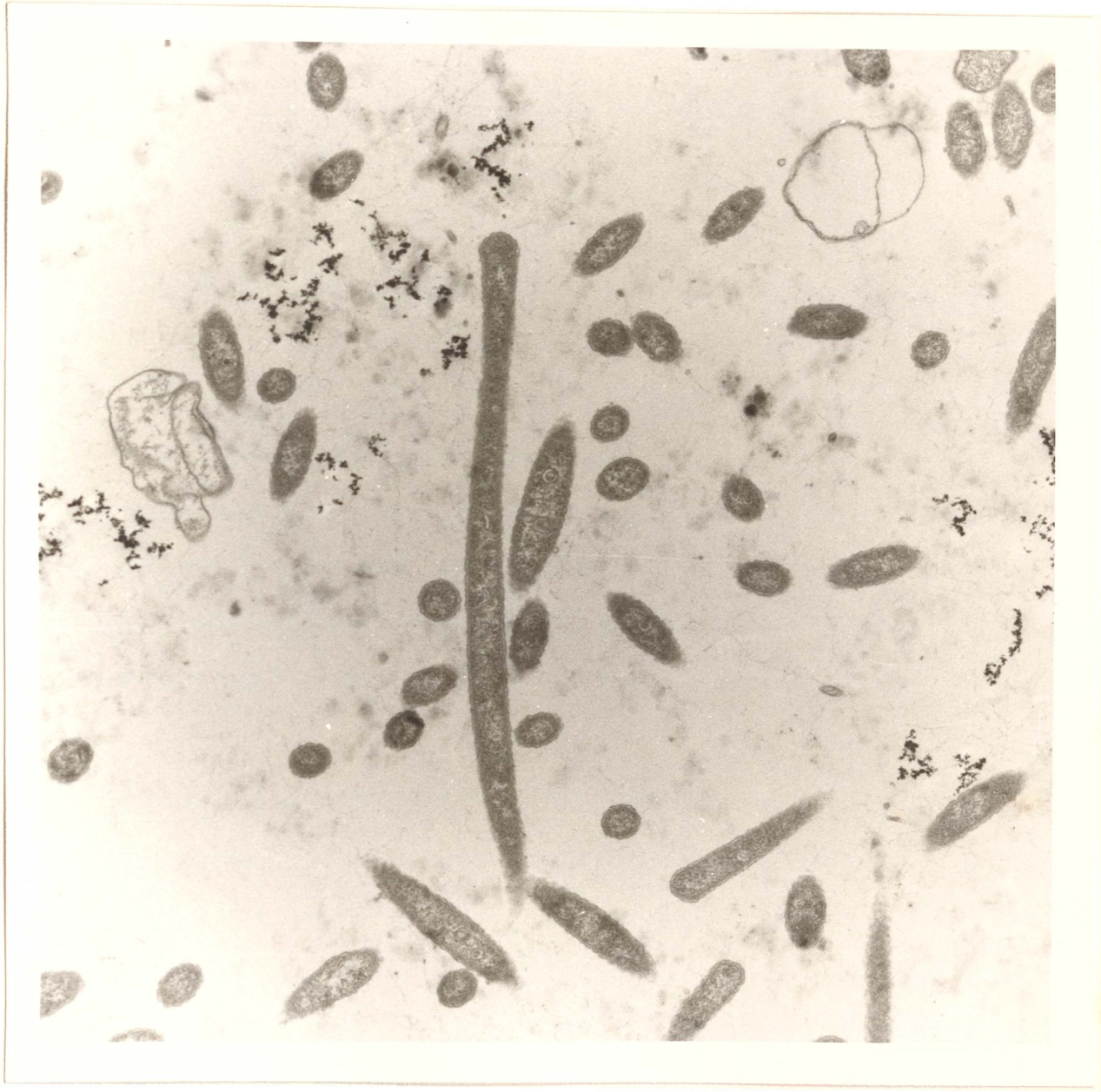


Plate 1. An electron micrograph showing longitudinal and transverse sections of *Thermus* T-351 cells. (Photograph courtesy of Dr H. Morgan.)

concluded that the organisms studied all belonged to the same species, *T. aquaticus*. It is interesting to note that Degryse *et al.* (1978) reported that all organisms showed gelatinase but no esterase activity.

The organism (Plate 1.) on which this research is based was isolated from the Whakarewarewa Thermal Region, Rotorua, New Zealand, by Dr H.W. Morgan, and has been designated *Thermus* T-351 pending a determination of G-C ratios. It has a growth maximum of 79°C to 80°C and is similar in characteristics to *T. aquaticus*. Pigmentation is variable, ranging from fawn to orange. A study of the electron transport system (Hickey & Daniel, 1979) has shown some interesting features, including an unusual a-type cytochrome which has not been observed in *Thermus aquaticus* (McFeters & Ulrich (1972)). Growth characteristics of *Thermus* T-351 have been described by Hickey & Daniel (1979).

3-2 Enzymes isolated from Thermus-like organisms

A wide range of enzymes has been isolated from the various strains and species of *Thermus* (Table 3-2). Almost without exception, these enzymes demonstrate a greater degree of thermostability than homologues isolated from mesophilic sources. In many cases, they are the most stable examples yet isolated.

Although extracellular gelatinase activity has been reported in a number of strains of *Thermus* (Degryse *et al.*, 1978), no isolation and characterisation studies have hitherto been carried out on *Thermus* extracellular proteases.

TABLE 3-2 Some enzymes isolated from Thermus-like organisms.

Enzyme	T_{opt} (°C)	T_m	Source	Reference
Acetohydroxyacid synthetase	75		*Thermus aquaticus*	Chin & Trela (1973)
Aldolase	95		*T. aquaticus*	Freeze & Brock (1970)
w-Amidase	80		*T. aquaticus*	Fernald & Ramaley (1972)
D-Asparaginase	75		*Thermus* T-351	Guy & Daniel (unpubl. results)
L.Asparaginase	75		*Thermus* T-351	Guy & Daniel (unpubl. results)
Aspartokinase			*Thermus* species	Saiki & Arima (1970)
ATPase		75	*Thermus*-like PS3	Yoshida *et al.* (1975)
Citrate synthetase			*T. aquaticus*	Weitzman (1978)
DNA polymerase		80	*T. aquaticus*	Chien *et al.* (1976)
Elongation factors		80	*Thermus thermophilus*	Arai *et al.* (1978)
Endonuclease			*T. thermophilus*	Sato & Shinomiya (1978)
Enolase	90	100	*T. aquaticus*	Stelwagen & Barnes (1976)
Enolase	70	88	*Thermus* X-1	Stelwagen & Barnes (1976)
Fructose 1, 6-bisphosphatase			*T. thermophilus*	Yoshida & Oshima (1971)
Fumarase		83	*T. aquaticus*	Cook & Ramaley (1976)
Fumarase	83		*Thermus* X-1	Cook & Ramaley (1976)
β-Galactosidase	80		*T. aquaticus*	Ulrich *et al.* (1972)
Glyceraldehyde-3-phosphate dehydrogenase		100	*T. aquaticus*	Hocking & Harris (1973)
Glyceraldehyde-3-phosphate dehydrogenase		90	*T. thermophilus*	Fujita *et al.* (1976)
Homoserine dehydrogenase			*Thermus flavus*	Saiki *et al.* (1973)
Isoleucyl t-RNA synthetase			*T. thermophilus*	Wakagi *et al.* (1975)
Lactate dehydrogenase			*Thermus* species	Weerkamp *et al.* (1971)
Malate dehydrogenase	70	95	*T. aquaticus*	Biffen & Williams (1976)
Malate dehydrogenase			*T. flavus*	Saiki *et al.* (1978)
$NADP^+$ isocitrate dehydrogenase			*T. aquaticus*	Ramaley & Hudock (1973)
$NADP^+$ isocitrate dehydrogenase		83	*T. flavus*	Saiki *et al.* (1978)
Alkaline phosphatase	75 - 80	78	*T. aquaticus*	Yeh & Trela (1976)
Phosphoenol pyruvate carboxylase			*T. aquaticus*	Bridger & Sumdaram (1976)
Phosphofructokinase	>80		*T. aquaticus*	Hengartner *et al.* (1976)
Phosphofructokinase	85		*T. thermophilus*	Yoshida (1972)
Phosphofructokinase	80		*Thermus* X-1	Cass & Stellwagen (1975)
Phosphoglucomutase			*T. thermophilus*	Yoshizaki *et al.* (1971)
Phosphoglycerate kinase			*T. thermophilus*	Nojima *et al.* (1978)
Ribonucleotide reductase	70	75	*Thermus* X-1	Sando & Hogenkamp (1973)
DNA-dependent RNA polymerase			*T. aquaticus*	Air & Harris (1974)
DNA-dependent RNA polymerase			*T. thermophilus*	Date *et al.* (1975)
Superoxide dismutase			*T. thermophilus*	Sato & Nakazawa (1978)
Threonine deaminase			*Thermus* X-1	Higa & Ramaley (1973)

CHAPTER 4

METHODS and MATERIALS

4-1 Growth and Maintenance of *Thermus* T-351 Cultures

In 1975, an organism with Thermus-like morphology and properties was isolated from the Whakarewarewa thermal region, Rotorua, New Zealand by Dr H. Morgan[#]. The organism has been designated *Thermus* T-351, pending exact classification. The properties of this organism are discussed briefly in Hickey & Daniel (1979).

Pure cultures were stored either freeze-dried and sealed in evacuated ampoules, or at -18^{o}C as a frozen suspension of cells in 10% maltose. To obtain an inoculum for liquid culture, the contents of an ampoule or maltose suspension were transferred aseptically into a 500 ml conical flask containing 200 ml of standard growth medium (composition given in Table 4-1).

Small-scale growth of *Thermus* was carried out in 2 litre conical flasks containing 500 ml of medium. These were incubated in a Gallenkamp orbital incubator set at 75^{o}C and 200 revolutions per minute, after addition of a 50 ml inoculum.

Medium-scale growth of *Thermus* T-351 was carried out in Quickfit^{R} 20 litre glass bottles up to three being used simultaneously. 18 litre batches of standard peptone media (0.3%) were prepared in the bottles and autoclaved for four hours. Aeroators, fermentor caps, and other attachements were autoclaved separately. The complete apparatus was assembled (see Plate 2.) once the fermentors were positioned in the water-bath, a metal-lined, 2.25 cubic meter "hot-pool", heated to

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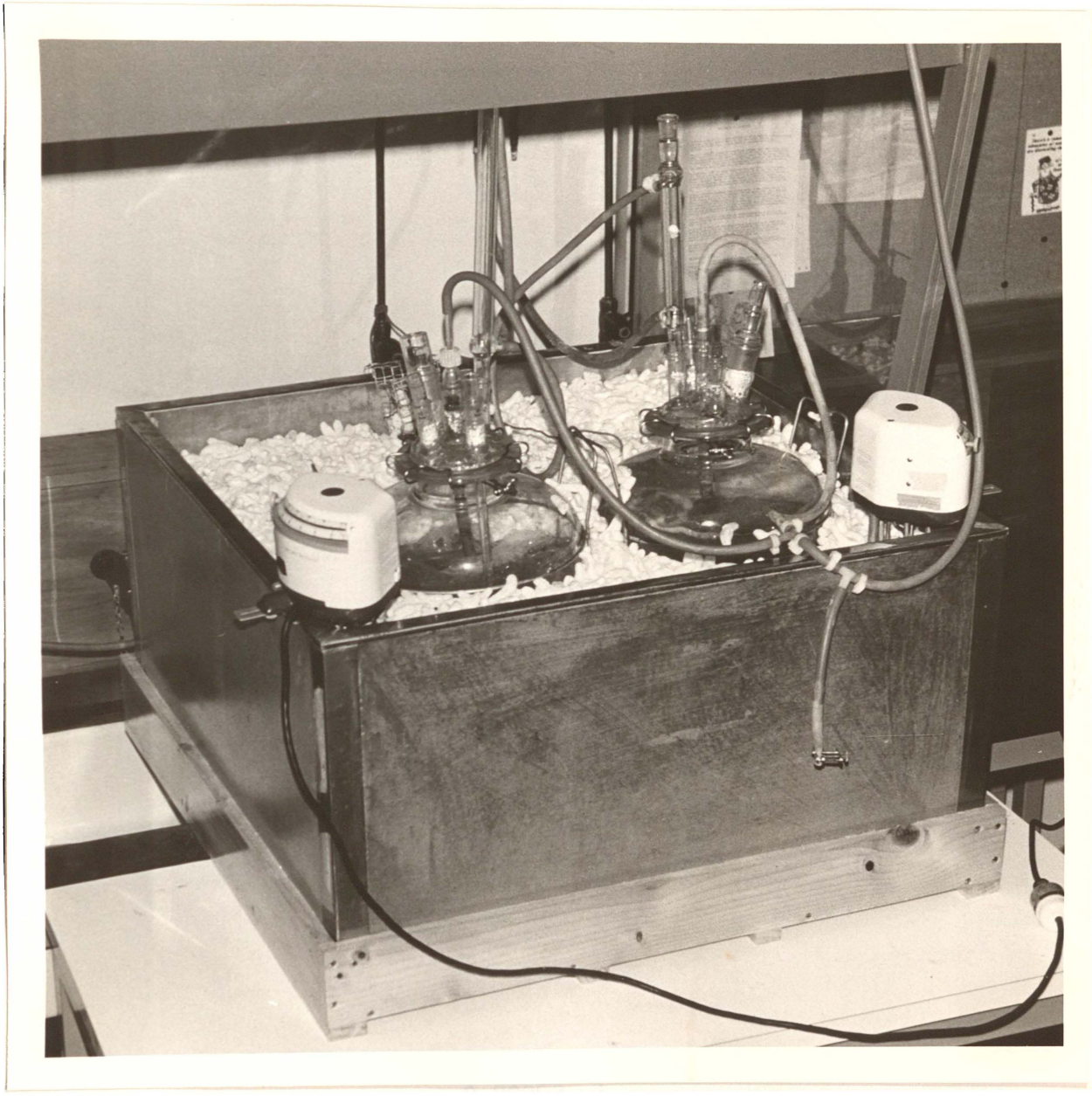


Plate 2. Assembled 20l glass fermentors in an artificial hot-pool.

78-79°C by two thermostatted immersion heaters. Evaporation loss from the hot-pool surface was minimised by addition of a layer of polystyrene chips or by a layer of paraffin oil.

TABLE 4-1 Composition of standard *Thermus* growth medium (from Hickey & Daniel, 1979)

Component	Grams per litre of distilled water	
Yeast extract	3.0	
Trypticase peptone	3.0	
$(NH_4)_2SO_4$	1.3	
KH_2PO_4	0.28	
$CaCl_2.2H_2O$	0.074	
$MgSO_4.7H_2O$	0.247	
$FeCl_3.6H_2O$	0.019	Premixed as 1000X concentrate. (1 ml of concentrate added per litre of medium).
$MnCl_2.4H_2O$	0.0018	
$Na_2B_4O_7.10H_2O$	0.0044	
$ZnSO_4.7H_2O$	0.00022	
$CuCl_2.H_2O$	0.00005	
$NaMoO_4$	0.00003	
VCl_2	0.00003	

Each volume of growth medium was adjusted to pH 8.1, autoclaved, and cooled to 75°C before inoculation with *Thermus*.

A period of approximately four hours was needed to raise the temperature of the fermentor vessels from 20°C to 75°C. Once equilibrated at water bath temperature, 2 litres of an active *Thermus* culture (pregrown as described above) was added to each vessel, and air circulation was

started. Aeration was maintained at 10-12 litres per minute per vessel from an Edwards ECB1 compressor.

Evaporation loss from each vessel was reduced by positioning a water-cooled condenser on the cap assembly. In monitoring the temperature during culture growth, a 2°C difference between water bath and vessel temperature was noted, presumably a result of the air passage. Occasional foaming in the fermentors was controlled by addition of a single drop of sterile Silicone anti-foaming agent (Sigma) to the culture.

Under the conditions described above, cultures could be grown to stationary phase ($A_{650} \sim 1.4$) within 12 to 20 hours. For optimal protease production, cultures were harvested at an absorbance of 0.9 to 1.1 after 9 - 15 hours.

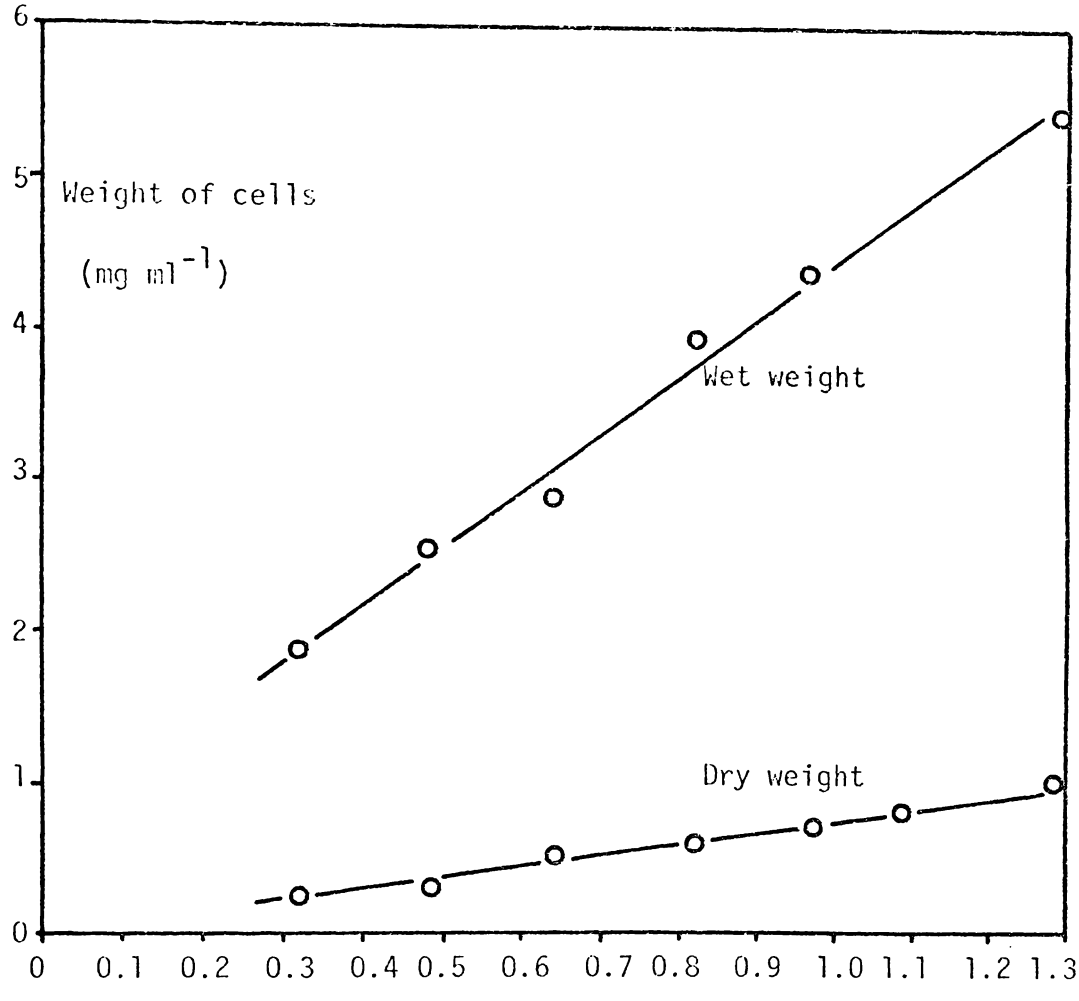
Cells and cell-free supernatant were harvested by continuous-flow centrifugation (Sorvall RC-2B with SS-34 continuous-flow head) at 15,000 rpm. At a flow rate of 100 - 150 ml $minute^{-1}$, yields of 20 to 60 g of wet weight cells per 20 litre vessel were routinely obtained.

Cell-free supernatants were cooled during and after centrifugation. Calcium chloride was added to a concentration of 5 mM to stabilise the extracellular protease. Addition of calcium always resulted in the appearance of a white precipitate (probably $Ca_3(PO_4)_2$).

4-2 Cell mass-absorbance relationships in *Thermus* T-351 cultures

To determine the corelation between cell mass and A_{650} (absorbance at 650 nm) in *Thermus* cultures, a standard gravimetric procedure was carried out. 500 ml cultures of *Thermus* T-351 were grown as previously described, harvested at intervals during the growth period, and wet and dry cell weights estimated gravimetrically.

Fig. 4-1. Relationships between A_{650} of *Thermus* cultures and wet and dry cell weights.



When cell weights (expressed as milligrams of cells per millilitre of culture fluid) are plotted against A_{650}, linear relationships are obtained (Fig. 4-1).

It is reasoned that in 'young' cultures such as the ones from which this data is derived, little cell lysis should have occurred, thus avoiding the major factor potentially responsible for the loss of linearity between cell mass and absorbance. The empirical relationships:

Dry weight of cells (mg ml^{-1}) = 0.76 (A_{650}-Blank)

and Wet weight of cells (mg ml^{-1}) = 3.63 (A_{650}-Blank)

have been calculated from Fig. 4-1.

These relationships are used in subsequent experiments for the conversion of *Thermus* culture absorbance data to cell mass.

4-3 Protease assays: Introduction

A variety of different protease assays are reported in the literature, largely a result of the diversity of proteases, and the requirement for differing degrees of sensitivity. Quantitative assays can be grouped into the general categories of:

a) Hydrolysis of high molecular weight substrates
   - Native proteins
   - Dye-bound proteins
   - Isotope-labelled proteins

b) Hydrolysis of low molecular weight ester, peptide, and amide substrates.

The most commonly used assays are those based on the hydrolysis of casein (Kunitz, 1947) and denatured haemoglobin (Anson, 1938). In these, as in almost all assays utilising native proteins, the degree of hydrolysis is determined by the measurement of trichloroacetic acid (TCA)-soluble material remaining in solution after the removal of the unhydrolysed

precipitated protein. Quantitative determination of TCA-solubles can be achieved by measuring the absorbance at 280 nm (spectrophotometric determination of the aromatic amino acid content: Reimerdes & Klostermeyer, 1976); by reaction of free α-amino groups with ninhydrin (Moore & Stein, 1954); with the Folin-Ciocalteau reagent (Banga *et al.*, 1959), with the Biuret reagent (Hall, 1955); or by reaction with an amino-binding fluorescent compound such as fluorescamine (Garesse *et al.*, 1979).

A number of objections to the use of casein and A_{280} measurements have been published. These are largely based on the heterogeneity of casein as a substrate, the variable susceptibility of the casein components to different proteases (Reimerdes & Klostermeyer, 1976), and the presence of artifacts in some tissues (Barrett, 1977). Nevertheless, casein is frequently used as a substrate and the determination of A_{280} data is one of the most rapid quantitative assay methods.

A variety of dye-bound protein substrates are commercially available (Table 4-2). These are based on the same principle as the Congo-red//fibrin substrate complex synthesised by Roaf (1908). All rely on the optical estimation of the chromophore remaining in solution after precipitation of unhydrolysed protein.

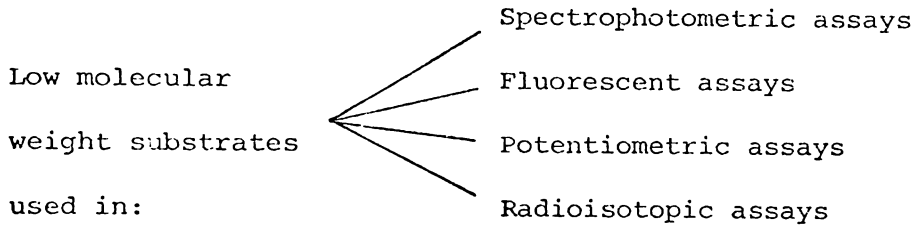
TABLE 4-2 Dye-bound proteins used as protease substrates.

Substrate	Reference
Congo-red//elastin	Naughton & Sanger, 1961
Azo-elastin	Robert & Samuel, 1957
Orcein-elastin	Sachar *et al.*, 1955
Azo-collagen	Ensign & Wolfe, 1965
Azo-albumin	-
Azo-casein	Starkey, 1977
Remazol blue Hide powder	Reinderknecht *et al.*, 1970
Fluorescein-haemoglobin [a]	DeLumen & Tappel, 1970

a. Fluorescence increases on release of dye.

A number of assays utilising isotopically labelled proteins have been reported: e.g. ^{3}H-elastin (Starkey, 1977); ^{3}H-acetyl-haemoglobin (Hille *et al.*, 1970; ^{131}I-casein (Katchman *et al.*, 1960); ^{14}C-N, N-dimethyl casein (Drucker, 1972); ^{131}I-albumin (Klotz & Duval, 1957); ^{131}I-fibrin (Sawyer *et al.*, 1960); and ^{14}C-collagen (Seifter & Harper, 1970). These assays are based on the same principle as those previously described.

The determination of proteolysis using low molecular weight substrates can be divided into four general categories.



The range of spectrophotometric assay substrates is enormous. Spectrally determinable leaving-groups include those listed below (Table 4-3). Only a few of the more common examples are quoted.

TABLE 4-3 Peptide analogues as protease substrates

Leaving-group	Substrate	Reference
Methanol	Tosyl-L-arginine methyl ester	Kezdy & Kaiser, 1970
Ethanol	Benzoyl-L-arginine ethyl ester	Schwert & Takenaka, 1955
o-nitrophenol	Benzoyl-L-arginine nitrophenyl ester	Kezdy & Kaiser, 1970
p-nitroaniline	Benzoxycarbonyl-glycine-L-proline-L-arginine-p-nitroanilide	Denker & Fritz, 1979

The range of N-terminal blocking groups and amino acids is large, producing many possible permutations.

A number of fluorimetric assays have been derived from the β-naphthyl amides, β-naphthyl esters, and methylumbelliferyl esters of CBZ-blocked amino acids (Coleman *et al.*, 1976). These assays are based on the release of the intramolecular quenching of the fluorigenic substrate when proteolysis occurs (Yaron *et al.*, 1979).

The hydrolysis of substrates such as benzoyl arginine ethyl ester can be quantitatively estimated by monitoring the rate of acid production (potentiometric titration) as the ester bond is cleaved (Walsh & Wilcox, 1970). This procedure necessitated the use of a temperature controlled pH-stat for accurate measurement of very small pH changes.

Isotopically labelled tosyl-L-arginine-(^{3}H)methyl ester has been used as a protease assay substrate (Roffman *et al.*, 1970). The detection of released (^{3}H)-methanol depends on the partition of substrate and hydrolysis products by their different solubilities in aqueous and toluene phases of the scintillation counting liquid. This assay is reported to be more than 100 times as sensitive as the equivalent spectrophotometric or potentiometric procedure (Coleman *et al.*, 1976). Hydrolysis of low molecular weight substrates is used predominantly for the assay of proteases which possess esterase or amidase activity (e.g. trypsin). Metalloproteases, which generally do not possess either, must be determined with larger peptide substrates such as Benzoyl-Phe-Val-Arg-p-nitroanilide (Bergstrom, 1977).

The assay requirements of this project were as follows. During the preliminary screening and isolation work, a rapid, convenient semi-quantitative assay was needed, firstly to detect the low levels of protease in culture media, and secondly to monitor the numerous eluted fractions from ion exchange, affinity, and gel filtration chromatography columns. This requirement was fulfilled by development of the casein-agar plate assay (section 4-7).

For almost all quantitative analysis, the Kunitz assay was found to yield reproducible results both quickly and simply. Where chemical interference at 280 nm by substances in the assay mixture occurred, the substrate azo-casein was found to provide good results.

In cases where high molecular weight (protein) substrates were not desirable, the peptide Benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma) was utilised. Owing to the high cost of this substrate, it was used infrequently and in select experiments only.

4-4 Protease assays: the Kunitz assay

A modification of the casein hydrolysis assay designed by Kunitz (1947) was routinely used for quantitative estimates of protease activity.

Reagents:

Casein (Hammersten quality)

Tris acetic acid buffer, 0.1M, pH 8.1. 50 ml of stock 2M Tris and 60 ml of stock 1M acetic acid were diluted to 1 litre with distilled water.

5% aqueous trichloroacetic acid

The substrate was prepared by adding 0.5 g of casein to 100 ml of Tris-acetic acid buffer and by heating the suspension to boiling point with continual stirring.

All quantitative assays were performed in duplicate or triplicate. In the latter case, each assay utilised six 15 ml glass centrifuge tubes, each containing 2 ml of 0.5% substrate. These were pre-incubated for at least 3 minutes at 75°C in a thermostatted water bath. The reaction was initiated by addition of 100µl of enzyme to each of the 'reaction' tubes (i.e. 3 of the 6), and allowed to continue for a measured period. The assay was terminated by addition of 3 ml of 5% TCA to all tubes, after which 100µl of enzyme was then added to each of the 'controls'.

Precipitated protein was allowed to flocculate for 10 minutes, after which all tubes were centrifuged at 5000 xg (MSE-Super Minor) for 10 minutes. The absorbance of each supernatant was measured spectrophotometrically at 280 nm (Beckman 24 duel-beam spectrophotometer) against a distilled water Blank. The degree of hydrolysis was calculated from the mean Sample absorbance less the mean Control absorbance. The ΔA_{280} min^{-1} was converted into µg tyrosine released $minute^{-1}$ by comparison with a standard tyrosine curve. (It is assumed that tyrosine is responsible for all A_{280} absorption.) Proteolytic activity was expressed as Proteolytic Units, where:

1 PU = 1 µg tyrosine released $minute^{-1}$ from 0.5% casein at $75^{o}C$.

Specific activity was defined as PU per mg protein, and is denoted by:

$$(PU)\ {}^{cas.\ 75^{o}}_{280\ \mu g\ tyr.}\ mg^{-1}\ \text{enzyme}$$

4-5 Protease assays: The hydrolysis of Dye-bound proteins

When substances absorbing at 280 nm were included in the assay mixture, (e.g. o-phenanthroline) azo-dye-bound proteins were used as substrates.

Azo-albumin and azo-casein were dissolved in 0.1M pH 8.0 Tris acetic acid buffer at a concentration of 0.1%. Assays were carried out as described in section 4-4, with the exception that TCA-solubles were spectrophotometrically determined at 440 nm.

Enzyme activity was expressed as ΔA_{440} min^{-1}.

Specific activity was expressed as ΔA_{440} min^{-1} mg $protein^{-1}$.

While this assay could be readily related to the Kunitz assay, this was not found to be necessary.

4-6 A continuous protease assay

A kinetic assay for Caldolysin using the peptide analogue substrate Benzoyl-L-phenylalanine-L-valine-L-arginine-p-nitroanilide (BPVAP) was performed according to the method of Bergstrom (1977).

BPVAP was dissolved in pH 8.1 0.01M Tris acetic acid buffer containing 10 mM calcium and 10% methoxyethanol, at a concentration of 0.3 mg ml^{-1} ($\sim$0.5 mM). Prior to each set of assays, the substrate was pre-incubated at 75°C to deaerate the solution. For a single assay, 0.9 ml of substrate was pipetted into a thermostatted semi-micro cuvette and the reaction initiated by the addition of 100 µl of enzyme. The progress of the reaction was continuously monitored at 405 nm on a Beckman 24 spectrophotometer with a chart recorder.

The rate of substrate hydrolysis was calculated from the molar absorbtion coefficient of p-nitroaniline (ε_M^{440} = 9400 $M^{-1}cm^{-1}$). Enzyme activity was expressed as mM min^{-1} substrate utilised.

The very high cost of BPVAP precluded its use as a routine assay substrate.

4-7 Protease assays: The semi-quantitative casein-agar plate assay

A number of qualitative and semi-quantitative protease assays have been reported, based either on the milky precipitation of partially hydrolysed casein, or on the clarification of an opaque protein-gel background around the site of protease application. These include gelatin digestion on old photographic plate (Jonsson & Martin, 1964), proteolytic clarification of collagen-containing gels (Hausmann & Kaufman, 1969; Robbertse *et al.*, 1978), clarification of fibrin-containing gels (Christman *et al.*, 1977), and precipitation of para-caseins (Grimont *et al.*, 1977). A semi-quantitative assay was developed, based

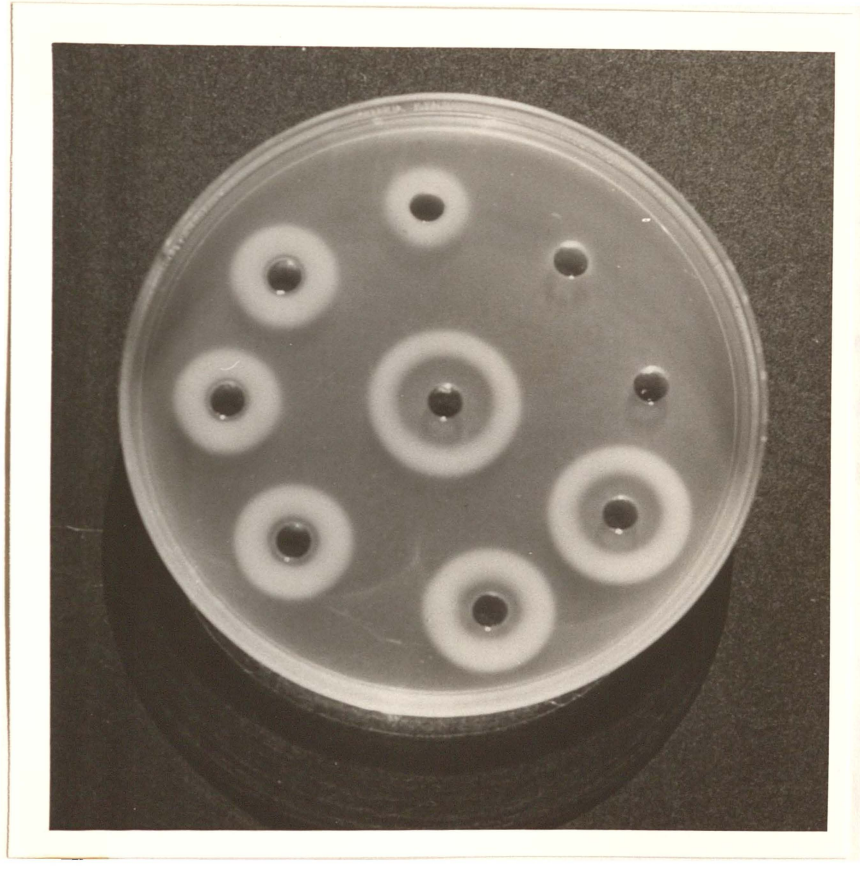


Plate 3. The casein-agar plate assay. White rings of precipitated para-caseins are indicative of proteolytic activity.

on the precipitation of para-caseins by partial proteolysis as described by Grimont *et al.*, (1977) and Scott (1973).

24 g of milk agar (Oxoid) and 10 g of casein (BDH, technical) were added to 1 litre of distilled water. This was heated with stirring to just below boiling point with periodic adjustments of the pH to 7.5. The solution was autoclaved, cooled to about 60°C, and poured into plastic petri plates (in approximately 20 ml aliquots) under aseptic conditions. After solidification of the gel, plates could be stored inverted and were used within four weeks.

Assays were performed after excision of wells in the gel using a 5 mm cork-borer. Wells were found to hold a maximum volume of 40 ± 5µl, and up to 18 could be cut in a single plate. A 30 µl volume of a test sample was pipetted into each well, the plate was marked for identification, and wrapped in Gladwrap^{R}. Assay plates were then incubated at 55°C for a period of 24 hours, after which the degree of enzyme activity could be determined by measurement of the diameter of the milky ring of precipitated p-caseins around each well (Plate 3). Enzyme activity was recorded as the diameter of the ring of hydrolysis minus 5 mm (the well diameter), and has been related to the quantitative Kunitz assay (see section 5-2).

4-8 Qualitative assay of cell-wall lysis

The lytic activity of Caldolysin was determined qualitatively and semi-quantitatively by a modification of the "lysoplate" method, first described in detail by Osserman & Lawlor (1966) and improved by Gosnell *et al.* (1975).

Lysoplates were prepared as described below. 10 ml of nutrient broth (0.1%) containing an active culture of the desired organism (see section 8-6 for a list of organisms used) was added to 200 ml of auto-

claved 1% agarose in pH 7.5 phosphate buffer at 40°C. Lysoplates were immediately poured under aseptic conditions (approximately 20 ml of seeded agarose gel was added to each plate). After cooling, seeded cultures were pregrown by incubation at 37°C for 24 hours, resulting in a visible lawn of bacterial colonies. Plates were then stored at 4°C until required.

Assays were performed by the application of 50 µl volumes of enzyme solution to 8 mm diameter wells cut in the gel. After addition of samples and standards, the lysoplates were marked for identification, wrapped in Gladwrap^{R}, and incubated at 75°C for 24 hours.

Active lysis was visible as a region of clearing in the lawn of bacterial colonies around the wells.

4-9 Determination of protein concentration

As a result of the very high specific activity of Caldolysin, working solutions normally contained a very low protein concentration (<20 µg ml^{-1}). It was found necessary to use a protein assay method of very high sensitivity. The Coomassie Blue assay of Bradford (1976) fulfilled this requirement. The assay solution was composed of 100 mg Coomassie Blue G250 in 50 ml 95% ethanol and 100 ml 85% phosphoric acid, diluted to 1 litre with distilled water.

For routine protein determinations, 100 µl of protein solution was mixed with 5 ml of the Bradford reagent. This was allowed to stand for five minutes then determined spectrophotometrically at 595 nm against a Blank containing the reagent and 100 µl of distilled water. All protein determinations were carried out in triplicate.

It was found necessary to use scrupulously cleaned test-tubes to obtain reproducible results when assaying very low protein concentrations. All test-tubes were routinely washed, and then rinsed, first with

distilled water and then with aliquots of the Bradford reagent, immediately prior to use for assaying.

4-10 Gel Slab Electrophoresis

The method described by Grimont *et al.*, (1977) was used as a basis for the electrophoretic separation of proteases.

Electrophoresis gels were prepared by dissolving 1.5% Oxoid pure electrophoretic agar in 75 ml volumes of 0.05M Tris acetic acid buffer, pH 8.1. The solution was autoclaved, poured hot onto a 20 cm x 20 cm glass plate with 4 mm raised sides, and allowed to solidify. 50 µl enzyme and standard protein samples were pipetted into 3 mm diameter sample wells cut along the central line of the gel. A maximum of 12 samples were applied per plate.

Connection between the gel and the electrophoresis buffer (0.1M Tris acetic acid, pH 8.1) was made with a strip of Whatman's 3MM chromatography paper. Proteins were electrophoresed at 150V to 200V for 6 - 12 hours and the buffer was renewed at four hourly intervals.

Electrophoretically separated proteases were detected by layering 50 - 70 ml of liquid casein/agar (0.7% w/v/ standard agar; 1% w/v casein, dissolved in 0.05M Tris HCl buffer, pH 7.1, and autoclaved) onto the surface of the electrophoresis gel. When solidified, the double-layer gel was wrapped in polythene and incubated at 55^{o}C for 24 hours.

Proteases were detected by the presence of milky spots in the upper gel layer.

4-11 SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gels in either 7 cm x 7 cm slabs or 8 cm x 0.5 c tubes were prepared by a method similar to that described by Weber &

Osborne (1969) and Davis & Stark (1970). 80 ml batches of gel with the following composition were prepared: 40 ml of 0.1M pH 7.1 phosphate buffer containing 0.1% SDS, 0.4 ml of 20% aqueous Tetramethyl-1, 2-diamino ethane (Sigma) 4 ml of a freshly prepared ammonium persulphate solution (100 mg/15 ml H_2O), and 36 ml of 30% aqueous acrylamide solution. The components were mixed and pipetted quickly into tubes (previously cleaned in chromic acid, washed in distilled water, rinsed in Photoflow^R, and set in inverted rubber septum caps), or separated glass plates (set in Plasticine^R). A layer of water was carefully applied to the surface of the gel solution with a Pasteur pipette. It was found that a Pasteur pipette with the tip drawn out finely and turned at a right angle was ideal for the application of water to the tubes without disturbing the gel-water interface. Gels normally set within 60 minutes of mixing.

24 hours prior to electrophoresis, protein samples were dissolved in small aliquots of 'sample buffer' (pH 7.1 phosphate buffer containing 0.1% SDS and 0.1% mercaptoethanol), and incubated at 37^oC. Just prior to electrophoresis, samples were mixed with glycerol (1:1 v/v) to ensure a high solution density, thus preventing mixing with the buffer during loading. 5 µl or 10 µl samples were loaded on to the gels with a Ziptrol^R micropipette. Sigma SDS-molecular weight marker proteins (containing bromophenol blue) were applied as standards.

Gel slabs and tubes were electrophoresed at 40mA for 6 - 8 hours or until the marker dye was within 1 cm of the end of the gel. Tube gels were then extracted by injecting water between the glass and gel using a fine syringe needle. Once loosened, gels were forced out with a plunger. Gel slabs were exposed by removal of the upper glass plate, leaving the lower plate as a support. In both cases, the position of the dye band was marked with non-soluble ink.

Staining and destaining were carried out by modifications of the methods described by Weber & Osborne (1969). A 12 hour immersion in 50% aqueous methanol: acetic acid (glacial): Coomassie Blue R250 (454:46:1.25, v/v/w) was followed by destaining for 36 hours or more in 5% aqueous methanol: 7.5% aqueous acetic acid.

Molecular weight values were determined by graphical comparison with standard proteins as described in the Sigma technical bulletin No. MWS-877 (1977).

4-12 Gradipore electrophoresis

Purified samples of Caldolysin were electrophoresed on Gradipore^{R} gel slabs using a modification of the procedure described by Manwell (1977). The pH 4.8 -alanine:acetic acid electrophoresis buffer of Reisfold *et al.* (1962) was routinely used. 5 µl or 10 µl volumes of enzyme samples (0.1 - 1 mg/ml protein) and standard proteins (30 mg/ml) were applied to the gel.

Gels were electrophoresed at 40mA until the marker dye (Bromophenol blue) was within 1 cm of the lower edge of the gel (40-60 minutes) and then fixed by immersion in 20% TCA for 15 minutes.

Protein bands were visualised by staining and destaining as described in Section 4-11. Molecular weight values were obtained by the graphic comparison of enzyme and standard protein migration distances. A linear relationship is obtained between the inverse of the relative migration distance and the cube-root of the molecular weight (Manwell, 1977).

4-13 Isoelectric focussing

Isoelectric focussing was carried out on commercially prepared pH 3.5 - 9.5 PAG-plates (LKB Ltd.). Purified and concentrated Caldolysin (0.1 - 0.6 mg ml^{-1}) was applied to the gel using multiple filter-paper

applicator strips. Loaded PAG-plates were electrophoresed on a Pharmacia Flat-bed electrophoresis unit connected to a Watson-Victor Constant Wattage power supply. A voltage of 800V was applied to the gel for 80 minutes.

After focussing, the pH gradient generated was determined with the use of a Pye surface-electrode.

The position of the enzyme bands was determined by placing a strip of gel on a pre-prepared casein-agar plate, which was then incubated at 55^{o}C for 24 hours (see section 4-7 for agar plate composition). Proteolytic activity was observed as a milky precipitation of p-caseins. Since casein was immediately precipitated by the acidic portion of the gel (pH 3.5 - 6.5) the detection of proteases with low isoelectric points was not possible by this method. Caldolysin, however, was shown to possess cationic mobility.

Protein bands were visualised by the staining and destaining methods outlined in section 4-11.

CHAPTER 5

PROTEASE ASSAYS

5-1 Development of the optimal gel composition for the casein-agar plate assay

In preliminary tests during the development of the casein-agar substrate, it was noted that the precipitation of p-caseins was influenced by the pH of the medium, and that the amount of casein present in the agar effected the gel properties.

Experiments were subsequently devised to determine (a) the pH, and (b) the casein content for optimal assay characteristics.

(a) 2.4 g of milk agar and 1g of casein were dissolved in 100 ml of distilled water by heating to boiling point. Five similar batches were prepared, but were maintained at pH values of 6.0, 7.0, 7.5, 8.0, and 9.0 respectively, during heating. Five plates of approximately 20 ml volumes were poured from each batch.

(b) Six 100 ml volumes of gel were prepared, each containting 2.4g of milk agar, and 5g, 2g, 1g, 0.5g, 0.1g, and 0g of casein respectively. During heating, a pH of 7.5 was maintained.

The quality of each test substrate was determined by the excision of five wells in each plate and subsequent treatment as described in section 4-7. After incubation, the diameters of hydrolysis rings were measured, and both the density of the precipitate and the resolution of the precipitate/gel boundary were scored for each plate on a scale of 1 to 5 (Table 5-1).

TABLE 5-1. Estimation of optimal gel composition.

Gel characteristic (pH)	Diameter of hydrolysis ring [a]	Density of precipitate [b]	Resolution [c]
6.0	25.60 ± 1.15	5	5
7.0	25.44 ± 1.14	4	5
7.9	23.59 ± 0.35	4	5
8.0	24.65 ± 0.57	2	3
9.0	20.64 ± 0.93	1	1
(% casein content)			
0	25.00 ± 0.56	1	1
0.1	24.32 ± 0.24	1	1
0.5	25.57 ± 0.69	2	1
1.0	23.59 ± 0.35	4	5
2.0	23.86 ± 0.57	5	5
5.0	∿15	3	1

a. Average of five measurements.

b. 5 - very heavy ppt.; 4 - heavy ppt.; 3 - moderate ppt.; 2 - slight ppt.; 1 - no ppt.

c. 5 - clearly defined; 3 - indistinct boundary; 1 - no ppt. -gel boundary apparent.

Essential criteria for accurate qualitative and quantitative plate assays are clarity of precipitation (for the determination of low levels of activity), and resolution of the precipitate boundaries (for accurate measurement). However, gel setting was inhibited at casein concentrations of 2% and over, precluding their use. On the assumption that *Thermus* extracellular proteases would have alkaline pH optima, the gel composition eventually selected was 1% casein at pH 7.5.

5-2 Quantification of the casein-agar plate assay

Caldolysin (93µg ml^{-1}) was diluted with 0.1M pH 8.1 Tris acetic buffer to give protein concentrations of 47, 33, 25, 18,6, 9.3, 4.7, and 0.9µg ml^{-1}. These enzyme solutions were assayed on casein-agar plates as described in section 4.7. At intervals throughout the 24-hour incubation period, the diameters of the milky hydrolysis rings were measured. Examples of the time-dependent growth of p-casein precipitates are shown in Fig. 5-1, and exhibit a logarithmic relationship, as would be predicted from an equation of radial diffusion derived from Fick's Second Law (Moore, 1962).

The range of enzyme dilutions was also assayed by the Kunitz method (section 4-4). These values, and those derived from a 24-hour incubation of the casein-agar plates, are presented in Table 5-2.

TABLE 5-2 A comparison of proteolytic activities determined by the Casein-agar plate and Kunitz methods. (± ( ) values indicate standard deviation with number of samples).

Enzyme protein concentration (µg ml^{-1})	Enzyme activities Casein-agar assay [a]	Kunitz assay [b]
93	17.72 ± 0.33 (9)	3.57 ± 0.13
47	15.82 ± 0.43 (9)	1.84 ± 0.25
33	14.00 ± 0.16 (3)	1.22 ± 0.11
26	12.63 ± 0.24 (3)	0.79 ± 0.06
18.6	11.67 ± 0.29 (3)	0.65 ± 0.05
9.3	11.20 ± 0.42 (6)	0.27 ± 0.03
4.7	8.34 ± 0.55 (6)	0.14 ± 0.01
0.9	4.58 ± 0.32 (6)	0.032 ± 0.002

a. Diameter of hydrolysis ring (mm) minus 'well' diameter after 24-hour incubation at 55°C (30µl enzyme samples).

b. mg tryosine released $min.^{-1}$ ml $enzyme^{-1}$ from 0.5% casein at 75°C.

Fig. 5-1. Time-dependent growth of hydrolysis rings in casein-agar protease assays.
(30µl of enzyme solution per well, incubated at 55°C)
Enzyme concentrations specified below.

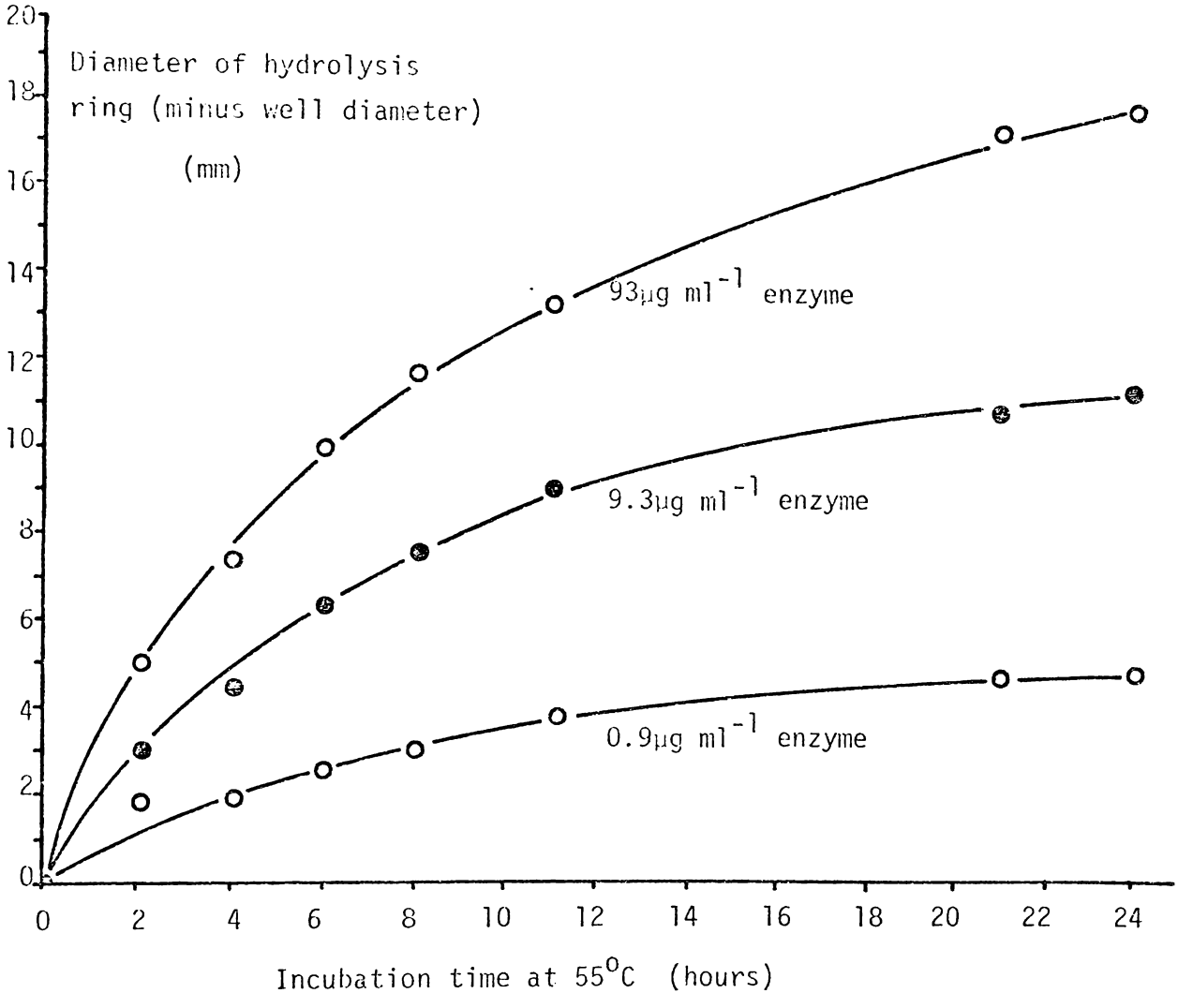
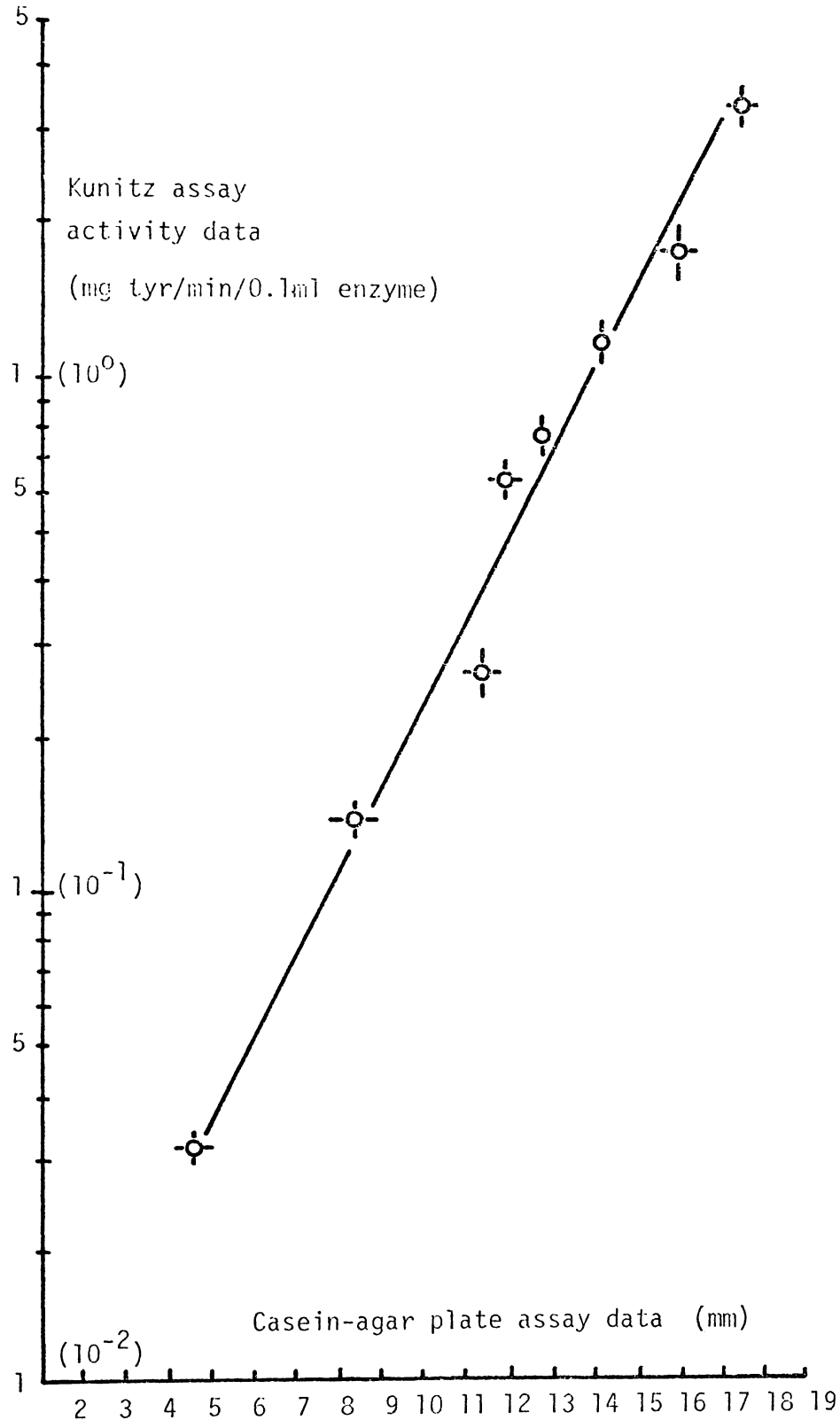


Fig. 5-2. Relationship between Kunitz and casein-agar assays.



When this data is plotted on a semi-logarithmic scale, a linear relationship between results from the two assays is obtained (Fig. 5-2), indicating that the latter is an acceptable and accurate method for semi-quantitative determination of proteolytic activity. The graphic relationship shown in Fig. 5-2 has been used for the conversion of casein-agar assay data to standardised Proteolytic Units.

5-3 The Kunitz assay; Time-dependence of enzyme activity

The apparent rate of proteolysis (i.e. the output of TCA-soluble, 280nm absorbing protein fragments) was determined during the complete hydrolysis of a sample of casein.

A series of Kunitz assays were started simultaneously, each with 2 ml of substrate and 100µl of identical enzyme solution. Individual assays were terminated after periods ranging from 5 to 100 minutes. It was thus possible to determine the apparent rate of proteolysis during the reaction. Results, expressed as the total absorbance at 280 nm as a function of the reaction time, are presented in Fig. 5-3. It is assumed that the rate of autolysis is negligable at the incubation temperature (see section 9-1). The effects of autolysis, if present, should be observed as negative deviations from the plot shown in Fig. 5-3. This does not occur.

The apparent increase in the rate of proteolysis above an optical density of 0.15 (corresponding to the release 0.209 mg tryosine ml^{-1}) could result from:

1. Increases in the susceptibility of the substrate. Available cleavage sites on the exterior of the casein molecules are presumably limited while the globular tertiary structures persist. However, endopeptidic hydrolysis would rapidly fragment the macro-

Fig. 5-3. Variation of apparent rate of enzyme activity with time.

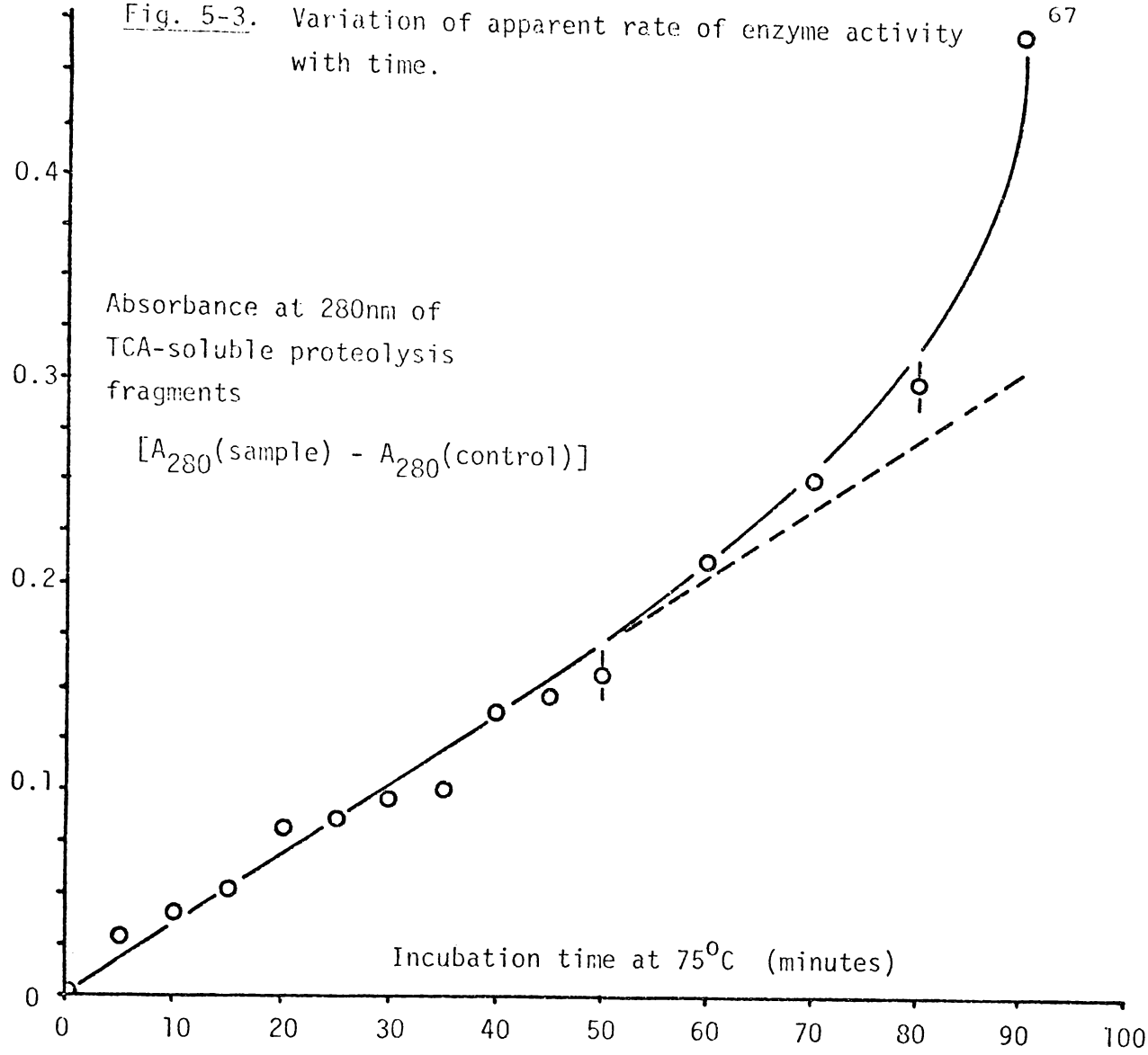
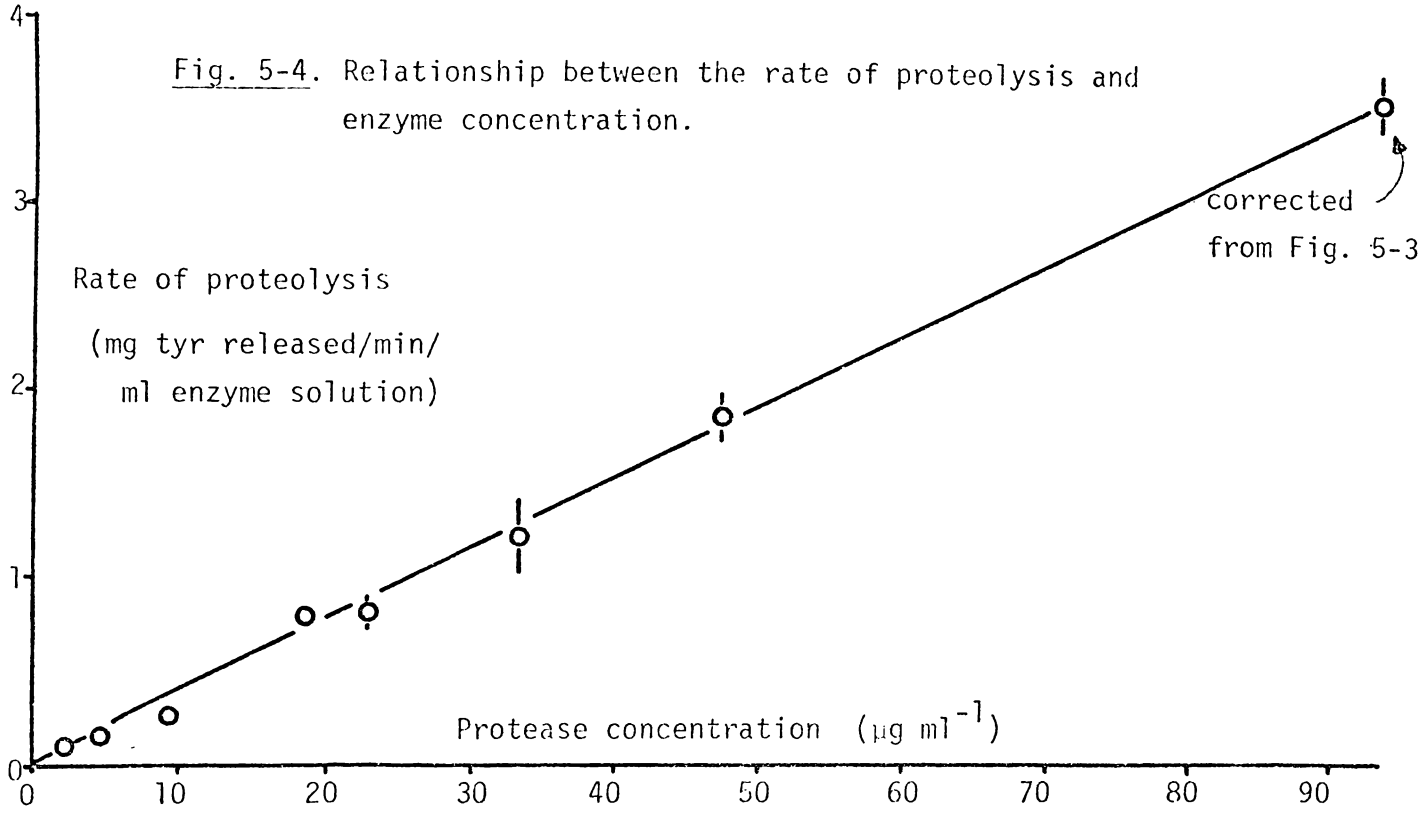


Fig. 5-4. Relationship between the rate of proteolysis and enzyme concentration.



molecule into denatured segments. Thus the availability of suitable cleavage sites should increase as the reaction progresses, resulting in an increase in apparent reaction rate.

2. Activation of the enzyme. This phenomenon, which has been observed at 75°C, is discussed in Chapter 10.

3. Heterogeneity of the distribution of aromatic residues in the casein substrate. Internal segments of the polypeptide (which would initially be protected from proteolysis) may contain higher proportions of aromatic amino acids than external fragments.

As a result of this experiment, a value of 0.15 absorbance units (A_{280} sample - A_{280} control) was arbitrarily set as a maximum acceptable level for Kunitz assay data, to avoid potential complications and errors resulting from non-linearity.

5-4 The Kunitz Assay; The rate of proteolysis as a function of enzyme concentration

Enzyme concentrations between 1 and 100µg ml^{-1} were assayed by the Kunitz method, (Fig. 5-4), and a linear relationship obtained. (The data point at 93µg ml^{-1} has been corrected for the non-linearity discussed in section 5-3. In a 5 minute assay, an absorbance of 0.328 was measured, and was subsequently corrected to 0.250 from Fig. 5-3).

Because of the very high specific activity of Caldolysin, accurate measurements of the rate of proteolysis of enzyme concentrations above 100µg ml^{-1} are difficult to obtain by the Kunitz method, very short assay times being necessary to avoid exceeding the A_{280} limit set in section 5-3.

CHAPTER 6

EXTRACELLULAR PROTEASE PRODUCTION BY *THERMUS* T-351 CULTURE

6-1 Growth phase and protease production

One litre cultures of *Thermus* T-351 were grown as described in section 4-1. Growth was monitored by the periodic removal of 2 ml aliquots for pH and A_{650} determination. Samples were then centrifuged (6000 rpm, 10 minutes) and the cell-free supernatant assayed for proteolytic activity.

A typical growth curve, with related changes in pH and protease production is presented in Fig. 6-1. The amount of proteolytic enzyme in the bacterial culture fluid is maximal during late logarithmic to early stationary phase, but decreases rapidly after the cessation of cell growth. When the data of Fig. 6-1 is replotted as the amount of protease per milligram of cells, it is observed that the rate of enzyme excretion varies considerably during the culture growth cycle (Fig. 6-2).

The rapid loss of activity during stationary phase is probably the result of autolytic degradation. The rate of autolysis is known to be dependent on both the calcium concentration of the medium and the concentration of the enzyme (section 9-2). At 75°C and a calcium concentration of approximately 1.5 mM, it is calculated that the half-life of the activity would be in the order of 200 minutes (assuming that Caldolysin is responsible for all proteolytic activity present). This corresponds well with the half-life of about 220 minutes estimated from Fig. 6-1, which is based on the assumption that protease production ceases almost completely at 17 hours, just prior to the cessation of growth.

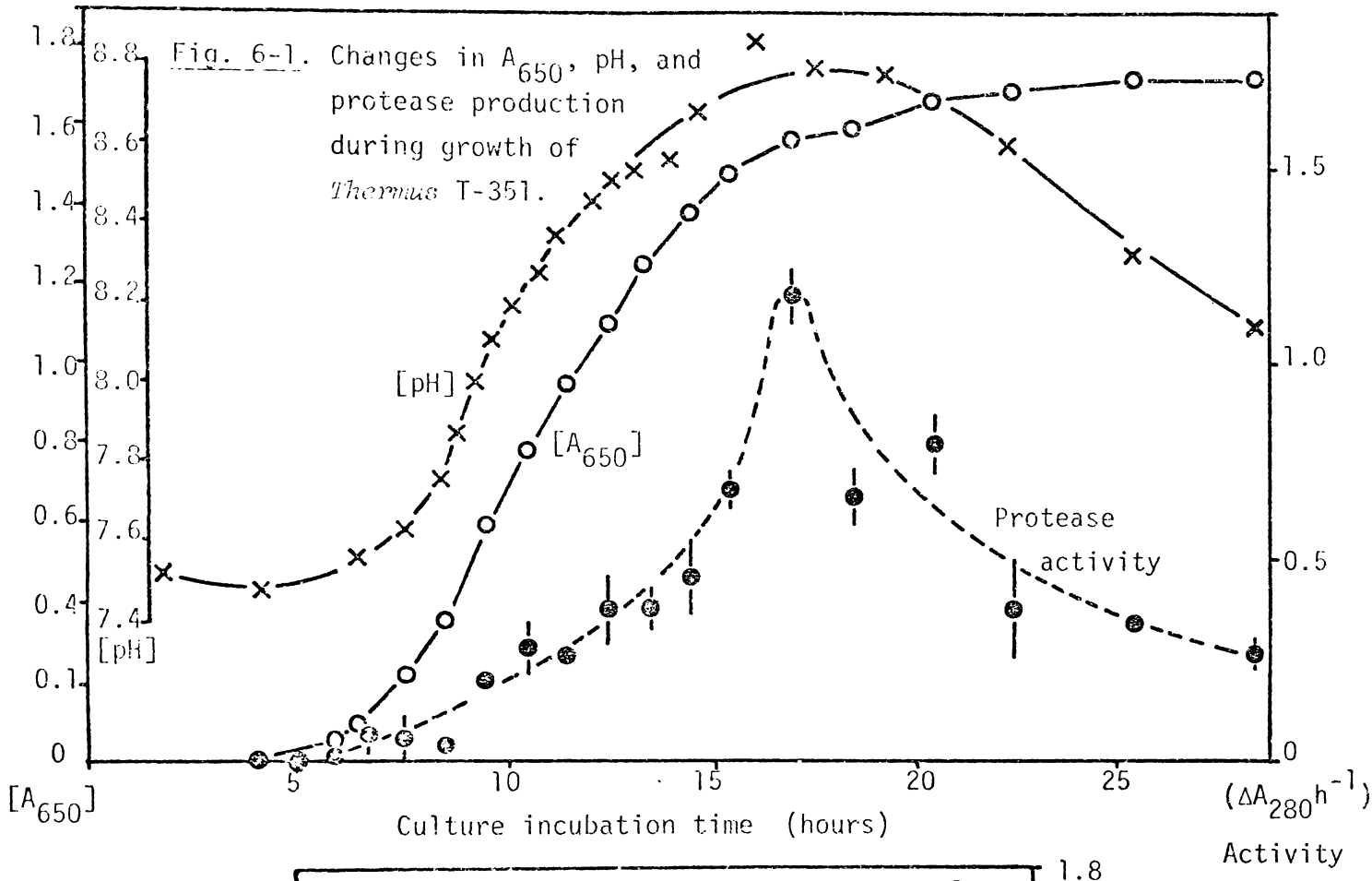


Fig. 6-1. Changes in A_{650}, pH, and protease production during growth of *Thermus* T-351.

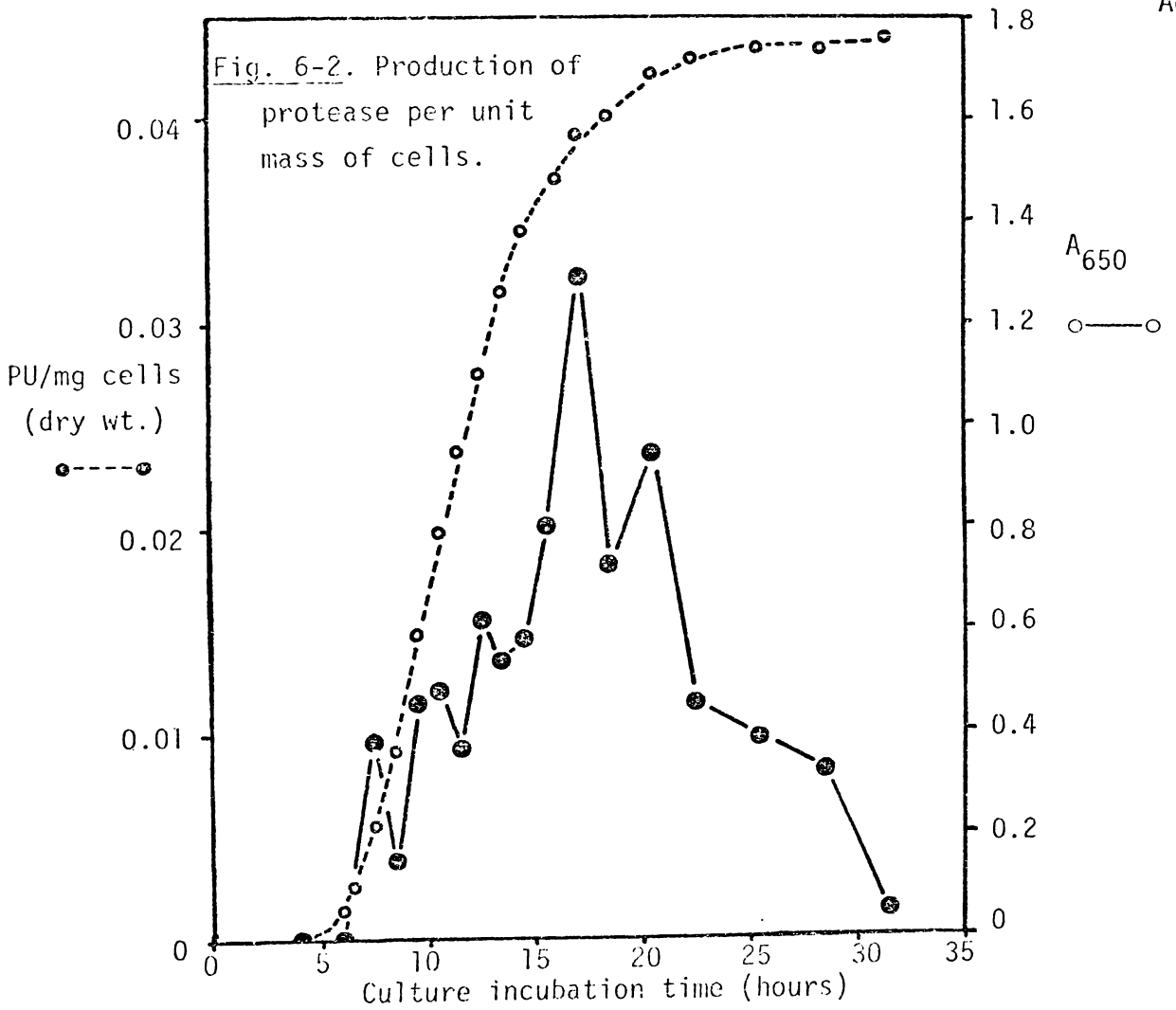


Fig. 6-2. Production of protease per unit mass of cells.

6-2 The effect of complex substrates on protease production

Extracellular proteolytic enzymes from a variety of microorganisms can be induced or repressed by addition of suitable nutrients to the growth medium. For instance, collagenase production in *Achromobacter iophagus* cultures was found to be subject to repression by ammonium ions and various amino acids (Reid *et al.*, 1978), while the production of extracellular protease by *Serratia marcescens* was repressed by some amino acids (asn, arg, ser, and pro) but induced by others (asp, glu, leu, phe, val, ala and his) (Loriya *et al.*, 1977a). Bjorklind & Arvidson (1978) showed that the synthesis of extracellular proteases in *Staphylococcus aureus* was repressed by low concentrations of some amino acids, but fully induced by higher concentrations.

It is considered by some authors that the inhibition of the synthesis of extracellular proteases is an example of catabolite repression, in that amino acids are readily used as a carbon source by many microorganisms (Loriya *et al.*, 1977a; Kean & Williams, 1967; Litchfield, 1970). However, others consider that the effect is not derived directly from an amino acid-mediated mechanism, but results from repression by ammonium ions formed during the catabolism of the amino acids (Pinghui & Hsieh, 1969).

The experiments below attempt to relate the production of extracellular proteolytic enzymes by *Thermus* T-351 to the nutrient status of the growth medium, in a determination of the optimal growth medium for maximum protease production.

Growth and proteolytic activity was monitored throughout the growth cycles of *Thermus* cultures containing different concentrations of the complex growth substrates, Trypticase Peptone and Yeast Extract. Results, expressed as the amount of proteolytic enzyme present per milligram of cells (dry weight) at intervals during the growth cycle, are presented in Table 6-1.

Fig. 6-3. Relationship between peptone concentration and maximum protease production per unit weight of *Thermus* cells. (Peptones: Yeast extract; Trypticase peptone)

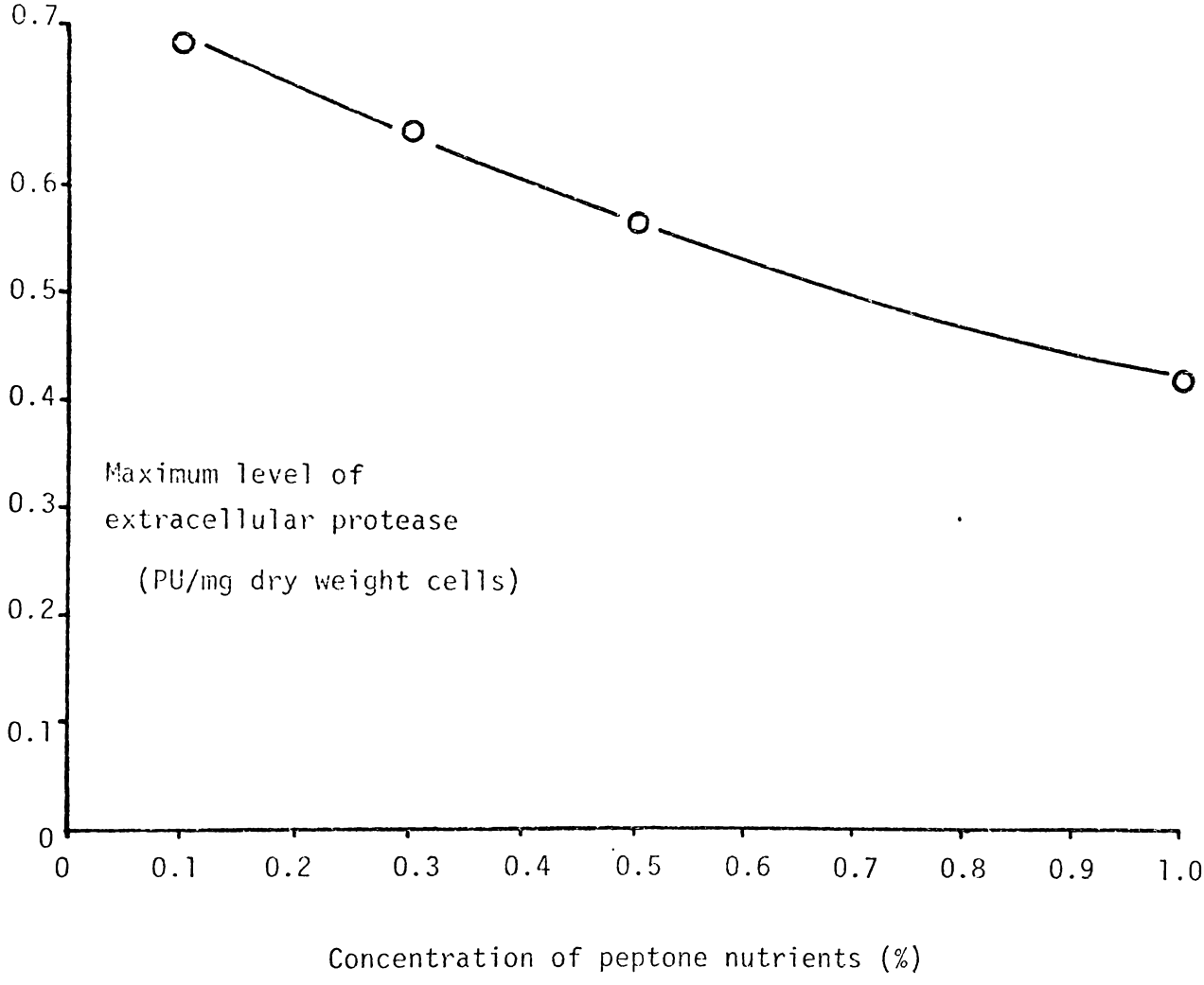


TABLE 6-1 Extracellular protease production in complex growth media

Concentration of trypticase peptone and yeast extract	Proteolytic activity (PU) per milligram dry weight of cells during growth. Time after inoculations (hours).									
%	2	3	5	7	8.5	12	23	28.5	32	46.5
0.1	0	.030	.063	-	.048	.073	.023	.013	0	0
0.3	0	0	.033	.032	.065	.055	.034	.024	.013	0
0.5	0	0	.032	.024	.043	.057	.029	.020	.011	0
1.0	0	0	.016	.023	.038	.042	.021	.017	.012	0

In Table 6-1, it is shown that increasing peptone content can be related to the maximum level of production of extracellular protease (Fig. 6-3), and to a progressive delay in the initial secretion of the proteolytic enzyme. Since the production of protease per cell is higher at lower concentrations of peptone, it is concluded that inhibition has occurred at some level (e.g. synthesis, excretion, or activity).

The delay in protease excretion during lag and early logarithmic growth phases (2 to 5 hours), must indicate repression of enzyme production, since culture growth rates over this period were very similar.

6-3 The effect of Nitrogenous substrates on extracellular protease production.

It was noted in section 6-2 that the production of extracellular protease was influenced by the presence of complex nitrogenous substrates. Further experiments were carried out to determine the influence of a variety of simple nitrogen salts, amino acids, and protein substrates (Tables 6-2 and 6-3). Proteins have been shown to induce extracellular protease synthesis, but only under conditions of nitrogen starvation (Bromke & Hammel, 1979; Cohen & Drucker, 1977).

TABLE 6-2 The effect of nitrogen source on *Thermus* growth and protease production

Composition of culture medium	Cell growth[a]	Protease output[b]
Salts[c] + 0.3% YE + 0.3% TP	+++	++
Salts + 0.3% YE + 0.3% TP + 1.3% ammonium sulphate [d]	+++	++
Salts + 0.3% YE + 0.3% TP + 1% casein	+++	++++
Salts + 0.3% YE + 0.3% TP + 1% gelatin	+++	++++
Salts + 1% casein	++	+++
Salts + 0.5% casein	++	+++
Salts + 0.5% gelatin	++	+++

a. Approximate maximum A_{650} obtained: ++ 0.900; +++ 1.400

b. ++ moderate production; +++ high protease production; ++++ very high protease production.

c. Composition given in section 4-1 (ammonium sulphate omitted).

d. Composition of standard growth medium.

It is concluded from Table 6-2 that, firstly, NH_4^+ ions show no significant repressive effect, and secondly, that the presence of protein substrates increases the output of extracellular protease. The effect of various added substrates on the production of proteolytic enzyme in the presence of protein is presented in Table 6-3.

Since the growth rates of all cultures were similar, the data shown in Table 6-3 confirm that the presence of casein results in a considerable increase in protease output. Furthermore, the addition of either ammonium sulphate or glutamic acid does not significantly repress protease production, while the addition of peptones results in a marked increase. The greatest enhancement of protease production results from the addition

of both casein and peptones (a synergistic effect).

TABLE 6-3 Substrate control of protease production

Culture medium	Culture growth: A_{650} at:		Maximum level of protease produced (PU/mg dry weight of cells)
	5 hours	23 hours	
Salts + 0.3% YE + 0.3% TP (standard medium)	0.840	1.342	0.08
Salts + 1% casein	0.916	1.364	0.34
Salts + 1% casein + 0.3% glutamic acid	0.853	1.249	0.26
Salts + 1% casein (dialysed)	0.732	-	0.54
Salts + 1% casein + 1.3% ammonium sulphate	0.968	1.472	0.41
Salts + 1% casein + 0.3% YE + 0.3% TP	0.758	1.186	0.77

When dialysed casein (low molecular weight substances removed) is used in the culture medium, a significantly greater level of protease production occurs than in the presence of non-dialysed casein. It is concluded that while protein substrates cause considerable protease induction, some repression results from low molecular weight nitrogen substrates (possibly amino acids and/or peptides), but little or none from free NH_4^+ ions.

Cultures containing salts and a variety of simple carbon/nitrogen sources (e.g. glutamate, aspartate, urea, ammonium sulphate and starch, and sodium nitrate and starch) showed no *Thermus* growth, and hence no protease excretion.

6-4 The effect of carbon substrates on protease production

It has been established that *Thermus* extracellular proteases are partially inducible enzymes. However, the transcription of the operons

of inducible enzymes is often regulated simultaneously by positive and negative mechanisms (Loriya *et al.*, 1977b). Catabolite repression of *Thermus* extracellular proteases by nitrogenous substrates is apparently insignificant (section 6-3). A series of experiments have been carried out in an attempt to clarify the role of carbon sources in protease production.

Cultures of *Thermus* were grown in media containing salts, 0.3% Trypticase peptone, 0.3% Yeast extract, and 2% carbohydrate (specified) Results, listing the production of protease and culture growth in relation to the carbon source, are presented below.

TABLE 6-5 Carbon source in relation to *Thermus* growth and protease production

Carbon source added	Maximum A_{650} of culture	Protease production (PU/mg dry wt. of cells)
None	1.415	0.047
Lactose	1.627	0.062
Starch	1.808	0.057
Sucrose	1.527	0.051
Glycogen	1.332	0.044
Sodium succinate	0.892	0.034
Mannitol	1.375	0.024
Maltose	1.656	0.019
Xylose	0	0
Arabinose	0	0
Glucose	0	0
Fructose	0	0
Glycerol	0.821	0
Sodium acetate	0.778	0
Sodium citrate	0	0
Sodium pyruvate	0.889	0

With some exceptions, culture growth can be directly corelated to protease production. However, in the presence of glycerol, acetate, or pyruvate, moderate growth occurred yet no proteolytic activity was detected. These may be examples of catabolite repression, while the levels of protease produced in the presence of mannitol and maltose represent parial repression. Since the levels of protease produced in the cultures listed in Table 6-5 are very low in comparison to those in Table 6-4, it is concluded that carbohydrate catabolite repression is a minor influence.

The absence of bacterial growth in the presence of xylose, arabinose, glucose, fructose, and citrate can probably be attributed to the presence of inhibitors in the growth medium. Degryse *et al.*, (1978) have noted that the addition of some carbohydrates to metal salt solutions can result in the formation of inhibitory hemiacetals.

6-5 The influence of calcium on protease production

Calcium has been implicated both in cellular survival (Ljunger, 1970; Ljunger, 1973; Stahl, 1978) and in the stability of intracellular components in thermophiles, (Amelunxen & Murdock, 1978a; 1978b; Stahl, 1978). (The role of calcium in the stabilisation of the major extracellular protease from *Thermus* T-351 is described in Chapter 9).

To determine the general influence of calcium on the protease output of *Thermus* cells, cultures containing calcium chloride at concentrations between 0.3 mM and 10 mM were monitored for cell growth and protease production (Table 6-6).

Several conclusions can be derived from the data of Table 6-6. Firstly, it is observed that calcium concentration has no gross effect on the maximum cell density although a small positive correlation exists. This could be a function of calcium stabilisation of the cells (via wall

TABLE 6-6 The effect of calcium concentration on the growth of *Thermus* and the extracellular protease level [a]

[Calcium]	Maximum A_{650} of culture [b]	Maximum proteolytic activity in solution [c]	Age of culture at optimum production (hours)	% of maximum activity retained in 28h old culture
0.3	1.667	0.0565	17	7
0.35	1.677	0.087	17	11
0.4	1.699	0.099	17	4
0.6	1.772	0.117	17	23
0.8	1.753	0.119	17	21
1.1	1.806	0.101	17	16
1.3	1.866	0.098	17	16
2.3	1.776	0.069	18	15
5.3	1.979	0.037	18.5	65
10.3	1.917	0.077	24	83

a. 400 ml batches of standard media (without calcium) used. Innoculated with 20 ml volumes of active *Thermus* culture (centrifuged and resuspended in distilled water).

b. Maximum A_{650} obtained after 25 - 28 hours incubation time.

c. Measured by Kunitz assay (A_{280} $hour^{-1}$).

Fig. 6-4. The effect of calcium concentration on the production of extracellular protease in *Thermus* T-351 cultures.

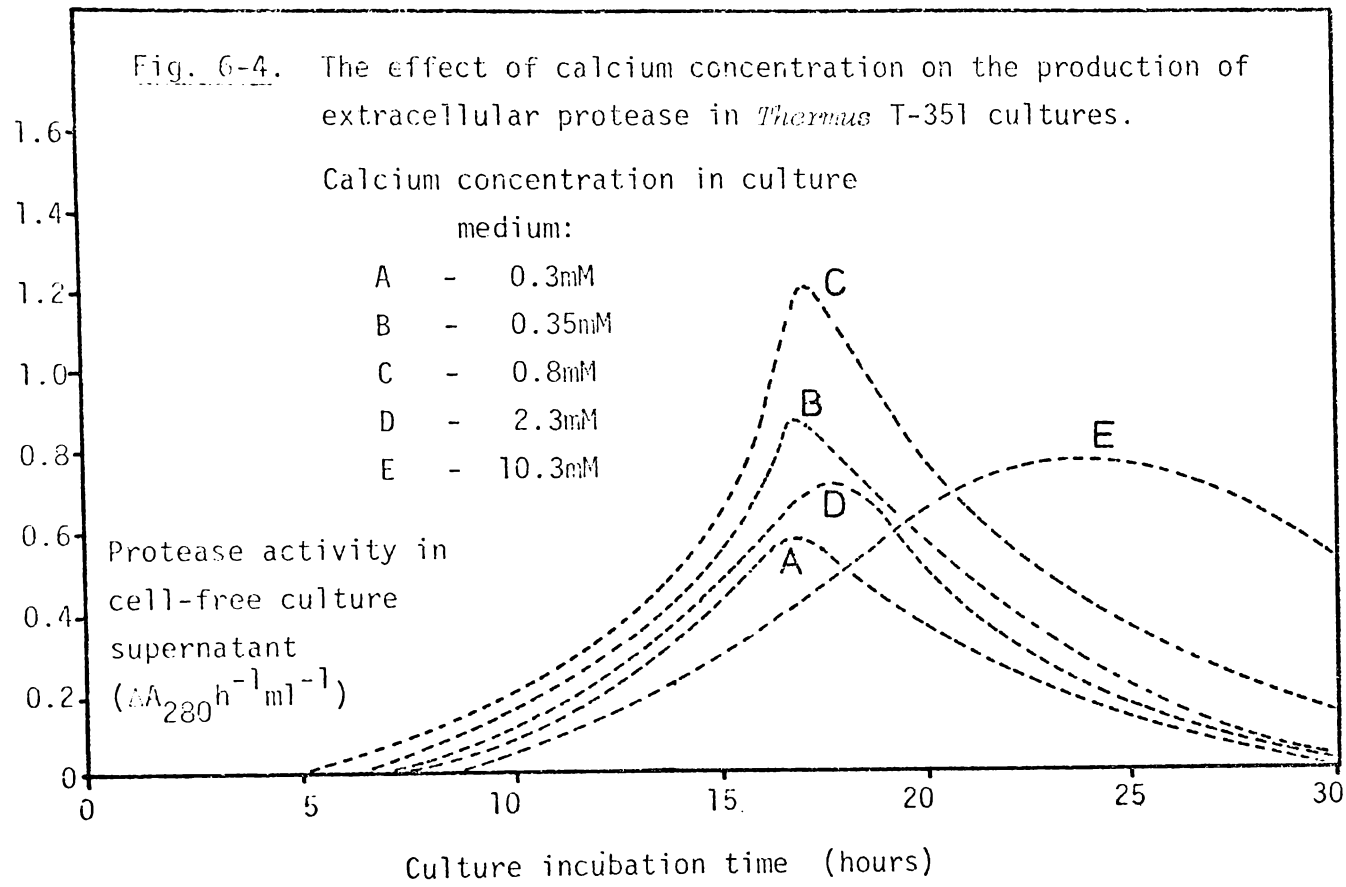
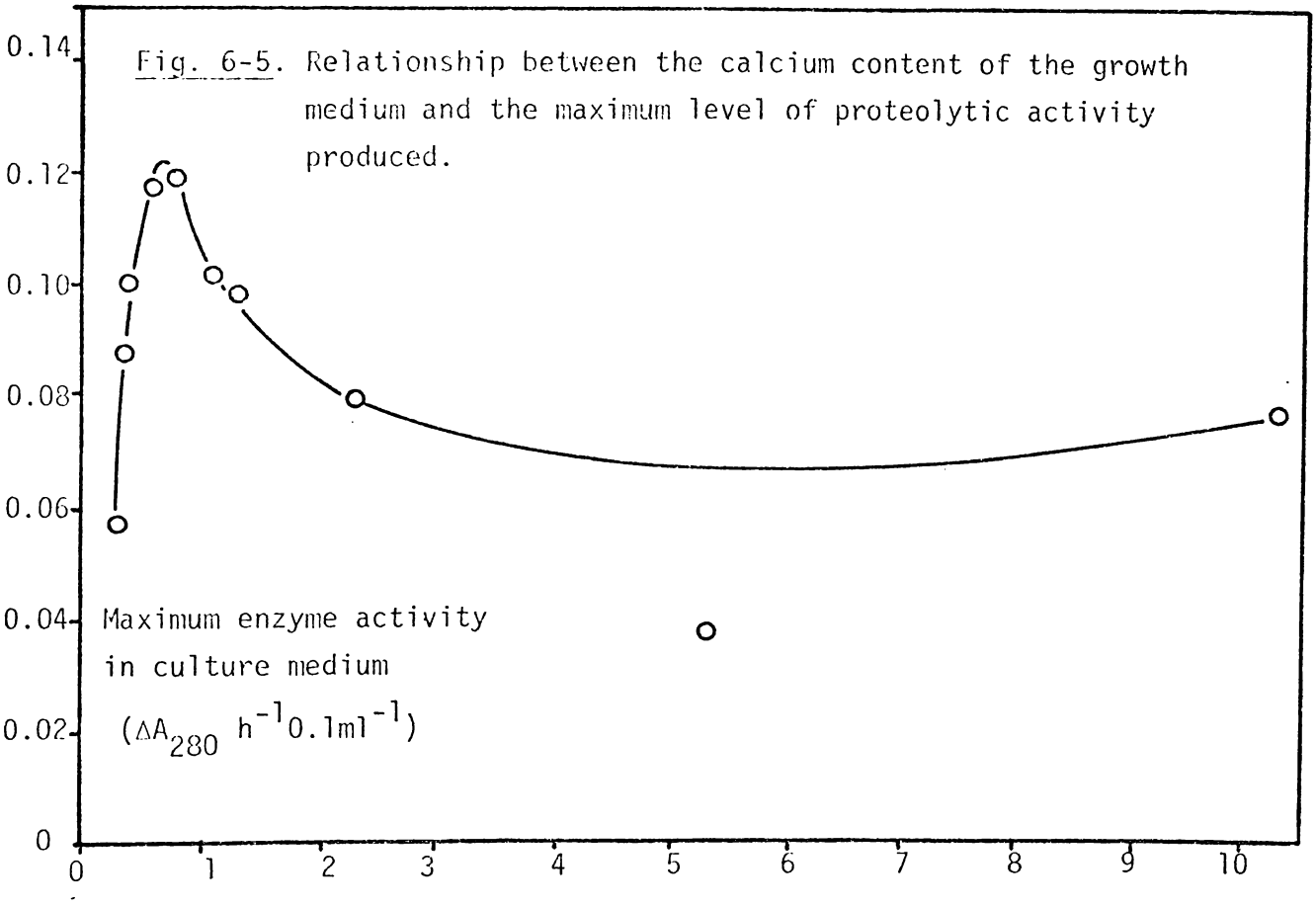


Fig. 6-5. Relationship between the calcium content of the growth medium and the maximum level of proteolytic activity produced.



or membrane) resulting in a slight increase in survival, or merely an increase in growth rate by provision of an essential mineral element. This is not a major effect as cells apparently grow and survive readily even at lower calcium concentrations.

Secondly, at high calcium concentrations the occurrence of peak protease levels is delayed, as shown graphically in Fig. 6-4.

A corelation is found to exist between maximum protease level and the amount of calcium present in the culture. When data from Table 6-6 is replotted as calcium concentration against optimal proteolytic activity (normalised to exclude differences in culture growth), it is observed that level of activity in the growth medium is maximised at a calcium concentration of 0.6 - 0.8 mM (Fig. 6-5). The proteolytic activity observed in a culture at any time is a function of two simultaneous processes; production (synthesis and excretion), and degradation (autolysis and denaturation). Increases in the calcium concentration will reduce autolysis and denaturation (see section 9-2). For example, it is noted in Table 6-6 that protease longevity is related to the level of calcium present. However, the increased stability of extracellular protease is balanced above 0.8 mM by an apparent reduction in the amount of protease excreted at higher calcium concentrations.

It is concluded that the optimal production of protease at approximately 0.8 mM calcium is detrimentally influenced at higher and lower calcium concentrations, by the inhibition of synthesis or excretion and by decreased stability, respectively.

CHAPTER 7. PART A

EXTRACTION AND PURIFICATION OF CALDOLYSIN

Localisation of the source of extracellular proteases

Extracellular proteases are excreted via the bacterial periplasmic space (the region between cell wall and cell membrane where enzyme pools are often retained), and usually passed through the permeable wall by passive diffusion (Costerton *et al.*, 1974). Most evidence suggests that extracellular proteases are excreted from the cell in active form (but see Drapeau, 1978). Furthermore, proteolytic activity present in bacterial growth media can be derived from the release of cell contents on disintegration.

Cell disrupting agents (10% toluene, 10% acetone, and 2% Tween 80) were added to cultures of *Thermus* cells of known activity levels. No increase in extracellular proteolytic activity was subsequently detected, suggesting that the pools of both extracellular and intracellular proteases retained within the cells are small.

It has been shown (section 13-1) that intracellular protease comprises less than 2% of the total protease content of a *Thermus* T-351 culture. Thus, it is concluded that:

a. extracellular protease is not derived from the release of intracellular proteases on cell lysis.

b. the pool size of any active extracellular protease retained within the bacterial cell is not large.

The observation that the maximum extracellular protease level occurs during mid to late exponential phase, a period during the growth cycle when lysis would be minimal, supports the contention that cell lysis does not contribute significantly to the extracellular protease pool.

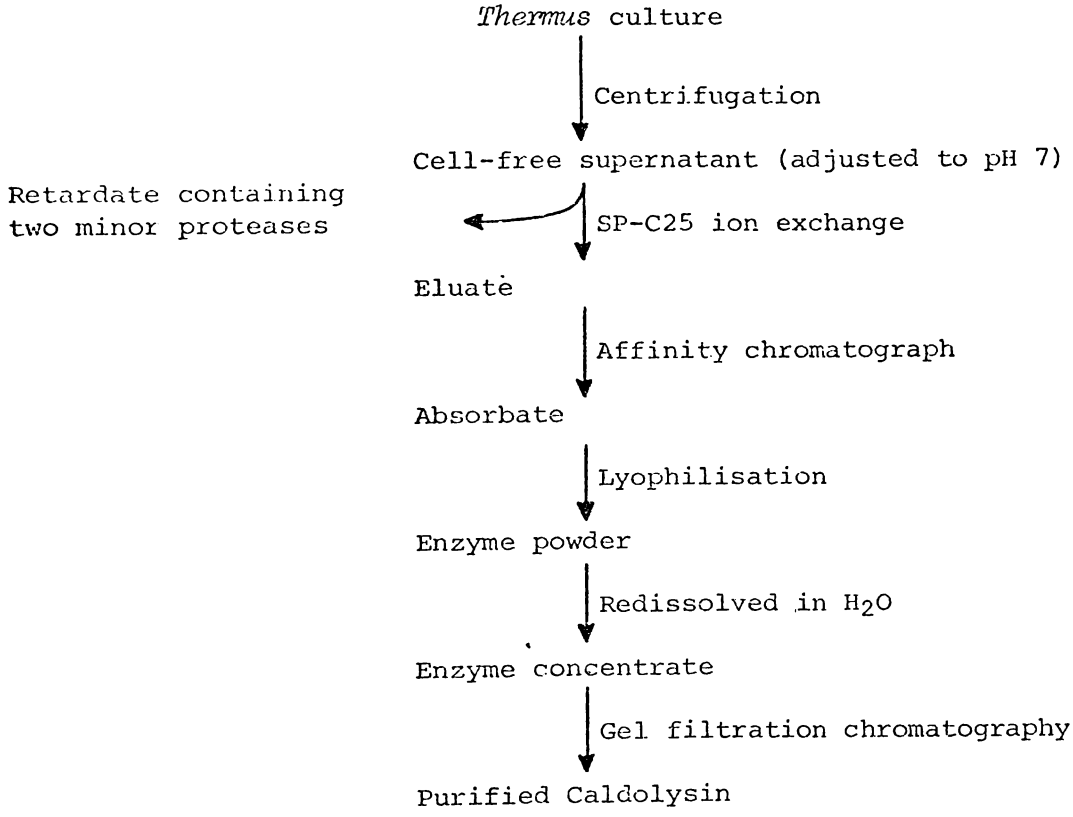
PART B.

PURIFICATION

7-1 Standardised purification sequence

The following standardised sequence was used routinely for the purification of Caldolysin.

Fig. 7-1. Steps in the purification of Caldolysin.



The determination and demonstration of the homogeneity of the purified enzyme is discussed in section 7-7. Quantitative data derived from the purification sequence outlined above is presented in Table 7-1.

TABLE 7-1 Purification of Caldolysin

Fraction	Volume (ml)	Total protein (mg)	Total activity (PU) [a] ($x10^{-3}$)	Specific activity (PU mg^{-1})	Yield %	Purification (-fold)
Cell-free supernatant	5000	400	590	1230	100	1
SP-C25 eluate	4950	274	403	1470	68.3	1.2
Affinity gel eluate	250	3.6	243	67500	41.2	54.9
Lyophilised concentrate	30	3.6	179	49716	30.3	40.4
G75 eluate	150	0.9	141	156667	23.9	127.4

a. PU = µg tyrosine released/minute at 75°C.

7-2 Ion exchange chromatography

Ion exchange chromatography was evaluated as the first purification and concentration step in the isolation sequence.

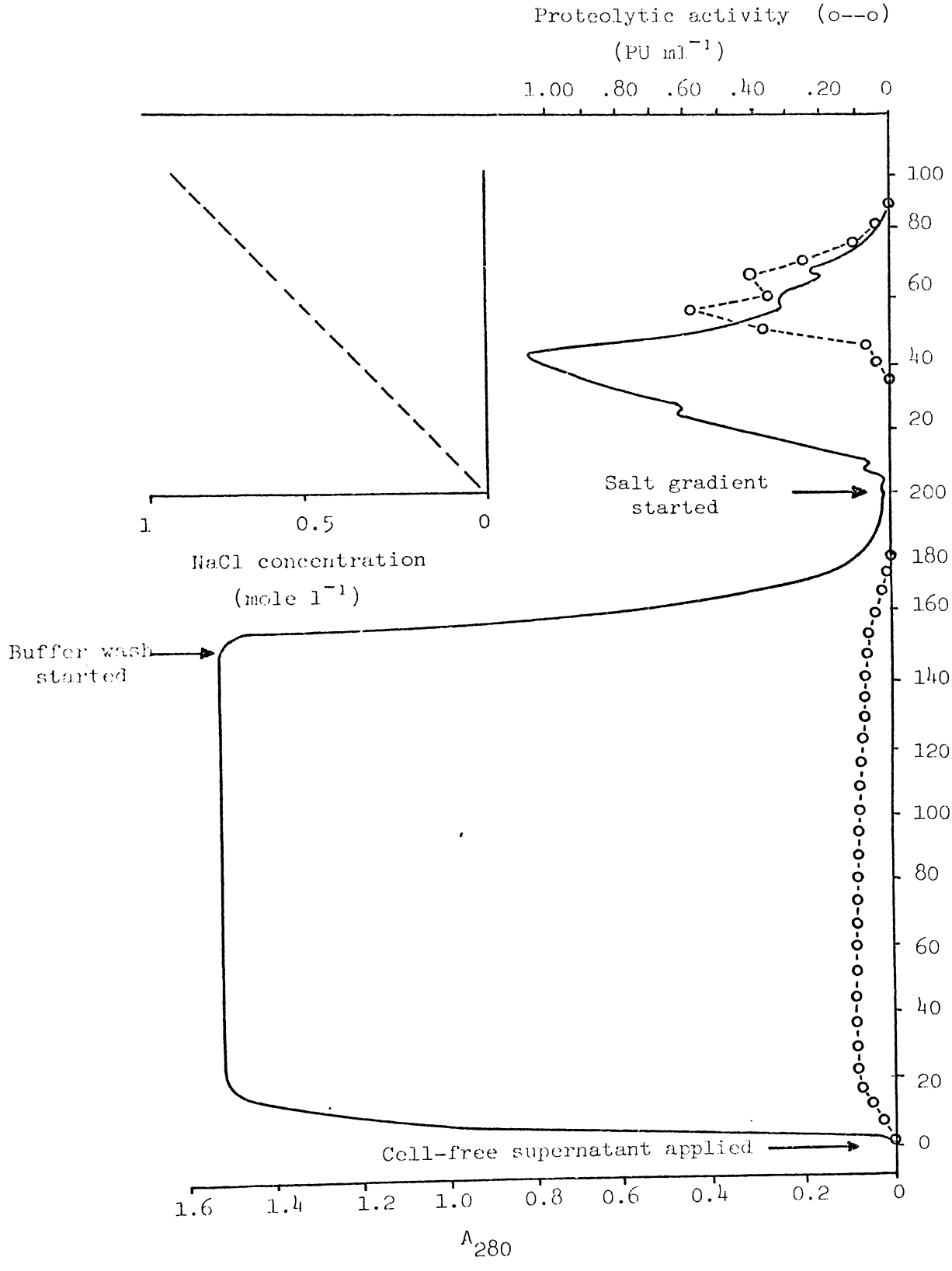
Small volumes (30 - 50 ml settled gel) of a variety of ion exchange gels (Sephadex CM-C25, SP-C25, QAE-A25 and DEAE-A50) were placed in Pharmacia K16 columns. After equilibration with appropriate buffers, the gels were loaded with a volume of protease-containing cell-free supernatant, adjusted to a desired pH, then rewashed with buffer. Adsorbed protein was eluted by application of a linear 0 - 1M sodium chloride gradient, monitored at 280 nm with an ISCO continuous-flow spectrophotometer, and collected in 5 ml aliquots by an LKB 2112 Redirac fraction collector.

After the elution of both cationic and anionic ion exchange gels at pH values ranging from 5.0 to 12.5, the major proportion of extracellular proteolytic activity was not bound. However, a proportion of the activity was adsorbed to SP-C25 gel at pH 7.1. There is no reasonable explanation other than the possibility that the ionic strengths of buffers used was too high, for the failure to adsorb the major protease fraction to any ion exchange gel. Such behaviour is considered to be anomalous. For instance, the absence of binding to anionic exchangers at pH 6.0 to pH 12.5 suggests that the protease or proteases present have very high isoelectric points. Under this assumption, proteases should bind readily to cationic gels at all pH levels below the isoelectric point. However, no more than 30% of total activity could be bound to SP-C25 at pH 7-8. It was concluded (see results in section 7-7) that adsorbed activity was derived from one or more minor proteases and that the major protease, Caldolysin, was eluted without retardation.

This conclusion was partly derived from the marked difference between the two fractions in their manner of hydrolysis on casein-agar assay plates. Caldolysin activity was readily detectable in cell-free supernatants by the appearance of milky rings of hydrolysis on casein-agar plates within 4 - 10 hours incubation at 55^{o}C, while the minor protease fraction gave opaque hydrolysis rings within minutes of application at room temperature. (The presence or absence of NaCl was found to have no effect on the mode of proteolysis of either fraction). That the latter type of enzyme activity was not observed previously in cell-free supernatants is assumed to be a result of the low concentrations present. The presence of minor proteases was subsequently confirmed by gel-slab electrophoresis (section 7-7).

As a result of the difficulties encountered in attempts to bind Caldolysin to an ion exchange gel, it was decided to utilise the absorption

Fig. 7-2. Elution profile from SP-C25 ion exchange chromatography. Cell-free supernatant (pH 7.1) loaded onto gel, washed with pH 7.1 Tris HCl buffer, and eluted with a linear 0 - 1M NaCl gradient.



behaviour of Sephadex SP-C25 as a method of removing minor protease contaminants. A typical elution profile is reproduced in Fig. 7-2. Quantitative data from the separation of major and minor proteases on Sephadex SP-C25 is presented in Table 7-2.

TABLE 7-2 Separation of proteases by ion-exchange chromatography.

Material	Volume (ml)	Total activity (PU)	Total protein (mg)	Specific activity (PU mg^{-1})
Cell-free supernatant	350	31.9	26.9	1.19
SP-C25 eluate	350	24.9	13.1	1.90
SP-C25 adsorbate	100	3.3	1.3	2.54

Yield of Caldolysin = 78.1%

Purification of Caldolysin = 1.6-fold

Despite the failure to bind Caldolysin to an ion exchange gel, the procedure used above forms a useful part of the purification sequence.

However, some other means of concentration of the major protease was necessary. Both millipore ultrafiltration and ammonium sulphate precipitation were attempted (see below).

7-3 Ammonium sulphate fractionation

Two fractionation series were performed. Finely ground ammonium sulphate (BDH, heavy metal-free) was added slowly with rapid stirring to cell-free supernatant cooled to 0°C. The solution was then allowed to stand for 15 minutes to permit protein flocculation, and centrifuged at 12000 x g for 20 minutes. Precipitated protein was redissolved in cold 0.1M pH 8.1 Tris acetic acid buffer, and the supernatant was cooled

to 0°C prior to the addition of the next ammonium sulphate fraction. Redissolved precipitates and supernatants were assayed for proteolytic activity at each step. The results of the two fractionation series are presented in Table 7-3.

TABLE 7-3 Precipitation of proteases by ammonium sulphate

Series 1			Series 2		
$[(NH_4)_2SO_4]$ (% saturation)	% of initial protease activity in:		$[(NH_4)_2SO_4]$ (% saturation)	% of initial protease activity in:	
	Precipitate	Supernatant		Precipitate	Supernatant
30	0	100	37	0	100
45	18	82	53	50	50
60	68	14	67	50	0
75	14	0	100	0	0
100	0	0			

Most of the soluble protease is precipitated between 37% and 67% ammonium sulphate saturation. The procedure was sometimes applied to small volumes of protease-containing *Thermus* culture supernatants, but for large volumes affinity chromatography (section 7-5) was preferred.

7-4 Ultrafiltration

Ultrafiltration was attempted as a means of reducing large volumes of culture fluid to manageable proportions. The apparatus available, a Millipore ultrafilter equipped with 5 sq. ft. membranes of both 1000 MW and 10 000 MW nominal exclusion limits, was used in conjunction with a peristaltic pump.

A volume of cell-free supernatant was initially passed through the 10 000 MW-cutoff membrane. Loss of activity led to attempts to dissociate membrane-bound enzyme.

TABLE 7-4 Recovery of enzyme activity during ultrafiltration

Sample	Volume (ml)	Enzyme activity (PU ml^{-1})	Total activity (PU)	Yield (%)	Protein concentration (µg ml^{-1})	Total protein (µg)	Yield of protein (%)
Supernatant	2000	0.0255	51.0	100	17	34000	100
Concentrate	116	0.0952	11.0	21.6	119	13804	40.6
Permeate	1850	0	0	0	∿6	∿11100	∿32.6
H_2O wash 1.	137	0.0102	1.4	2.7	∿4	∿500	∿1.5
H_2O wash 2.	90	0.0085	0.8	1.6	∿2	∿200	∿0.6
1M NaCl	134	0.0884	11.8	23.2	40	5360	15.8
H_2O wash 3.	114	0.0247	2.8	5.5	∿5	∿500	∿1.5
Total yields				54.6%			91.6%

A further two litre volume of cell-free supernatant was ultrafiltered after which the membrane was washed with both 1M NaCl and distilled water. Enzyme activities and protein concentrations from all fractions were monitored (Table 7-4).

Since about 90% of protein but only 55% of protease activity was recovered, selective adsorption, or denaturation of the protease had occurred. This could either be a feature of unusual charge characteristics of the enzyme protein, or might indicate specific enzyme-substrate binding to proteins irreversibly linked to or trapped within the membrane. It is also possible that physical entrapment within the membrane matrix is responsible for the loss of some activity.

As a result of the high losses of enzyme when using the 10 000 MW-cutoff membrane, this method was discarded as an efficient means for concentrating and purifying bulk culture fluids. The 1000 MW-cutoff membrane, while resulting in a greater recovery of protease during ultrafiltration, was found to be too slow (filtration rate $\sim$ 500 ml hour^{-1}) to conveniently handle the large volumes required.

7-5 Affinity chromatography

Use of the protease-specific affinity substrate, CBZ-D-phenylalanyl-TETA-//-Sepharose 4B (Pierce) has been shown to be useful in the extraction of proteases from cell extracts, and to further purify already 'pure' commercial protease preparations (Fujiwara *et al.*, 1975; Fujiwara & Tsuru, 1977).

A 10 ml volume of the affinity gel was used in the purification of Caldolysin by methods similar to those described in the publications cited above. Initially, protease-containing cell-free supernatant (treated by SP-C25 ion exchange and applied as a 10-times concentrate from Millipore ultrafiltration) was passed through the gel (held in a Pharmacia K 26

column) at a rate of approximately 150 ml $hour^{-1}$.

Initially, samples of eluate were collected at volume intervals of 100 ml and assayed to determine the gel binding capacity (Table 7-5).

TABLE 7-5 Protease-binding capacity of the affinity gel

Volume eluted through affinity gel	Enzyme activity in eluate [a] (PU ml^{-1})	% Binding
100 ml	0	100
200 ml	0	100
300 ml	0	100
400 ml	0	100
500 ml	0.016	95.9
600 ml	0.034	91.3
700 ml	0.066	83.1

a. Activity of solution applied: 0.39 PU ml^{-1}.

It can be calculated that the maximum holding capacity of 10 ml of affinity gel is approximately 314 PU. At a specific activity of 41.8 PU mg $protein^{-1}$ (calculated in Table 7-6 below), this corresponds to approximately 7.5 mg protein (0.75 mg protein ml gel^{-1}). This is rather lower than the commercially quoted figure of 2 mg ml^{-1} for trypsin.

During normal purification sequences (section 7-1), the following procedure was used. After the passage of a volume of protease-containing supernatant, the gel was washed with 0.1 M pH 7.1 Tris HCl buffer containing 10 mM Ca^{2+}, 0.1 M pH 2.8 acetic acid + 10 mM Ca^{2+}, 50 mM pH 9 borate buffer + 1 M NaCl + 10 mM Ca^{2+}, and Tris HCl buffer. All eluted material was monitored at 280 nm by an ISCO continuous-flow spectrophotometer (connected to a Serviscribe Chart Recorder) and collected in 5 ml fractions

by a LKB 2112 Redirac fraction collector. A typical elution profile demonstrating the distribution of protein and protease activity is reproduced in Fig. 7-3.

It was found that all adsorbed protease was eluted by the acetic acid wash. Despite the considerable amount of protein eluted with borate buffer (and with pH 7 1M guanidine HCl, when used), no proteolytic activity was ever detected in these fractions. It was noted that a considerable quantity of a purple pigment was bound to the gel during application of the media, and only eluted during the pH 9 wash. In addition, a grey/brown material became permanently absorbed to the gel, and could not be removed by buffers between pH 2.8 and 10.0 containing 1M concentrations of NaCl. However, accumulation of this material appeared to have no effect on the affinity capacity of the gel, which did not decrease with repeated applications.

Quantitative results obtained from the application of 750 ml of Millipore concentrate to the affinity gel are presented in Table 7-6.

TABLE 7-6 Purification of Caldolysin by affinity chromatography.

Sample	Enzyme activity (PU ml^{-1})	Volume (ml)	Total activity (PU)	[Protein] ($\mu g\ ml^{-1}$)	Total protein (mg)	Specific activity (PU mg^{-1})
Concentrate	0.39	750	292.5	94	70.5	4.15
Acetic acid eluate	3.88	56	217.3	92	5.2	41.8
Yield = 74.3%			Purification = 10.1-fold.			

a. Yields ranged from 69% to 75%.

The use of affinity chromatography resulted in a greater degree of purification than any other technique attempted.

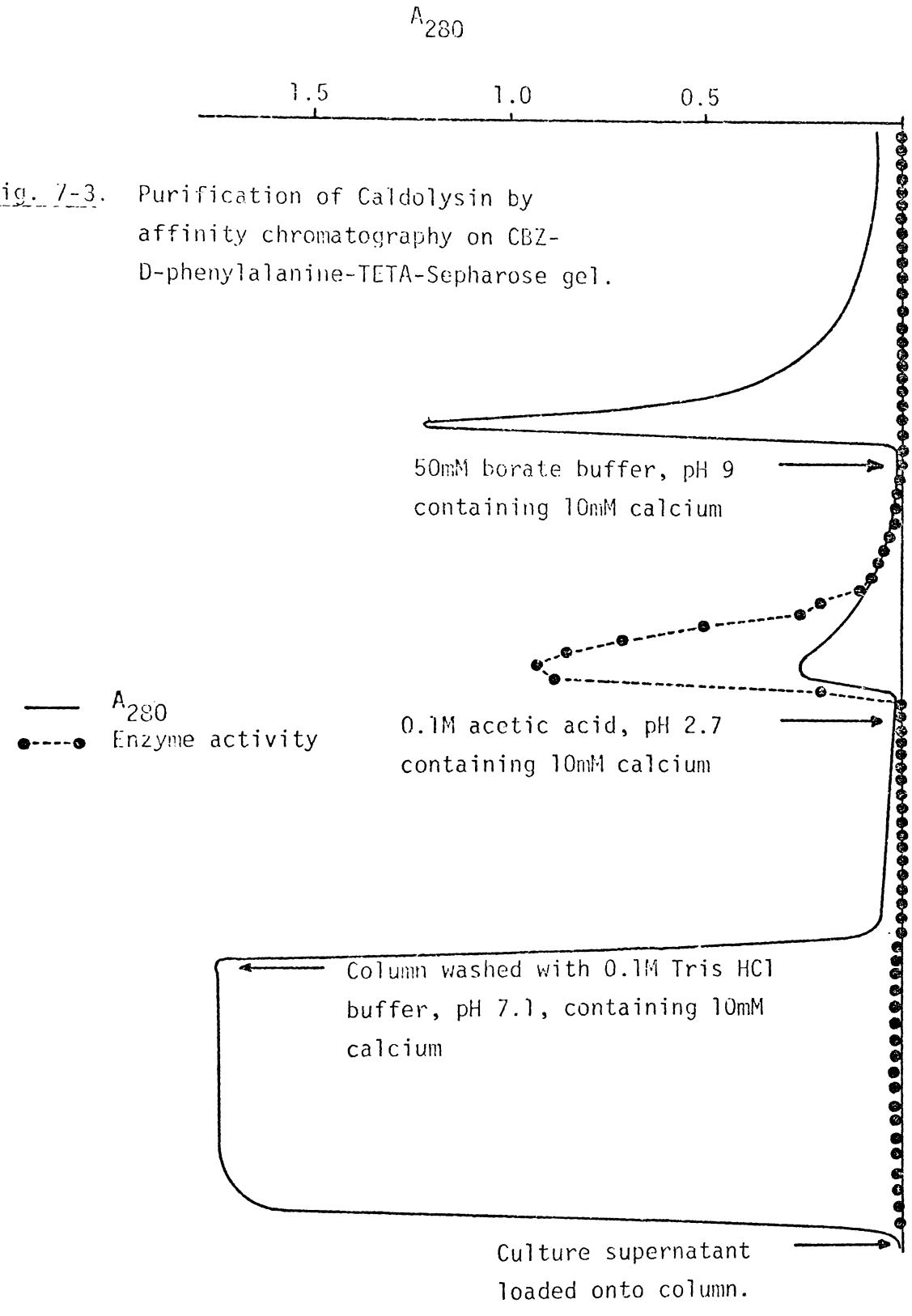


Fig. 7-3. Purification of Caldolysin by affinity chromatography on CBZ-D-phenylalanine-TETA-Sepharose gel.

7-6 Gel filtration chromatography

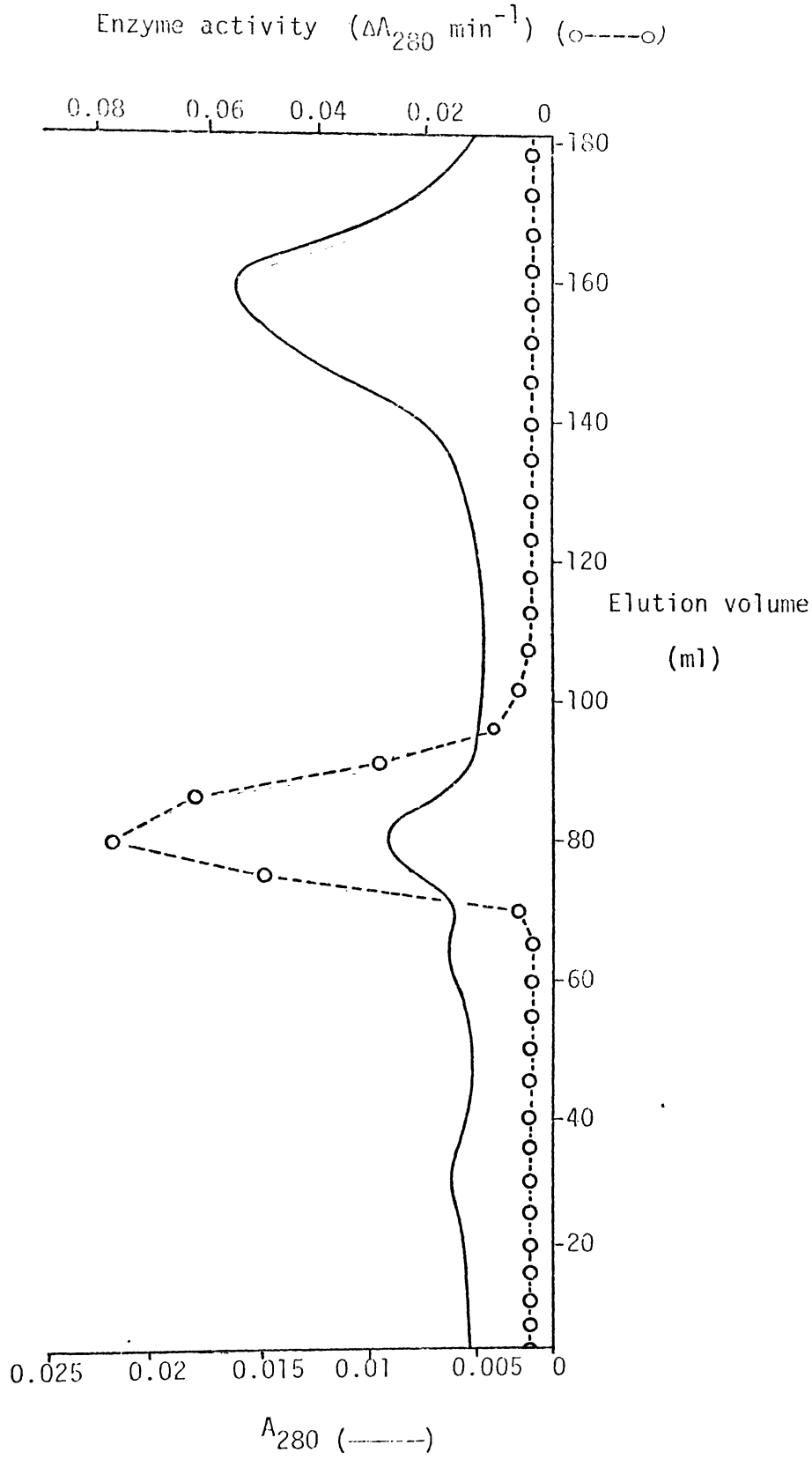
Sephadex gel filtration chromatography was routinely used as the final step in the purification sequence of Caldolysin (section 7-1).

Five millilitre volumes of enzyme concentrate (100 ml of affinity gel eluate lyophilised and redissolved in 5 ml of distilled water) were applied to the column (Sephadex G75 gel loaded in a 30 cm Pharmacia K26 column) and eluted with 0.01 M pH 8.1 Tris acetic acid buffer containing 5 mM $CaCl_2$. Eluted material was monitored by an ISCO continuous-flow spectrophotometer at 280 nm, and collected in 5 ml fractions by a LKB 2112 Redirac fraction collector. An elution profile is reproduced in Fig. 7-4, and shows a single activity peak corresponding to a peak of 280 nm absorbing material.

However, considerable variation in elution behaviour resulted in activity peaks ranging from V_e=80 ml to V_e=160 ml under similar conditions of pressure, flow rate, buffer, etc. By comparison with protein standards, molecular weights ranging from approximately 9000 to 24 000 were calculated. Similar behaviour with a number of extracellular proteases has been noted previously (Voorduow *et al.*, 1974a). While it has been suggested that the basic nature of a protein could result in chromatographic anomalies, such a hypothesis is untenable in the case of enzymes such as Thermomycolin (pI = 6). However, when an enzyme sample was chromatographed in a buffer containing 5 M NaCl, the elution volume at which the Caldolysin peak was eluted was considerably reduced (V_e (Tris buffer) = 160 ml; V_e (Tris buffer + 0.5 M NaCl) = 85 ml). The significance of the retardation of Caldolysin during gel chromatography is discussed more fully in Chapter 14.

Repeated gel chromatography runs gave an approximate molecular weight for Caldolysin of 22800 ± 2300, by comparison with the elution volumes of protein standards (Andrews, 1965). Typical quantitative data from the

Fig. 7-4. Purification of Caldolysin by G75 Sephadex gel chromatography. (Buffer: 0.01M Tris acetic acid, pH 8.1, + 5mM calcium)



purification of Caldolysin by gel filtration chromatography is shown in Table 7-7.

TABLE 7-7 Purification of Caldolysin by gel filtration chromatography

Sample	Volume (ml)	Activity (PU ml^{-1})	Total activity (PU)	Protein ($\mu g\ ml^{-1}$)	Total protein (mg)	Specific activity (PU mg^{-1})
Affinity eluate	125	1070	133 900	23	2.875	46 600
G75 eluate	42.5	2200	93 600	19	0.808	115 840
Yield = 70%			Purification = 2.49-fold			

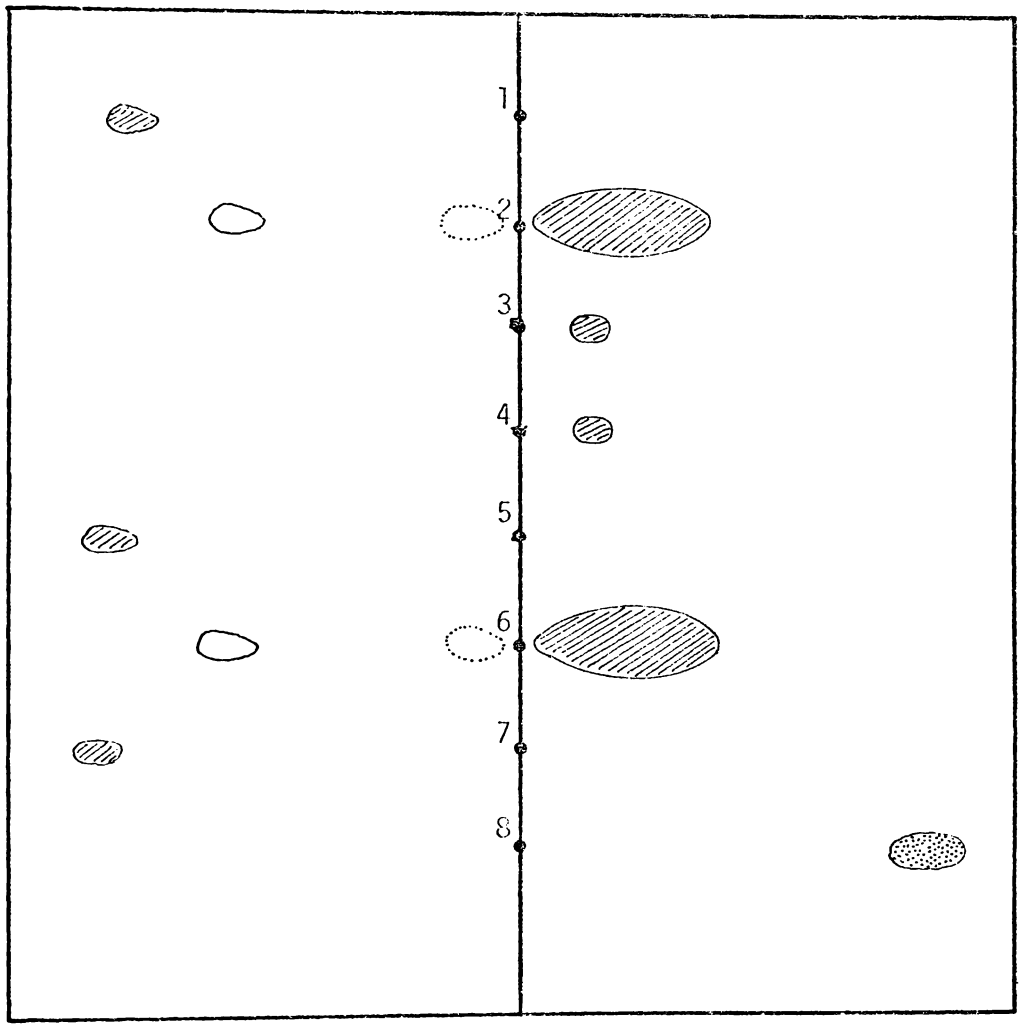
After gel chromatography, Caldolysin was found to be homogeneous by a number of electrophoretic methods (section 7-7). However, calculated specific activities were found to range from 75 000 to 160 000 µg tyr. $min^{-1}\ mg^{-1}$. It is assumed that this variation is derived from varying degrees of autolysis in the samples of purified enzyme tested.

7-7 Determination of homogeneity and molecular weight of Caldolysin

A variety of chromatographic and electrophoretic techniques were employed in the determination of the purity of the final enzyme preparation and its molecular weight.

a. The presence of two minor proteases in the cell-free supernatant, and their removal by SP-C25 ion exchange chromatography was demonstrated by gel-slab electrophoresis. A reproduction of a replica plate (Fig. 7-5) indicates the location of proteases only, since the technique of layering casein-agar gel onto the electrophoresis gel precludes subsequent protein staining.

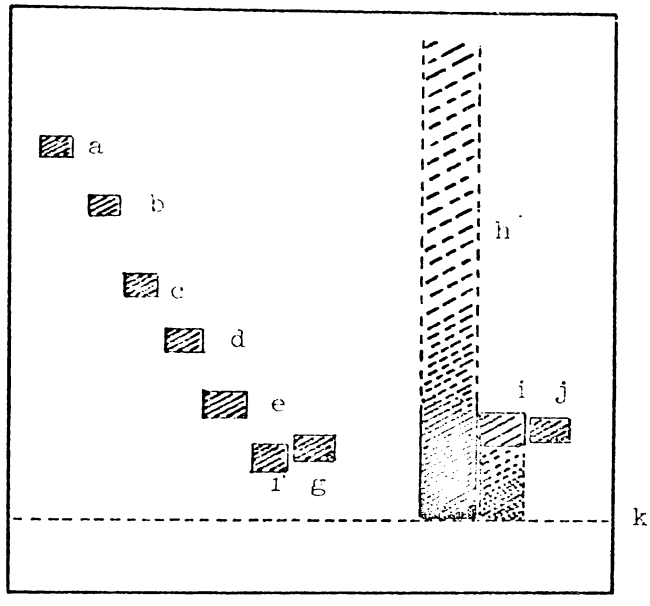
Fig. 7-5. Replica plate of proteolytic activity after electrophoresis of protease-containing fractions on a 20cm x 20cm agar gel slab.
(Buffer: pH 8.1 Tris acetic acid, 0.1M; 5µl sample applications)



1. 0.01 mg ml^{-1} Thermolysin
2. Cell-free *Thermus* culture supernatant
3. Ion exchange gel (SP-C25) eluate
4. as 3.
5. as 1.
6. as 2.
7. as 1.
8. 0.1% bromophenol blue (position apparent from colour)

b. SDS-polyarcylamide electrophoresis of different fractions from the purification sequence of Caldolysin indicated that the final enzyme preparation was homogeneous, within the detection limits of the protein stain (Fig. 7-6).

Fig. 7-6 SDS-gel slab electrophoresis



a. Bovine serum albumin
b. Ovalbumin
c. Pepsin
d. Soybean trypsin inhibitor
e. β-lactoglobulin
f. Lysozyme
g. Cytochrome C
h. Cell-free supernatant
i. Affinity gel eluate
j. Purified Caldolysin

k. Migration front

In a comparison of the R_f values of Caldolysin with those of standard proteins, a molecular weight of 16300 ± 1700 was estimated.

c. Molecular estimation by Gradipore electrophoresis (section 4-13) gave a value of 20450 ± 250. The mobilities of standard proteins were graphed against the cube root of the molecular weights as described in Manwell (1977).

d. The determiniation of molecular weight by Sephadex G75 gel filtration chromatography has been discussed briefly in section 7-6. The comparison of the mean elution volume of Caldolysin derived from a number of G75 chromatography runs with those of standard proteins gave a molecular weight estimate of 22800 ± 2 300.

From the three methods of molecular weight determination outlined above, it is estimated that the molecular weight of Caldolysin is approximately 20 000 ± 1 000.

CHAPTER 8

CHARACTERISATION OF CALDOLYSIN

8-1 The isoelectric point of Caldolysin

A preliminary determination was carried out by investigating the electrophoretic mobility of Caldolysin in comparison with that of cytochrome c (horse heart, Sigma), on cellulose acetate strips (Titan III, Helena) at selected pH's.

The apparent pI of cytochrome c (quoted by Righetti & Caravaggio (1976) as 9.4) was estimated to lie between pH 10.5 and pH 11.5, while that of Caldolysin was approximately 10.5. However, the charge composition of cellulose acetate results in a high degree of endosmosis, and consequently a considerable deviation from true electrophoretic behaviour (P.C. Molan, pers. comm.). Thus the pI of cytochrome c was apparently overestimated by one to two pH units, so that the true value of Caldolysin is probably between 8.5 and 9.5.

Polyacrylamide gels are less susceptible to electroendosmosis because of the low charge capacity of the gel. Such deviations can be eliminated by removal of the acrylic acid from the acrylamide (a by-product formed by oxidation during storage) prior to preparation of the gel (P.C. Molan, pers. comm.). Polyacrylamide gel slabs were prepared by pouring a mixture of 3 ml 30% acrylamide (eluted through a DEAE ion exchange column), 3 ml buffer, 10 µl TEMED, and 200 µl of 7% ammonium persulphate (dissolved in buffer and adjusted to desired pH with 10 M NaOH) into a 8 cm x 8 cm x 1.5 mm glass mould. Gels set within 5 minutes. 5 mm diameter wells were cut in the gel for sample application (4 µl). Gels were electrophoresed at 10 mM and 200 V for two hours. Protease activity was detected as described in section 4-10.

By this method, a pI of 8.5 was indicated for Caldolysin, but the value for cytochrome c was still about one pH unit higher than the published value.

A further determination was carried out by isoelectric focussing on a commercial PAG-plate (pH 3.5 - 9.5; LKB), as described in section 4-13. After electrophoresis, a single band of proteolytic activity was detected at the extreme cathodic margin of the gel. Since the pH gradients at either end of isoelectric focussing gel rise sharply, the pI could be estimated no more accurately than between pH's 8.5 and 9.5.

A simple technique for extending the upper pH range of commercially prepared pH 3.5 - 9.5 isoelectric focussing gels, described by R.R. Bürk, (unpublished results), involves the placement of a strip of filter paper, saturated with high pH ampholines, on the surface of the gel. After focussing cytochrome c and Caldolysin samples for 90 minutes at 250 V and 30 mA, the gel was treated as described in section 4-13. pI values for cytochrome c and Caldolysin of 9.5 and 8.5 respectively, were calculated. Since the published value for cytochrome c is 9.4, the pI of Caldolysin is estimated to be approximately 8.5

8-2 The response of Caldolysin to inhibitors

The reactions of proteases with well-defined classes of inhibitors often form the basis for initial classification (section 1-1).

The effect of a variety of such inhibitors on the enzyme activity of purified Caldolysin is shown in Table 8-1. These results were obtained by standard Kunitz assays of Caldolysin for periods of 30 to 90 minutes after the addition of the specified concentration of each inhibitor.

TABLE 8-1 The effect of inhibitors on Caldolysin activity

Class	Inhibitor	Concentration	% inhibition of proteolytic activity
Serine inhibitor	Phenyl methyl sulphonyl fluoride	2.0 mM	14%
		0.5 mM	0
		0.1 mM	0
Cysteine inhibitor	Iodoacetic acid	10.0 mM	75%
		2.5 mM	17%
		0.25mM	0
Cysteine inhibitor	p-Chloromercuribenzoate [a]	5.0 mM	0
		2.5 mM	0
Acid protease inhibitor	N-α-p-tosyl-L-lysine chloromethyl ketone	0.03mM	0
Trypsin inhibitor	Soybean trypsin inhibitor	1.0 mg ml^{-1}	0
Metal chelator	EDTA	10.0 mM	68%
		1.0 mM	40%
		0.13mM	0
Calcium-specific chelator	EGTA [b]	10.0 mM	45%
		1.0 mM	18%
Zinc-specific chelator	o-Phenanthroline	10.0 mM	0
		1.0 mM	0

a. Must be dissolved at pH 10-11, then adjusted to desired pH.

b. Ethylene glycol-bis-(β-amino ethyl ether) N, N'-tetra acetic acid (Sigma).

Since little or no inhibition results from the presence of phenyl methyl sulphonyl fluoride (PMSF), trypsin inhibitor, or tosyl-lysine chloromethyl ketone, it is assumed that Caldolysin does not belong to the classes of serine or acid proteases. The loss of 14% of enzyme activity after incubation for less than one hour in the presence of 2 mM PMSF is insufficient to indicate the presence of an active-site serine residue. For example, the serine protease, Thermomycolin, was completely inhibited after incubation at 30°C for 60 minutes in the presence of 0.05 mM PMSF (Ong & Gaucher, 1976). Furthermore, the pH optimum of Caldolysin (section 8-9) is incompatible with the category of acid proteases.

Contradictory results derived from the presence of cysteine inhibitors can be explained in terms of the broad specificity of iodoacetic acid. It has been established (Gundlach *et al.*, 1959) that iodoacetic acid can react with side-groups of amino acids other than cysteine, notably the imidizole group of histidine, the ε-amino group of lysine, the phenolic hydroxyl of tyrosine, and the heterocyclic atom of methionine. While being slow at neutral or acidic pH's these reactions, in particular that with lysine, occur at significant rates. Since inhibitor studies of Caldolysin were performed at pH 8.1, it is likely that reaction between iodoacetic acid and an active-site residue other than cysteine is responsible for the loss of enzyme activity. True sulphydryl proteases such as Cathepsin C are fully inhibited by 1 mM iodoacetic acid (Fruton & Mycek, 1956).

The inhibition of enzyme activity caused by EDTA and EGTA suggests that Caldolysin is a metalloenzyme, probably containing calcium ions. The lack of response to o-phenanthroline implies that zinc ions are not involved in the activity of the enzyme. These observations are discussed in more detail below.

8-3 The role of metal ions in Caldolysin

Proteases containing calcium and zinc are well documented (e.g. Thermolysin and *B. subtilis* neutral protease each contain one zinc and a number of calcium ions (Voorduow *et al.*, 1976a; Roche & Voorduow, 1978; McConn *et al.*, 1964)). However, it has been established in both cases that while zinc is bound within the active site cleft and is intimately involved in the catalytic mechanism, the calcium ions are bound in externally located sites and are involved in structural stabilisation. No documented cases of the location of calcium as an active-site cofactor in proteases have been found.

It is possible that an active-site located zinc ion might be tightly bound, and that a slow rate of chelation could account for the lack of inhibition noted in Table 8-1. To test this hypothesis, a sample of Caldolysin was incubated at 75°C in the presence of 25 mM o-phenanthroline. Enzyme activity loss was monitored during the incubation and compared with an inhibitor-free control (Fig. 8-1).

The similarity between the two activity profiles suggests that either; (a) Caldolysin contains no zinc ion cofactor, or (b) any zinc atom present is sufficiently tightly bound to defy chelation under the conditions employed. Since the zinc ion of Thermolysin was rapidly chelated by 0.1 mM o-phenanthroline at 25°C (Voorduow *et al.*, 1976) the simplest explanation is that zinc ions play no part in the activity or stabilisation of Caldolysin.

The loss of enzymic activity caused by the action of EDTA and EGTA could result from either of two processes:

a. The removal of an active-site bound cofactor essential for enzyme catalysis.

Fig. 8-1. The effect of o-phenanthroline on enzyme activity. ($29\mu g\ ml^{-1}$ enzyme, pH 8.1, 0.1M Tris acetic acid buffer, incubated at $75^{o}C$, assayed at intervals by Kunitz method)

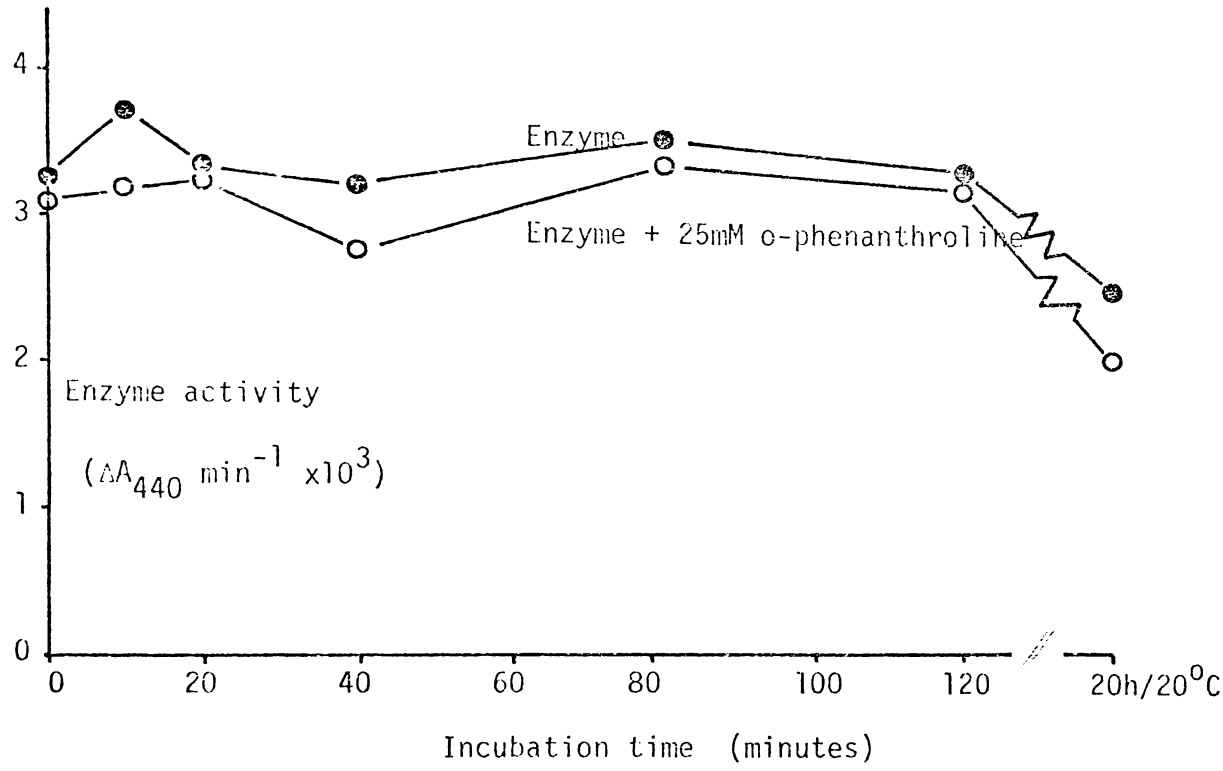
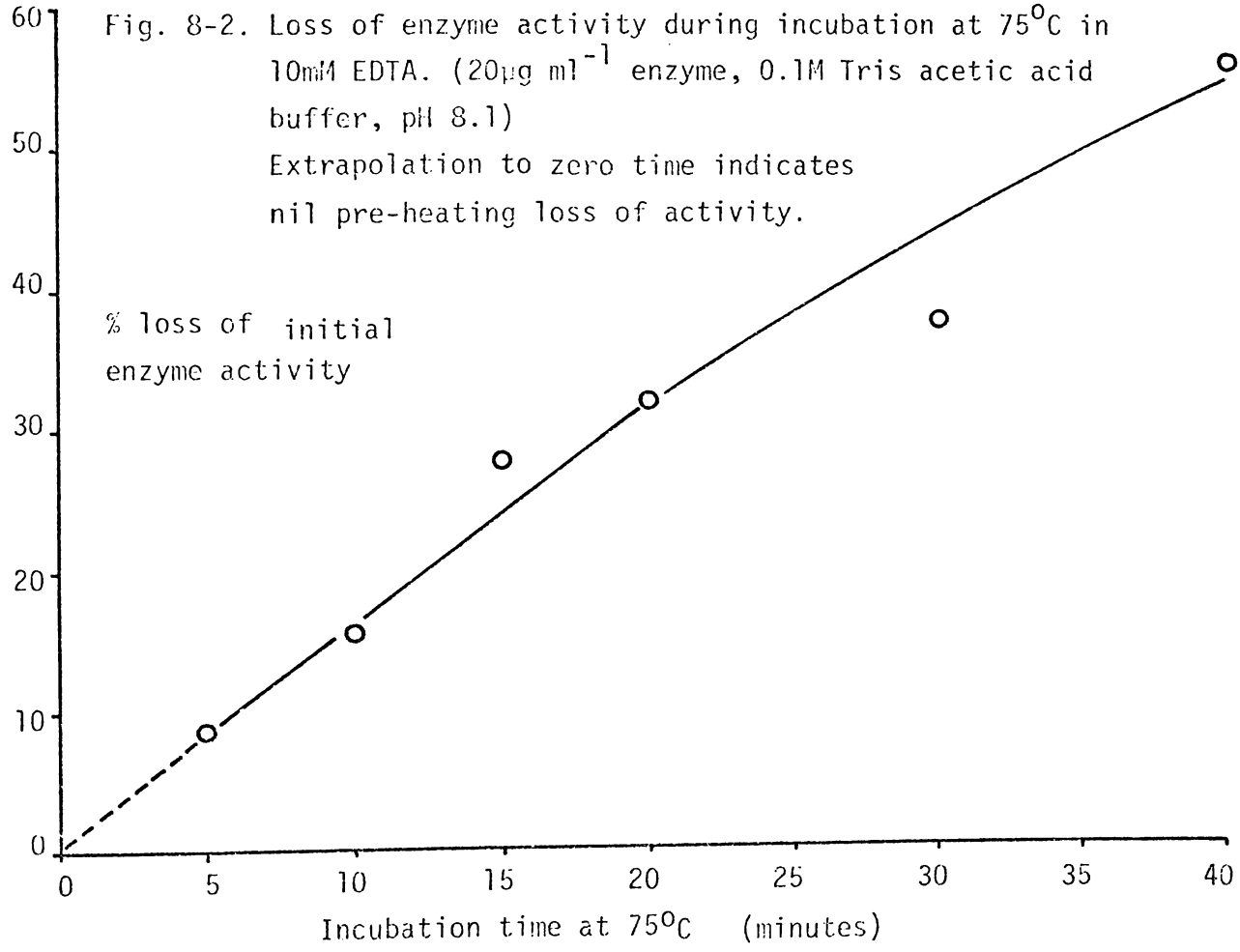


Fig. 8-2. Loss of enzyme activity during incubation at $75^{o}C$ in 10mM EDTA. ($20\mu g\ ml^{-1}$ enzyme, 0.1M Tris acetic acid buffer, pH 8.1)
Extrapolation to zero time indicates nil pre-heating loss of activity.



b. The abstraction of a metal ion from a site of structural stabilisation. The removal of such a component would result in the loss of enzyme activity as a product of accelerated autolysis or denaturation.

To determine whether calcium was important for enzyme activity or molecular stability, the following experiments were carried out.

A solution of Caldolysin of known activity was pre-incubated at 20°C for 60 minutes in the presence of 10 mM EDTA, at which temperature autolysis and denaturation have been shown to be insignificant (section 9-3). The enzyme solution was then heated to 75°C, and the loss of enzyme activity monitored over a period of 40 minutes. If calcium was involved in an active-site role, a substantial loss of enzyme activity should be observed at the beginning of the high temperature incubation period, resulting from the prior removal of the active-site cofactor. Alternatively, if only present in stabilisation sites, no initial loss of activity should be observed, but activity should decrease with subsequent heating as autolysis and denaturation progress.

The results from this experiment demonstrate clearly that no loss of enzyme activity has occurred prior to incubation at 75°C (Fig. 8-2). This suggests that calcium is involved only in the maintenance of molecular integrity, given the possible exception that ions in the active site might be inaccessible to EDTA.

In a further experiment (results not shown), EDTA-treated "apocaldolysin" was found to bind to and dissociate from the protease-specific affinity substrate (CBZ-phe-TETA-Sepharose 4B) in a manner identical to the holoenzyme. While there is no certainty that substrate binding would be effected by the removal of an active site metal ion, this result tends to support the contention that calcium is not involved in catalysis.

Results presented in section 9-2 on the influence of calcium concentration on the thermostability of Caldolysin provide further evidence of the structural rather than catalytic role of calcium. Thus, on the basis of inhibitor sensitivity, Caldolysin cannot be assigned to any of the categories of proteases outlined in Table 1-2.

8-4 Substrate specificity: Protein hydrolysis

Solutions or suspensions of a number of proteins and dye-labelled proteins (0.5%, pH 8.1) were assayed by the standard Kunitz procedure. Comparative data were obtained by the addition of 100 µl aliquots of a 20 µg ml^{-1} Caldolysin solution. Reaction tubes containing insoluble proteins were agitated throughout the assay period. Results are presented below.

TABLE 8-2 Hydrolysis of proteins by Caldolysin

Substrate	Rate of hydrolysis (ΔA_{280} min^{-1} x 10^3)	% of rate of casein hydrolysis
casein	3.33	100
ovalbumin	1.45	44
bovine serum albumin	1.33	40
heamoglobin	0.90	27
collagen	0.70	21
fibrin	0.65	18
	Rate of hydrolysis (ΔA_{440} min^{-1} x 10^3)	% of rate of azo-casein hydrolysis
azo-casein	2.75	100
azo-albumin	4.15	151
azo-collagen	0.87	32
	(ΔA_{395} min^{-1} x 10^3)	
elastin-congo red	0.25	approx. 7

Of the native proteins tested, casein shows the highest degree of susceptibility to proteolysis. The insoluble proteins, collagen, fibrin and heamoglobin (the latter precipitates rapidly at 75°C) are less susceptible, but nevertheless, still hydrolysed.

It is noted that while the rate of proteolysis of casein is twice that of albumin, the reverse is found for the azo-dye derivatives. Two possible explanations for this are:

a. Structural changes in the protein during azo-derivitisation resulting in increased accessibility of susceptible albumin peptide bonds, or the steric interference of others in casein.

b. An underestimation of the true rate of albumin hydrolysis resulting from an inhomogeneous distribution of aromatic groups in the molecule. (The reverse situation in casein could equally well apply). Calorimetric estimation of azo chromophores, which are bound to free amine moieties, avoids such inconsistencies.

The ability to hydrolyse one or more of the insoluble fibrous proteins is a characteristic of microbial proteases, especially the metal-chelator-sensitive neutral proteases (Matsubara & Feder, 1971).

8-5 Substrate specificy; Hydrolysis of peptides and low molecular weight substrates

The action of Caldolysin on individual peptide, ester, and amide substrates (Table 8-3) was determined either spectrophotometrically or chromatographically. The assay of chromogenic substrates was carried out as described in section 4-6. The chromatographic estimation of hydrolysis products was performed by single dimension descending chromatography on Whatmans 1 paper in a n-butanol: acetic acid: water (4:1:5 top-phase) liquid phase. Amino acid and peptide spots were visualised with ninhydrin. The splitting point (peptide bond hydrolysed)

was determined by comparison of ninhydrin-positive hydrolysis products with standard amino acids, dipeptides, and tripeptides.

TABLE 8-3 Hydrolysis of peptide and peptide analogues by Caldolysin

Substrate	Hydrolysis	Bond hydrolysed	Reference
Gly-gly	-	-	Nunokawa & McDonald (1968)
Gly-gly-gly	-	-	"
Gly-gly-gly-gly	+	gly-gly	"
Gly-gly-gly-gly-gly	+	gly-gly	"
D-leu-gly	-	-	"
L-leu-gly	-	-	"
BOC-ala-try-met-asp-phe-NH_2	-	-	"
CBZ-gly-phe-NH_2	-	-	Feder & Schuck (1970)
Acetyl-ala-ala-ala-OMe	+	ala-ala	Gertler & Hofman (1970)
CBZ-gly-pro-gly-gly-pro-ala	+	gly-pro	Schoellmann & Fisher (1966)
CBZ-gly-pro-leu-gly-pro	+	pro-leu	Matsubara & Feder (1971)
Benzoyl-arginine ethyl ester	-	-	Schwert & Takenaka (1955)
CBZ-gly-p-nitro-phenyl ester	-	-	Ong & Gaucher (1976)
Tosyl-arginine ethyl ester	-	-	Walsh (1970)
Benzoyl-arginine-p-nitroanilide	-	-	Erlanger *et al.* (1961)
Benzoyl-phe-val-arg-p-nitro-anilide	+	amide	Bergstrom (1977)
CBZ-gly-pro-arg-p-nitroanilide	-	-	Bergstrom (1977)

The following conclusions are derived from the data above:

a. Substrates containing less than four groups (amino acid residues and/or terminal blocking groups) are not hydrolysed by Caldolysin.

b. Caldolysin apparently possesses no esterase activity. The substrate Acetyl-ala-ala-ala-OMe was shown to be hydrolysed at an alanine-alanine linkage and no ester hydrolysis was detected spectrophotometrically.

c. Within the scope of the limited data available, a specificity for small neutral aliphatic amino acid residues (gly, ala, pro, leu) on either side of the splitting point is indicated.

Some anomalies exist in the data presented above. While the failure to hydrolyse BOC-ala-try-met-asp-phe-NH_2 might result from the absence of a suitable peptide linkage in the correct orientation (data suggest that preferentially hydrolysed bonds are third from C-terminus), the failure of Caldolysin to hydrolyse CBZ-gly-pro-arg-p-nitroanilide is unexplained.

The absence of esterase activity in Caldolysin is not conclusively proven since three of the four ester substrates would not fulfil the active site size requirements discussed above.

8-6 Lysis of microorganisms

The lytic activity of purified Caldolysin against a variety of microorganisms was tested by the lysoplate method described in section 4-8. The results are presented below.

Of the 18 strains or species of Gram-negative bacteria tested, all but *Agrobacterium tumefaciens* and *Cytophaga johnsonae* were lysed by Caldolysin. None of the 12 Gram-positive microorganisms were fully lysed, and only four showed partial lysis. Results from this limited range of microorganisms suggest a marked specificity for Gram-negative cell-wall structures. This specificity is uncommon since the 'outer

TABLE 8-4 Lysis of microorganisms at 75°C by Caldolysin (20 μg ml^{-1}, 0.1 M CH_3COONa, pH 7.5)

Microorganism	Gram reaction [a]	Complete lysis	Partial lysis	No lysis
Arthrobacter globiformis	+			+
Arthrobacter No. 9	+			+
Bacillus cereus	+		+	
Bacillus megaterium	+			+
Bacillus circulans No. 9374	+		+	
Micrococcus luteus	+			+
Micrococcus lysodeikticus	+		+	
Saccharomyces cerevisiae	+			+
Sarcina lutea	+			+
Sporeformer (unidentified *Bacillus*)	+			+
Staphylococcus aureus	+			+
Streptomyces griseus	+		+	
Agrobacterium tumefaciens	-			+
Alcaligenes faecilis	-	+		
Alcaligenes viscolactis	-	+		
Citrobacter freundii	-	+		
Cytophaga johnsonae C_4	-			+
Escherichia coli B	-	+		
Escherichia coli K_{12}	-	+		
Escherichia coli K_{12}Hfr	-	+		
Escherichia coli W	-	+		
Enterobacter aerogenes	-	+		
Enterobacter cloacae	-	+		
Klebsiella pneumoniae	-	+		
Proteus vulgaris	-	+		
Pseudomonas aerogenes	-	+		
Salmonella typhimurium	-	+		
Serratia marcescens	-	+		
Shigella flexneri	-	+		
Shigella sonnei	-	+		

a. Gram reactions quoted from Bergeys Manual of Determinative Bacteriology (1974), 8th edition, (Buchanan R.E. and Gibbons N.E., eds.) Williams & Wilkins Ltd.

multiple-track layer' of lipoprotein and lipopolysaccharide in Gram-negative cells prevents most lytic enzymes from reaching the underlying peptidoglycan (Ghuysen, 1968). However, Golinova *et al.* (1973) have isolated a thermophilic lytic enzyme from *Micromonospora vulgaris* which, while lysing both Gram-positive and negative cells, is more active against the latter. Although the peptidoglycan cleavage site of Caldolysin has not been established, the peptidase activity demonstrated against tetraglycine and pentaglycine may be significant. Pentaglycine cross-linkages between polysaccharide chains are common in the peptidoglycans of Gram-positive microorganisms and may occur in Gram-negatives (Strominger & Ghuysen, 1967).

The ability of Caldolysin to lyse bacterial cells enables the assignment of this enzyme to the category of lytic proteases (Table 1-2). The *Myxobacter* Al-1 protease (Jackson & Matsueda, 1970) shows some similarities to Caldolysin, in particular its substrate size requirement which precludes the use of any of the low molecular weight analogues as assay substrates (Jackson & Wolfe, 1968). In addition, it will hydrolyse penta- and tetraglycine but not triglycine or diglycine, as found for Caldolysin (section 8-5). However, Al-1 lytic protease has been shown to lyse Gram-positive bacteria almost exclusively (Ensign & Wolfe, 1965).

The high pI of α-lytic protease from a *Sorangium* species (Whitaker, 1970) and the low molecular weights of *Sorangium* α-and β-lytic proteases are comparable with data obtained for Caldolysin (section 7-9). The similarities and differences between Caldolysin and other bacterial lytic proteases will be discussed in more detail in Chapter 14. However, no documented lytic protease demonstrates thermostability comparable to that of Caldolysin.

8-7 Michaelis-Menten Kinetics

Kinetic data for Caldolysin (reaction velocity vs substrate concentration) were initially determined by Kunitz assays using protein substrates at concentrations ranging from 2% to 0.005%. Both Michaelis-Menten (v vs s) and Lineweaver-Burk (1/v vs 1/s) relationships are shown in Fig. 8-3. A Lineweaver-Burk plot of data from the hydrolysis of azo-albumin is presented in Fig. 8-4.

It is evident that strong substrate inhibition occurs in the hydrolysis of both casein and azo-albumin. A value of K_s' for casein, (the inhibition constant of the binding between enzyme and substrate) has been estimated from a plot of 1/v vs s (Fig. 8-5) (for a theoretical treatment of the derivation of K_s', see Dixon & Webb, 1964). A K_s' of 0.085% (i.e. 0.85 mg ml^{-1} casein) was determined from the intercept on the X-axis. The K_s' value is an indication of the lower limit of "competitive inhibition by the substrate itself", since the theoretical derivation of the term K_s' is based on the assumption that the reaction, $ES + S \rightarrow ES_2$ is responsible for the inhibition of activity. While it is assumed (Dixon & Webb, 1964) that the ES_2 complex consists of two substrate molecules simultaneously bound to different portions of the active site (competitive inhibition), it is considered that a binding site other than the active site (non-competitive inhibition) could produce a similar inhibitory effect. The mechanisms of substrate inhibition are discussed in more detail in Chapter 10.

The influence of deviations at both high and low substrate concentrations reduces the accuracy of K_m values (est. 0.28 mg ml^{-1} for azo-albumin) derived from the hydrolysis of protein substrates.

No substrate inhibition was observed when the low molecular weight synthetic substrate, Benzoyl-phe-val-arg-pNA, was used (Fig. 8-6). This suggests that substrate inhibition in Caldolysin may not be the result

Fig. 8-3a. Michaelis-Menten plot from the hydrolysis of casein by Caldolysin. (5μg ml^{-1} enzyme, substrate dissolved in pH 8.1, 0.1M Tris acetic acid buffer, assays at 75°C)

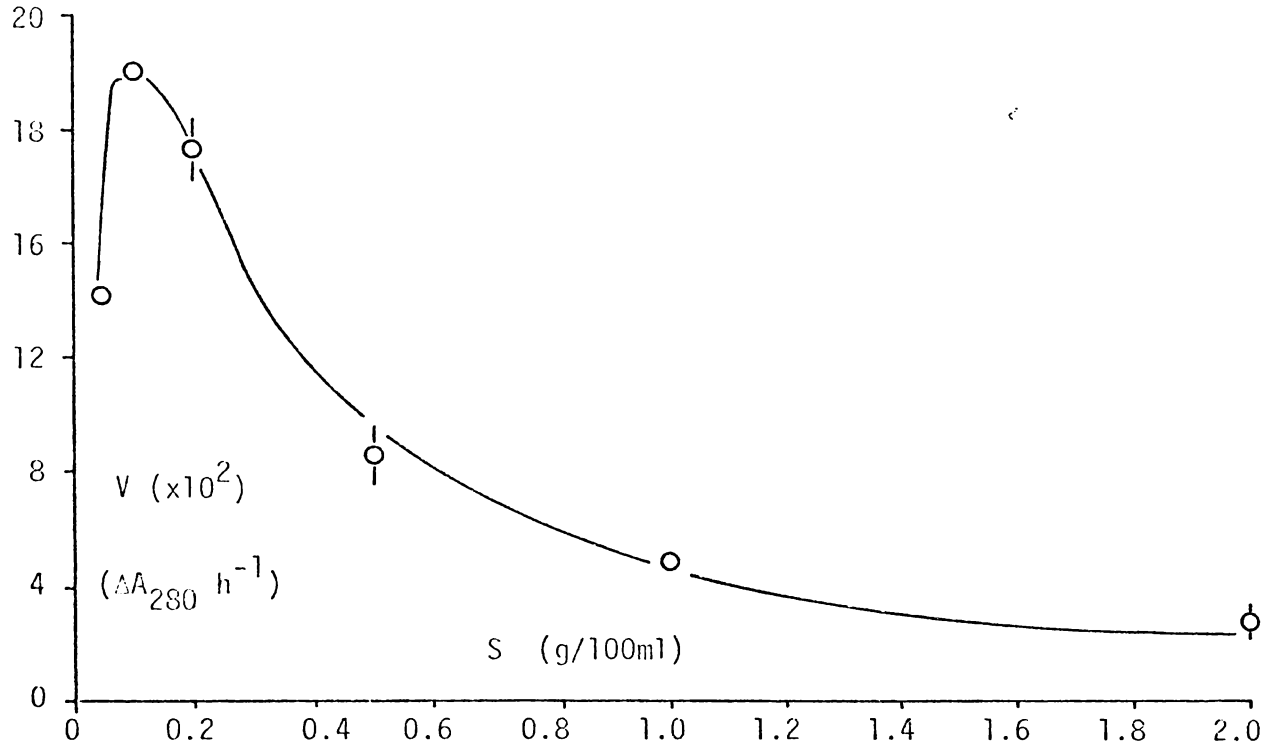


Fig. 8-3b. Lineweaver-Burk plot from the hydrolysis of casein by Caldolysin. (conditions as above)

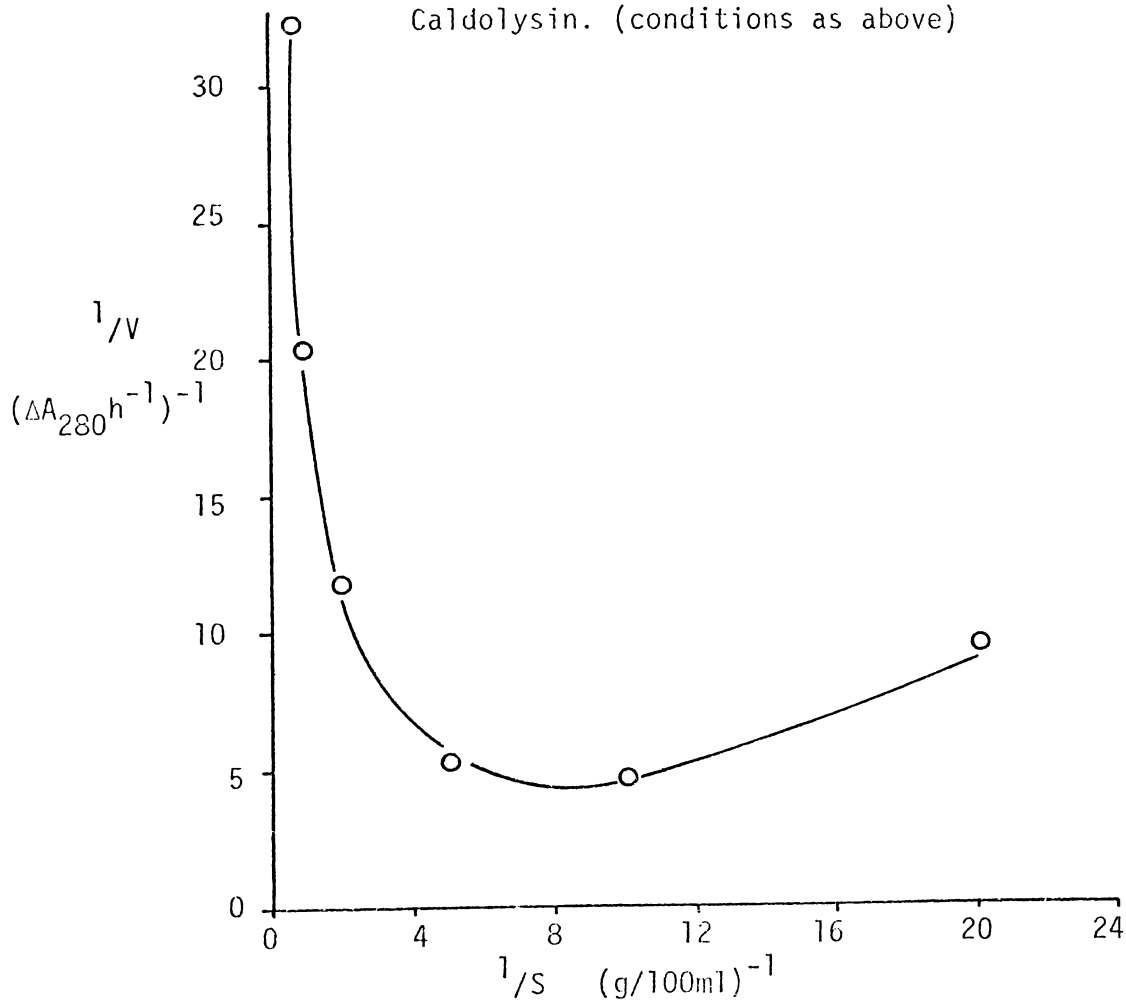


Fig. 8-4. Lineweaver-Burk for the hydrolysis of azoalbumin by Caldolysin. ($24\mu g\ ml^{-1}$ enzyme, substrate dissolved in pH 8.1, 0.1M Tris acetic acid + 10mM calcium)

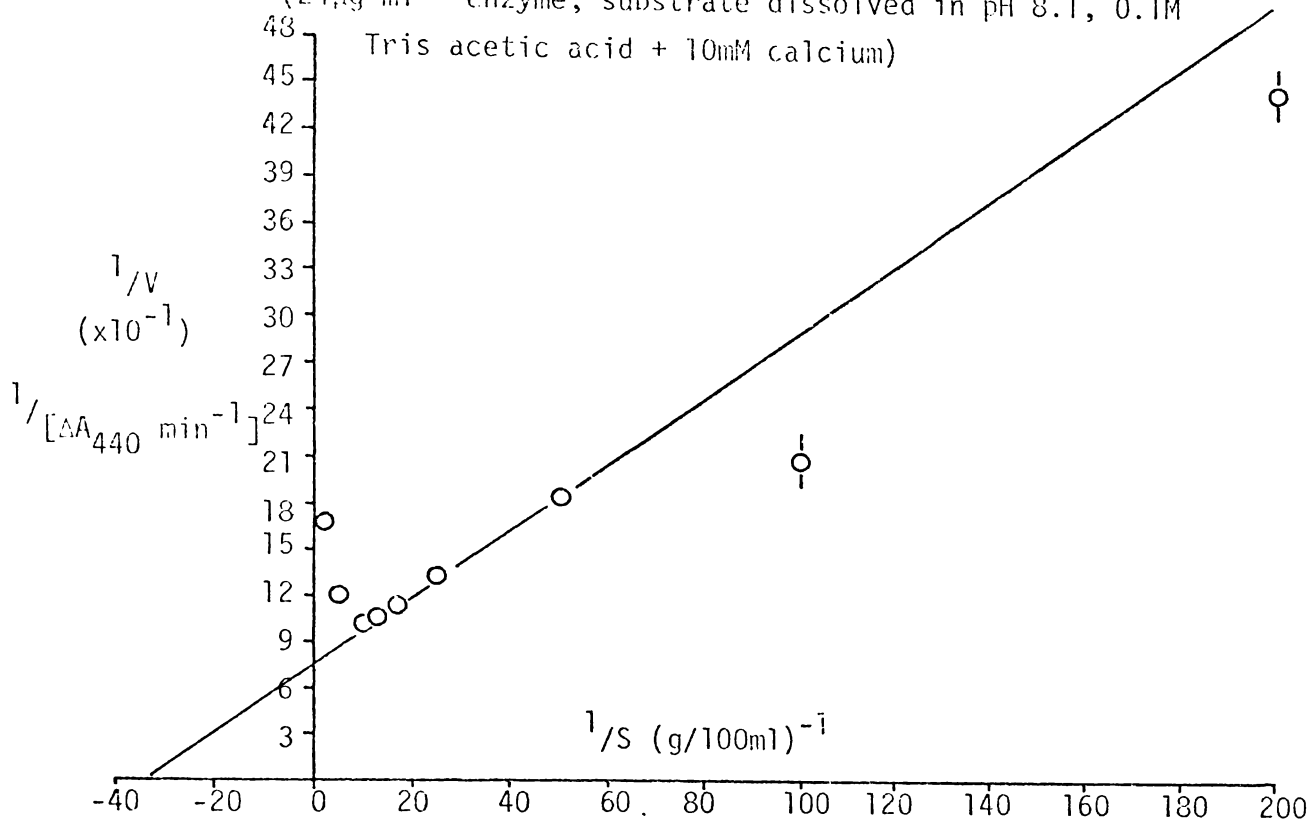


Fig. 8-5. Determination of the Inhibition Constant (K_s') from kinetic data. (hydrolysis of casein)

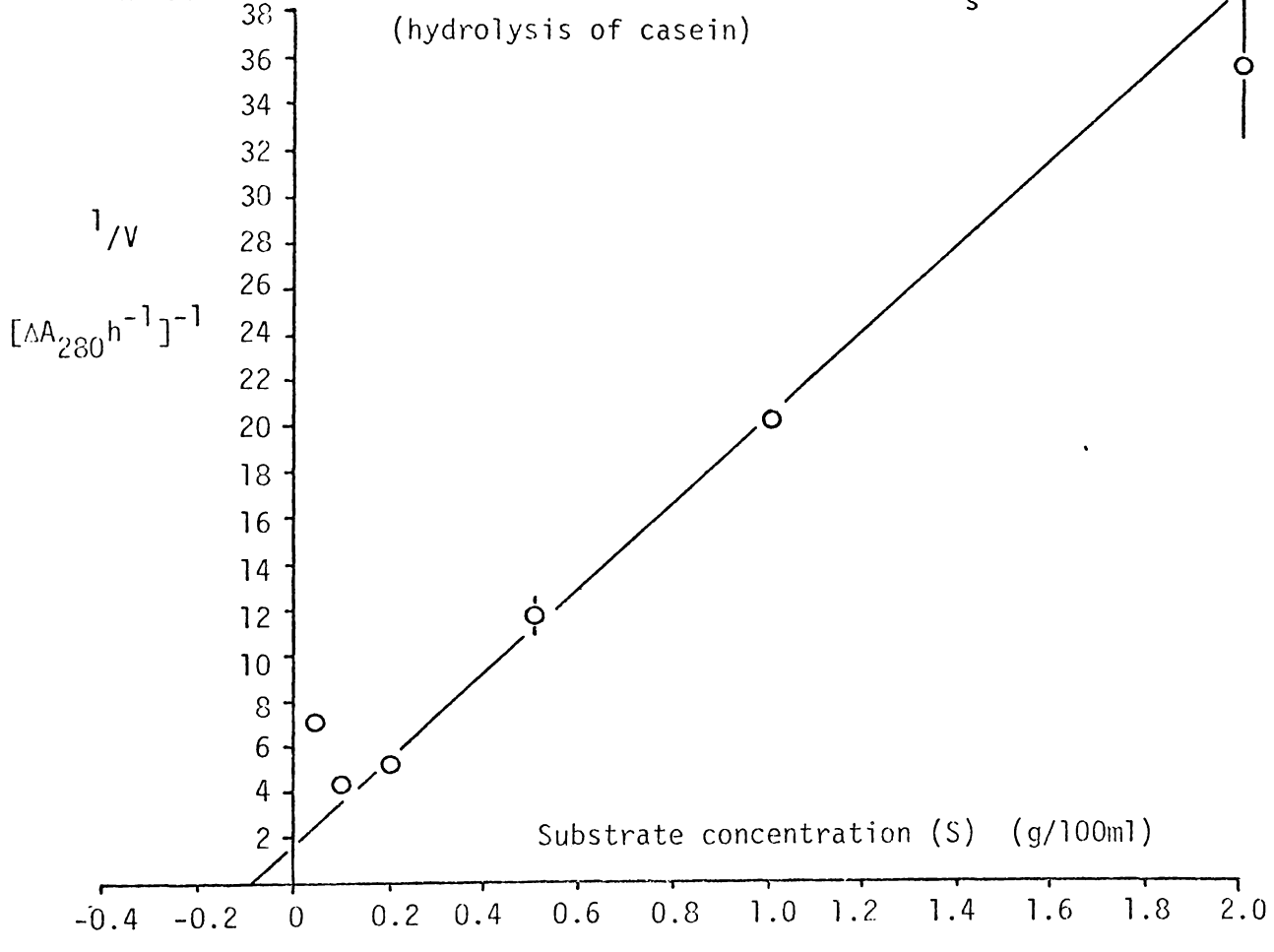
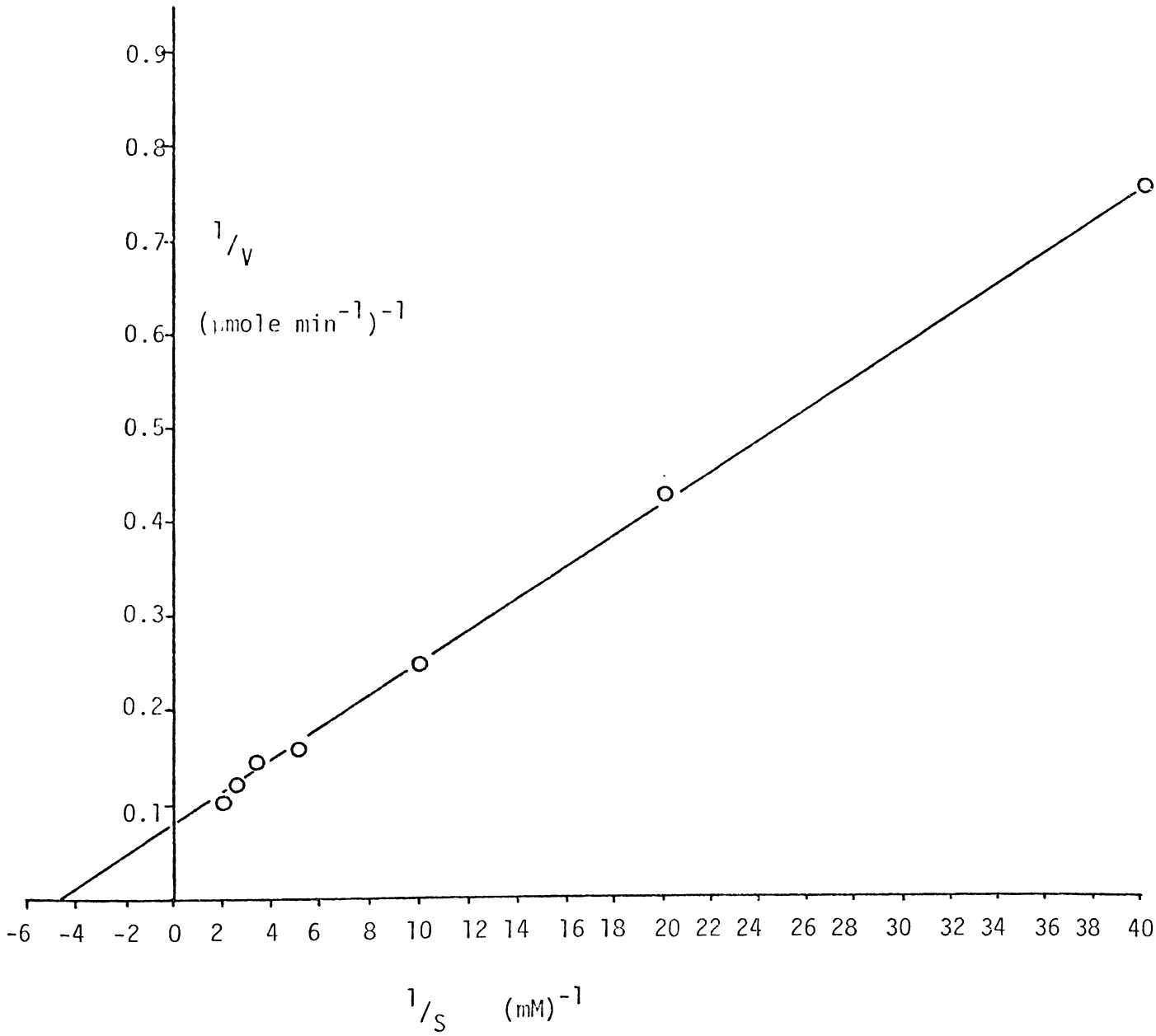


Fig. 8-6. Lineweaver-Burk plot from the hydrolysis of Benzoyl-L-phenylalanine-L-valine-L-arginine-p-nitroanilide by Caldolysin.
($5\mu g\ ml^{-1}$ enzyme: see section 4-7 for conditions of assay)



of dual binding to the active site, but may be due to steric interference.

A K_m calculated from Fig. 8-6 is compared with values obtained for Thermolysin and other proteases on a variety of substrates (Table 8-5).

TABLE 8-5 Michaelis constants for the proteolysis of synthetic substrates

Enzyme	Substrate	K_m	Reference
Caldolysin	Benzoyl-phe-val-arg-pNA	2.17×10^{-4}M	-
Thermolysin	CBZ-gly-pro-leu-gly-pro	1.92×10^{-2}M	a
Thermolysin	CBZ-gly-leu-amide	2.6×10^{-2}M	a
Thermomycolin	Ac-ala$_3$-OMe	1.5×10^{-3}M	b
Subtilisin BPN'	Ac-ala$_3$-OMe	4.5×10^{-4}M	b

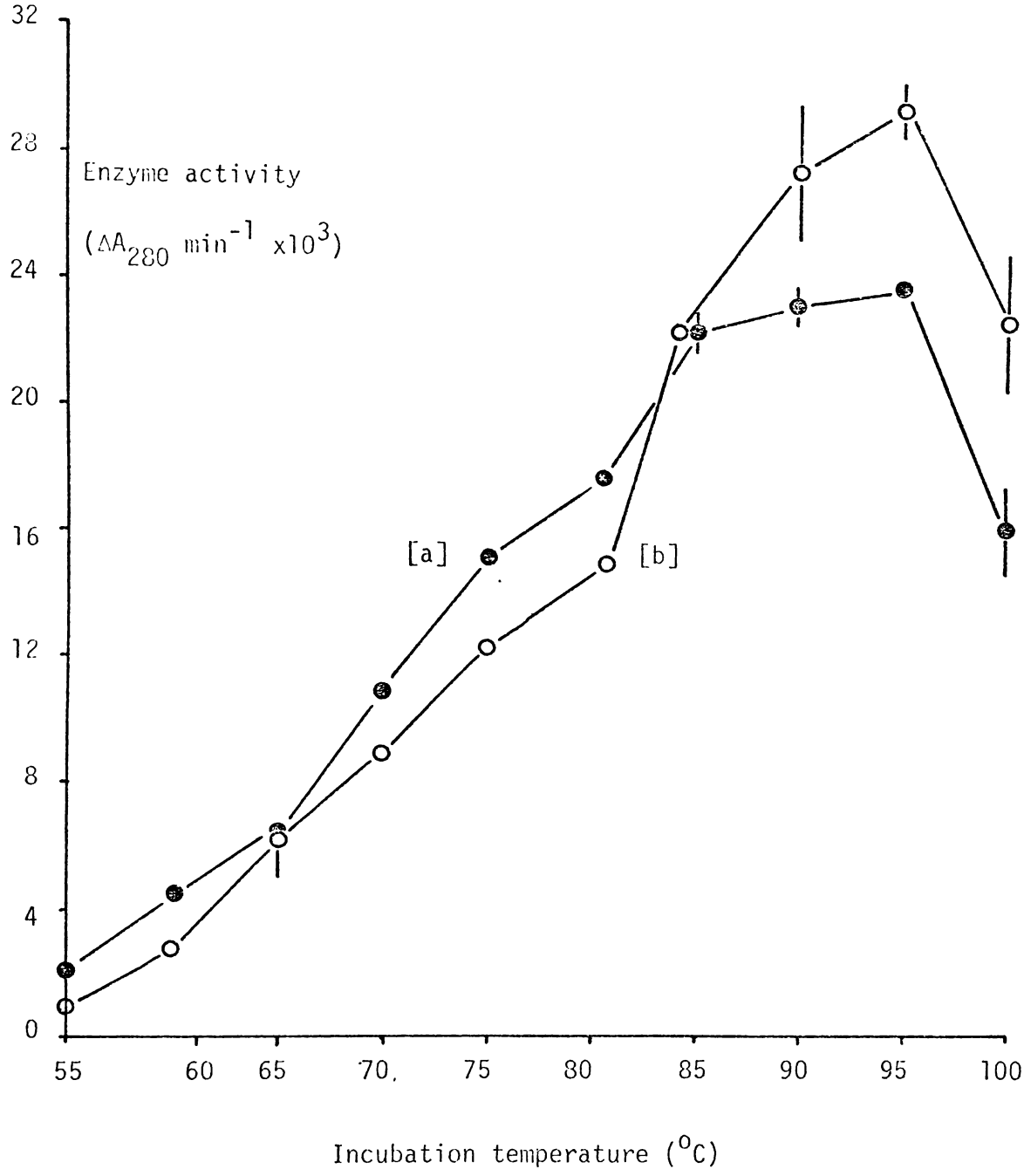
a. Matsubara, 1970.

b. Stevenson & Gaucher, 1975.

8-8 Temperature-activity optimum

Determinations of enzyme activity optima as a function of temperature, obtained by monitoring the amount of substrate reacted in a given time at a range of temperatures, are empirical functions, dependent on experimental conditions. The influence of denaturation (and, in proteolytic enzymes, autolysis) results in a negative shift in the apparent temperature optimum with increasing assay duration. However, with a reduction in assay time, an estimate can be obtained of the temperature above which rapid loss of enzyme activity occurs. This will only hold true where the 'thermal melting' process is very rapid in relation to the assay time used.

Fig. 8-7. The influence of temperature on the rate of proteolysis. ($20\mu g\ ml^{-1}$ enzyme, 0.5% casein substrate dissolved in 0.1M Tris acetic acid buffer, pH 8.1)
Incubation times at given temperatures:
[a] 10 minutes
[b] 5 minutes



The data displayed in Fig. 8-7 are the result of estimations of the rate of proteolysis using 10 minute (a) and 5 minute (b) incubations at each temperature. A longer incubation period decreases the apparent rate of proteolysis at high temperatures (a result of increased autolysis), but does not shift the optimum at 95°C. It is evident that rapid loss of enzyme activity occurs at temperatures in excess of 95°C, presumably the result of thermally induced unfolding of the Caldolysin polypeptide chain.

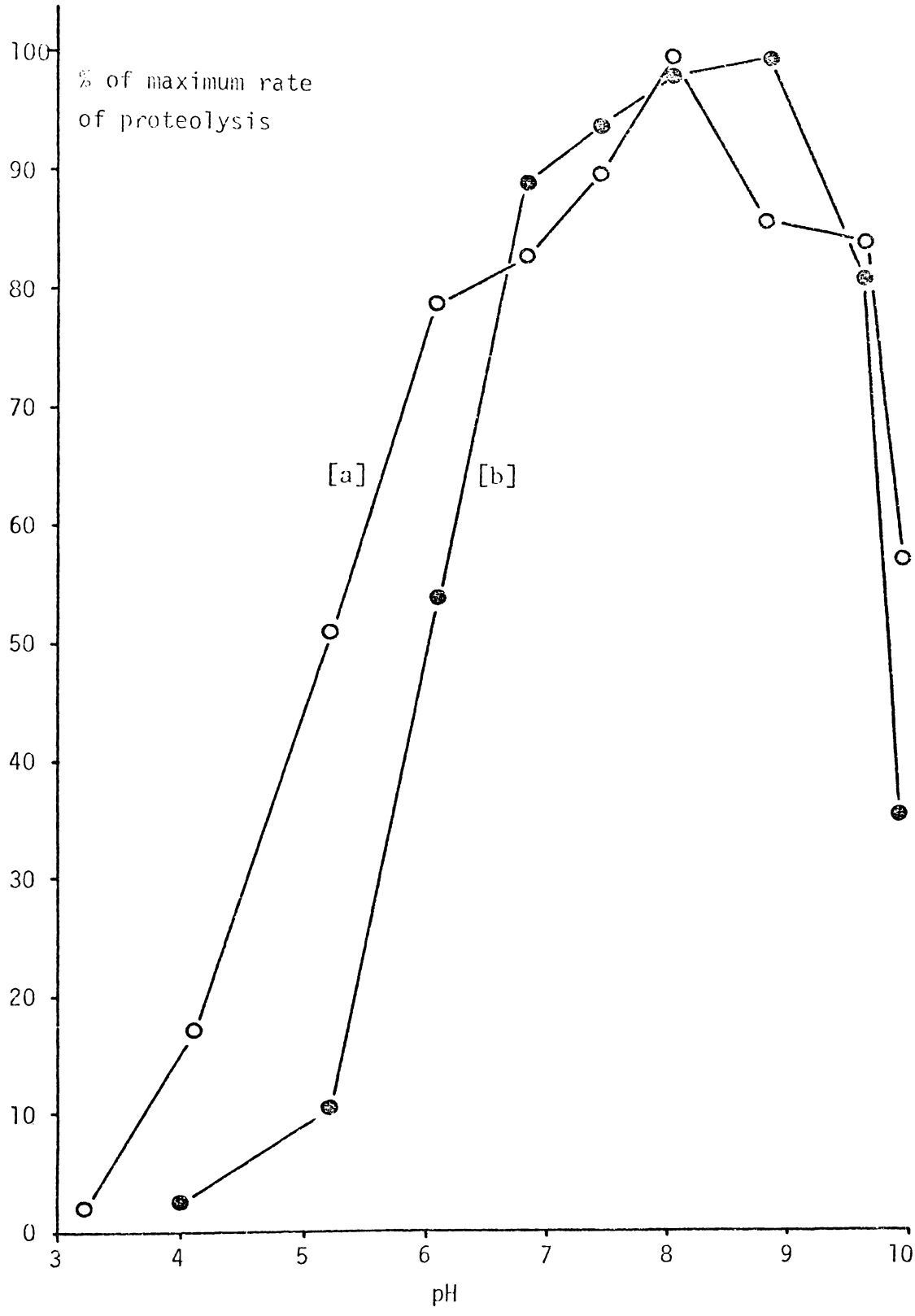
The melting temperatures (T_m) of a number of thermophilic enzymes have been measured by a variety of experimental methods. Stelwagen & Wilgus (1978) quote T_m values of *T. aquaticus* alkaline phosphatase, DNA polymerase, fumarase, and enolase as 78, 80, 83 and 90°C respectively. Extracellular proteases are, in general, very stable proteins. The thermal denaturation temperatures of Thermolysin and Thermomycolin are 80 - 88°C (Ohta *et al.*, 1966) and 69 - 75°C (Voorduow *et al.*, 1974b), respectively. Thus the estimation of a very high melting temperature for Caldolysin, which demonstrates higher thermostability than either Thermolysin or Thermomycolin, is to be expected (see also Chapter 9).

8-9 pH optimum of Caldolysin

Estimated pH optima for the substrates azo-casein and azo-albumin are 8.0 ± 0.2 and 8.5 ± 0.5, respectively (Fig. 8-8). The only major difference in the activity profiles of the two substrates is the substantially greater susceptibility of casein at low pH's.

The observed rate of proteolysis will be, in part, a function of the state of ionisation of both substrate residues and the enzyme active site groups at any given pH. Since the decrease in the rate of hydrolysis of both substrates is similar at high pH levels, it is suggested that this effect is a function of changes in the state of ionisation of

Fig. 8-8. The effect of pH on the rate of proteolysis by Caldolysin. (Substrates: 0.1% azocasein [a]; 0.1% azoalbumin [b]. assayed at 75°C, 18μg ml^{-1} enzyme)



the active site. The isoelectric point of Caldolysis implies that the molecule contains a number of basic amino acids with high K_b values (e.g. lysine and arginine). It is suggested that positively charged groups such as the ε-amino moiety of lysine (see section 11-4) and the guanidinium moiety of arginine in the active site are responsible for substrate recognition, and that above pH 9.5, deprotonation removes this facility.

The accuracy of estimates of proteolytic activity at various pH values relies on the assumption that activity loss resulting from pH-induced denaturation is negligible. It has been shown that at 75°C and pH values between 4 and 9, little activity loss occurred in 100 hours. Thus, it is estimated that denaturation during the 16 minute incubation periods of the above experiment would not have been significant. Whether extremes of pH influence the structure of the substrate and hence its susceptibility to proteolysis is unknown.

CHAPTER 9

STABILITY AND ACTIVITY RELATIONSHIPS

9-1 Thermostability of Native Caldolysin

In determinations of the "thermostability" of Caldolysin, no distinction is made between activity losses resulting from autolysis and from denaturation, since these two factors cannot be easily separated.

Each determination of thermostability involved incubation of the enzyme (20 - 40 $\mu g\ ml^{-1}$ in pH 8.1, 0.1 M Tris acetic acid buffer + 10 mM $CaCl_2$) at a given temperature in a vessel sealed with a rubber septum cap. Sodium azide (0.01%) was added to samples incubated at temperatures below 80°C. Aliquots of enzyme solution were removed at appropriate intervals and assayed at 75°C by the Kunitz method. The stability profiles derived from incubations at temperatures between 20°C and 100°C are presented in Fig. 9-1.

It was concluded that the most effective manner in which to express such data simply and clearly was in the form of half-lives ($t_{\frac{1}{2}}$), the time taken for the loss of 50% of the initial enzyme activity under the conditions specified (Table 9-1).

TABLE 9-1 Half-life data for Caldolysin at various temperatures. (0.1 M Tris acetic acid, pH 8.1, 10 mM $CaCl_2$)

Temperature (°C)	$t_{\frac{1}{2}}$
20	> 30 weeks
75	> 193 hours
80	∿ 30 hours
85	5 - 6 hours
90	56 - 60 minutes
95	28 - 30 minutes
100	3.5 minutes

Fig. 9-1. Thermostability profiles of Caldolysin at various temperatures. (experimental conditions- see text)
Incubation temperatures specified below.

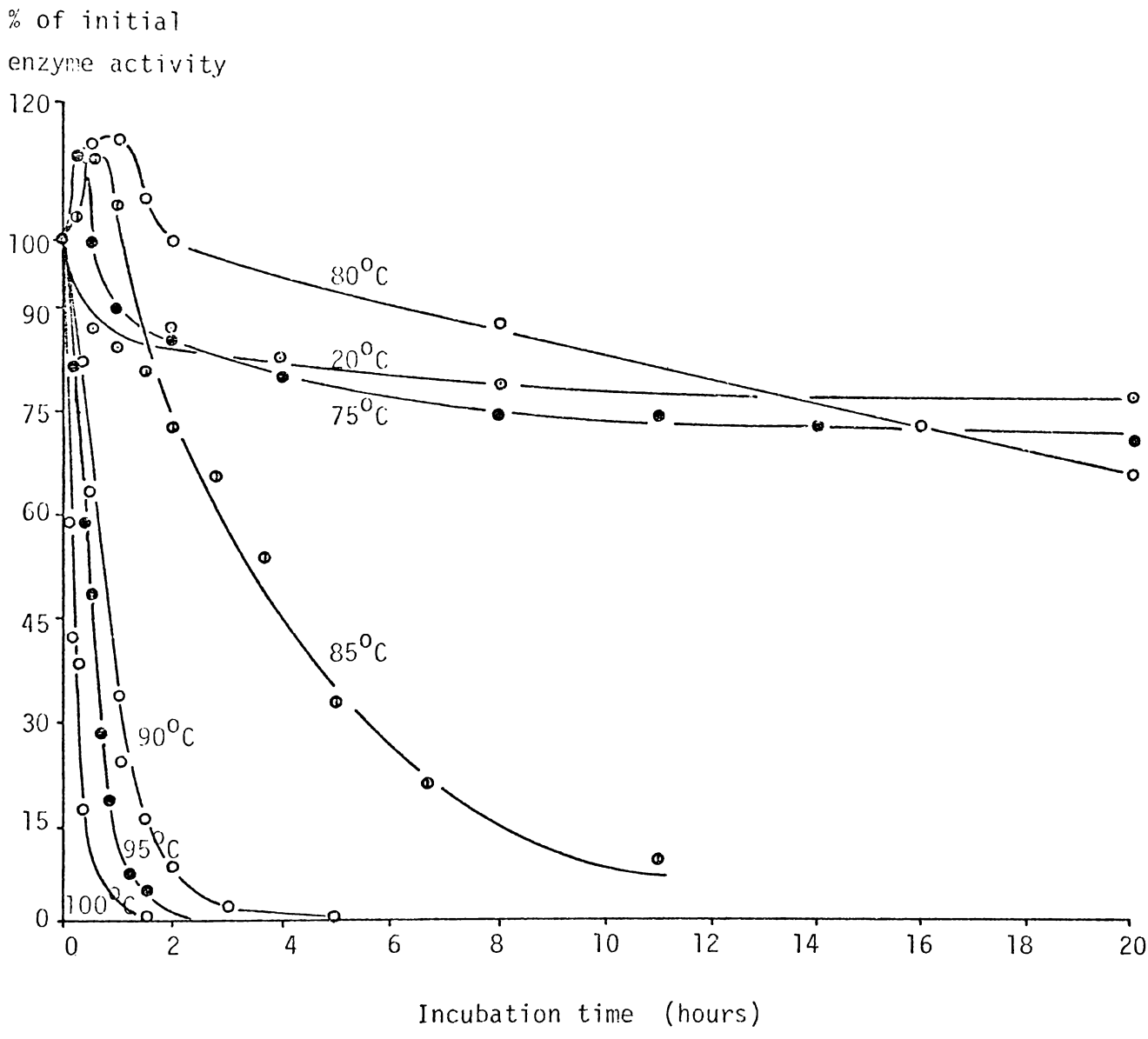


TABLE 9-2 Thermostability of other thermophilic proteases

Protease	Organism	Temperature of incubation (°C)	$t_{\frac{1}{2}}$ (hours)	Reference
Thermolysin	*B. thermoproteolyticus*	80	1	Matsubara (1970)
Thermomycolin	*Malbranchea pulchella*	73	<2	Gaucher & Stevenson (1976)
Protease	*B. caldolyticus*	80	>8	Heinen & Heinen (1972
Neutral protease	*B. stearothermophilus*	65	5	O'Brien & Campbell (1957)
Lytic protease	*Micromonospora vulgaris*	80	½ - ¾	Golinova *et al.* (1973)
Aminopeptidase 1	*B. stearothermophilus*	80	>15	Roncari *et al.* (1976)
Thermitase	*Thermoactinomyces vulgaris*	80	<½	Behnke *et al.* (1978)
Lytic protease	*Streptococcus lactis*	98	>1	Williamson *et al.* (1964)

When compared with thermostability data from other thermophilic proteases (Table 9-2), it is evident that Caldolysin is considerably more resistant to autolysis and/or denaturation than most other documented examples.

9-2 The effect of calcium concentration on the thermostability of Caldolysin

It has been established that calcium is important in the structure of Caldolysin (Chapter 8). Experiments to determine the nature and limits of the calcium ion stabilisation were carried out.

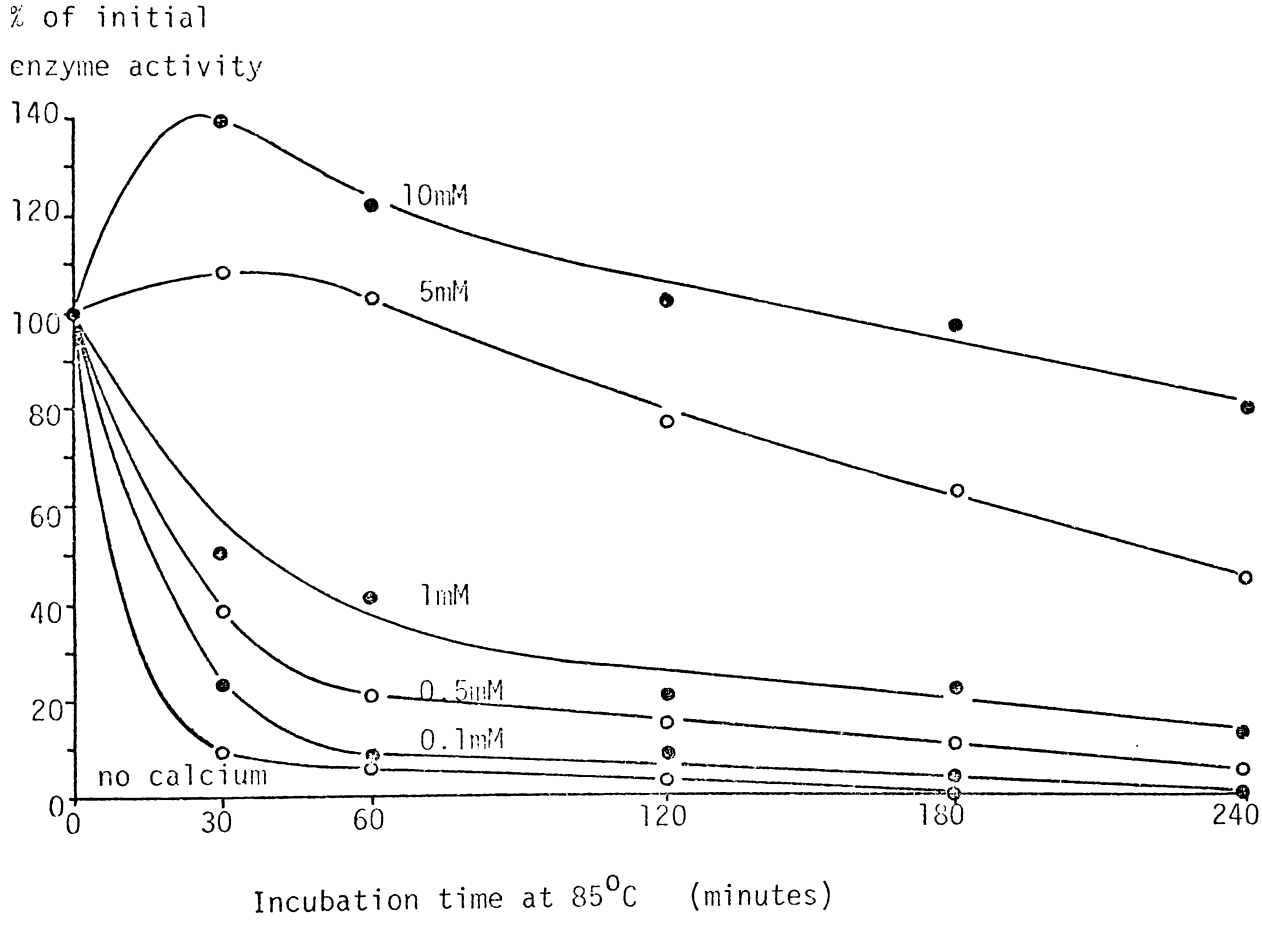
A solution of Caldolysin (10 μg ml^{-1}) was dialysed against 0.5 mM EDTA at 4°C for 18 hours. Samples were then incubated at 85°C (0.01 M Tris acetic acid, pH 8.1) after the addition of specified amounts of $CaCl_2$. Thermostability profiles (Fig. 9-2), from which the $t_{\frac{1}{2}}$ data in Table 9-3 were obtained, were determined by the periodical removal and assay of samples from each incubation tube.

TABLE 9-3 The effect of calcium on the thermostability of Caldolysin at 85°C

Calcium concentration (M)	(pCa^{2+}) [a]	$t_{\frac{1}{2}}$ (minutes)	k_{obsd} (M^{-1} sec^{-1})
0	-	<10	-
10^{-4}	4	15	4.31×10^5
5×10^{-4}	3.3	22	1.70×10^5
10^{-3}	3	38	9.00×10^4
5×10^{-3}	2.3	225	2.56×10^4
10^{-2}	2	300-360	8.89×10^3
5×10^{-2}		600	-
10^{-1}		780	-
5×10^{-1}		780	-

a. $pCa^{2+} = \log_{10} [Ca^{2+}]$

Fig. 9-2. The influence of calcium concentration on the thermostability of Caldolysin at 85°C.
($10\mu g\ ml^{-1}$ enzyme, pH 8.1, 0.01M Tris acetic acid buffer: calcium concentration specified below)



Although a number of factors may influence nature of the profiles in Fig. 9-2 (e.g. enzyme activation (see Chapter 10), autolysis, or denaturation), treatment of these data in the manner described by Voorduow & Roche (1975a) shows that activity loss is largely the result of autolysis.

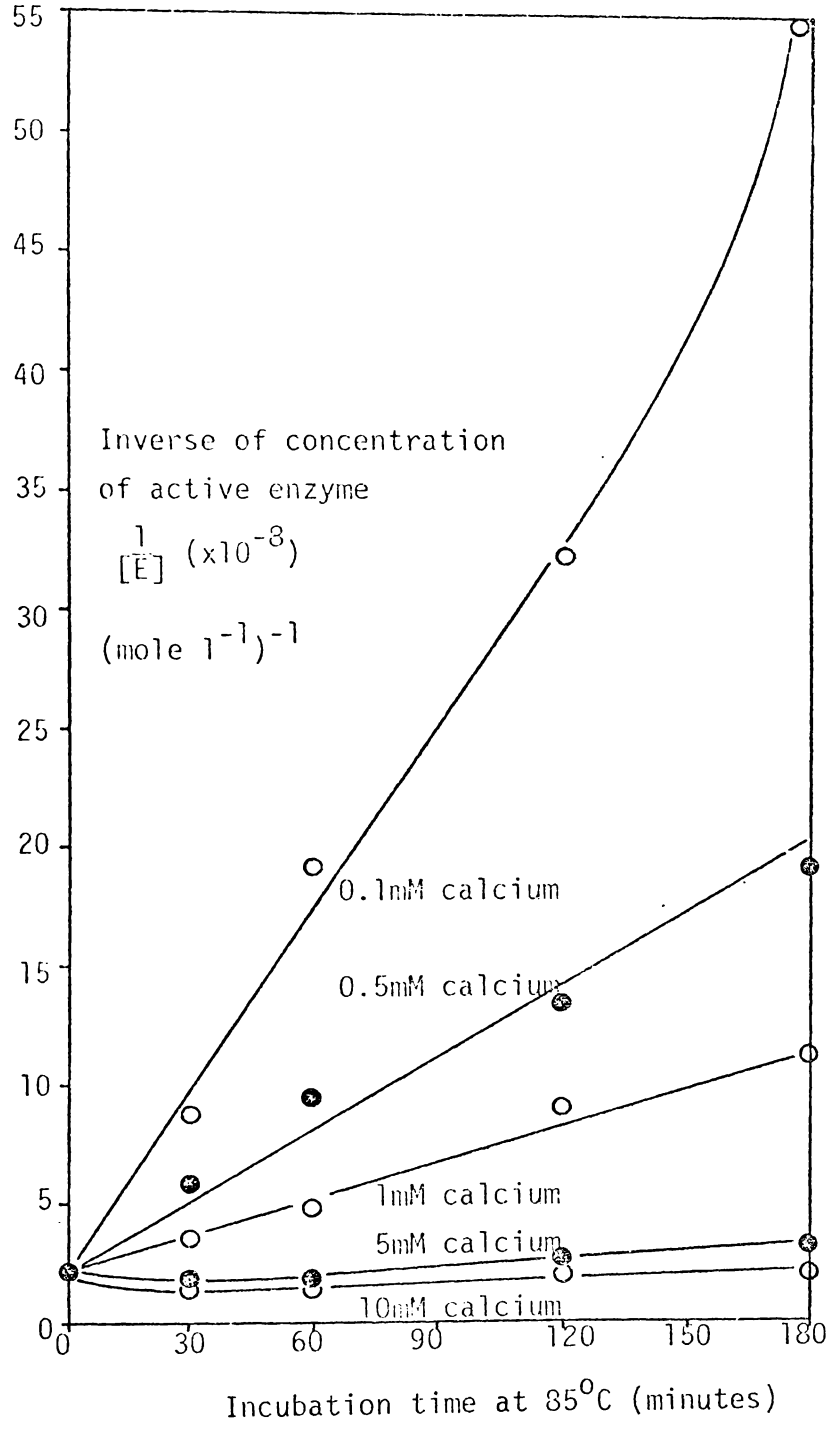
When the reciprocal of the concentration of the remaining active Caldolysin is plotted against time (at 85°C (Fig. 9-3)), the linear region of the relationship indicates the presence of second order reaction kinetics, and the gradient yields a value of the second order rate constant, k_{obsd} (Table 9-3). The presence of second order kinetics implies that autolysis is the major cause of the loss of enzyme activity, since thermal denaturation is a first order process (Voorduow & Roche, 1975a, 1975b).

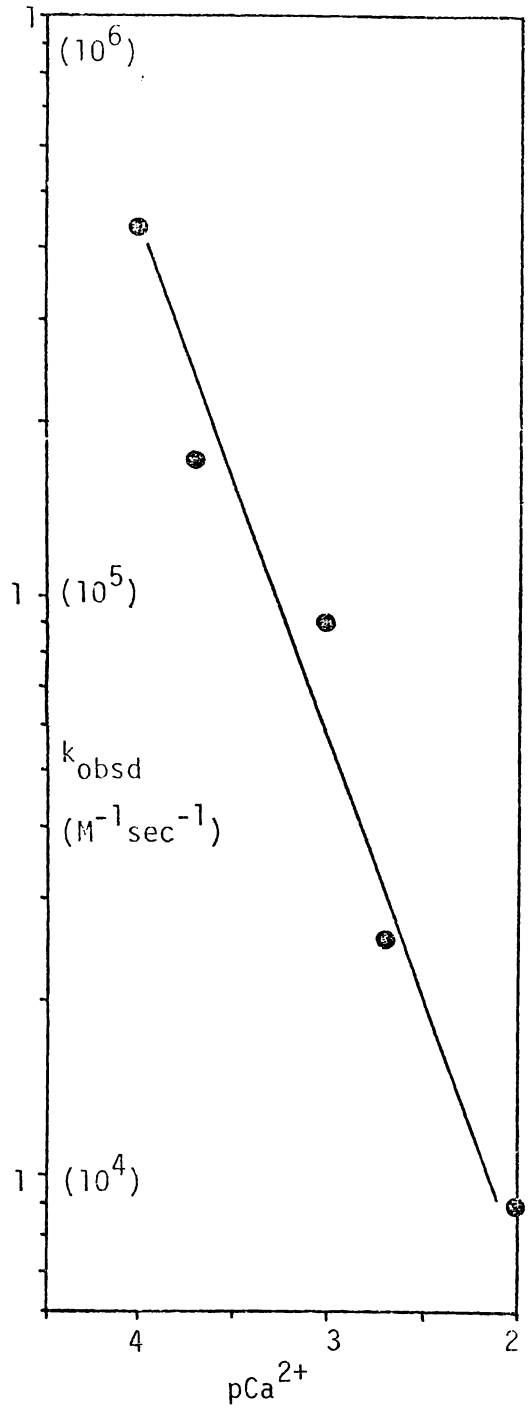
When values of log k_{obsd} are plotted against pCa^{2+}, a linear relationship is obtained (Fig. 9-4). According to the mathematical model of Voorduow & Roche (1975), this shows that only enzymes with unoccupied calcium sites serve as substrates for autolytic degradation. This is based on the assumption that full calcium-site occupancy is actually a state of equilibrium; $ECa \rightleftharpoons E + Ca$, where the duration spent in the apoenzyme state will decrease in proportion to the external calcium concentration. Thus, where the calcium sites are practically saturated, k_{obsd} is inversely proportional to $[Ca^{2+}]$, but where calcium sites are unoccupied, k_{obsd} should be independent of $[Ca^{2+}]$. That Fig. 9-4 conforms to the former category suggests that calcium sites in Caldolysin are essentially saturated at calcium concentrations of 10^{-4} M and above.

Thus, it is concluded that at 85°C, losses of enzyme activity are primarily derived from autolysis, both in the presence and absence of calcium.

Fig. 9-3. Activity loss in Caldolysin. (85°C, 0.1M buffer, pH 8.1, calcium concentration specified below) The slopes give k_{obsd}.

Fig. 9-4. Dependence of the second order rate constants (k_{obsd}) on the calcium concentration.





9-3 Thermostability of Apocaldolysin

Solutions of enzyme were prepared by extensive dialysis of the holoenzyme, firstly against 0.01 M Tris acetic acid containing 5 mM EDTA, and then against calcium-free Tris buffer. These preparations were designated 'apocaldolysin', although no tests were carried out to determine whether residual calcium was present. Apoenzyme thermostability was determined by procedures analagous to those used for holocaldolysin.

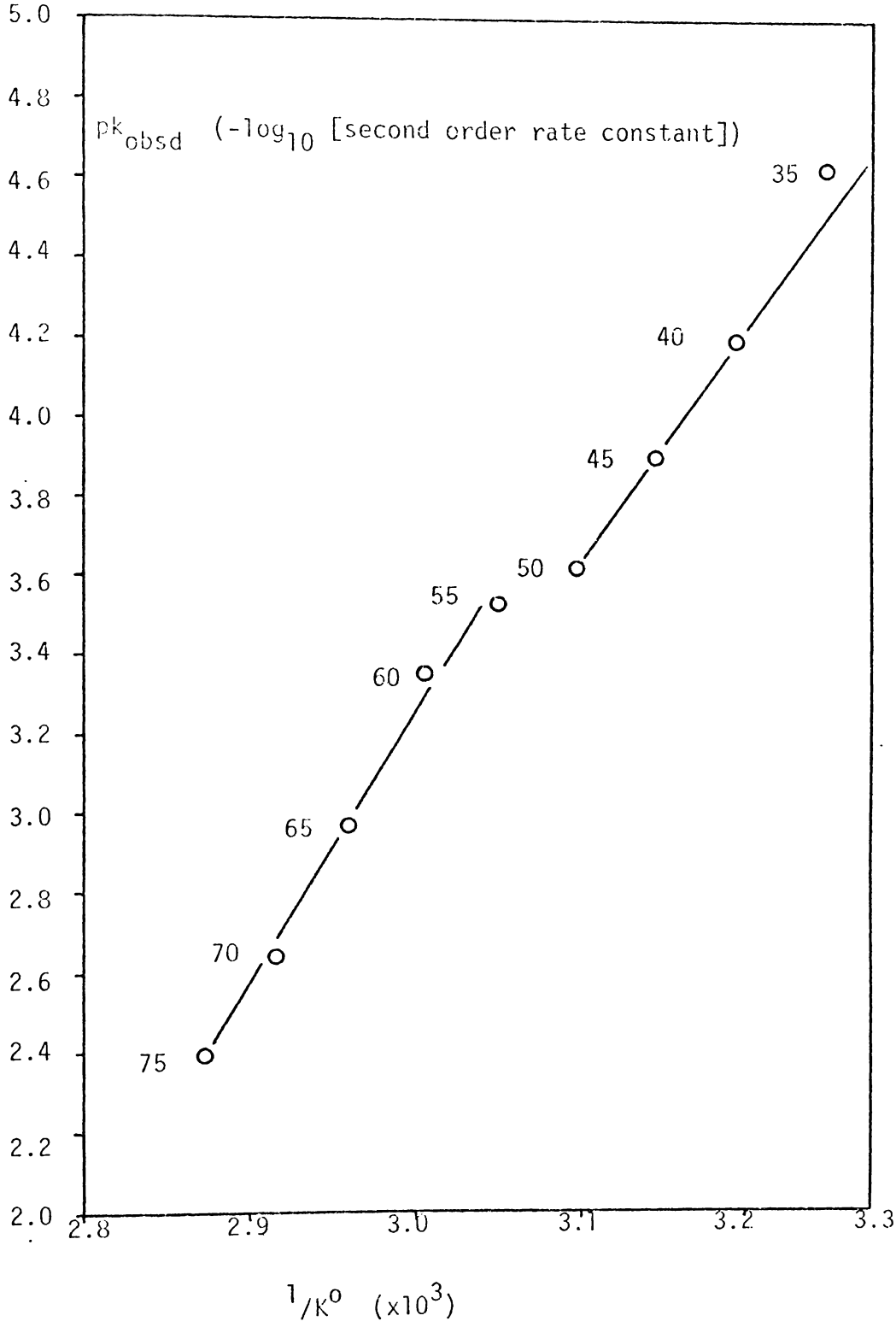
Half-life data resulting from the incubation of apocaldolysin (about 20 μg ml^{-1}) at temperatures between 35°C and 75°C are presented in Table 9-4.

TABLE 9-4 Thermostability of apocaldolysin

Temperature (°C)	$t_{\frac{1}{2}}$ (minutes)
35	330
40	260
45	85
50	60
55	40
60	25.5
65	13
70	5.5
75	4.8

The thermostability of Caldolysin is reduced drastically by the removal of stabilising calcium ions ($t_{\frac{1}{2}}$ at 75°C: apoenzyme = 4.8 minutes; holoenzyme > 193 hours). When the data from Table 9-4 are replotted in the

Fig. 9-5. Arrhenius plot for the autolysis of apocaldolysin. (temperature in ^{o}C indicated)



form; pK_{obsd} vs $1/T_{abs}$ (pK_{obsd} is calculated as described in section 9-2), the Arrhenius-type plot shows a discontinuity at approximately 60°C (Fig. 9-5). Above this temperature, autolysis proceeds at an accelerated rate, implying that some structural change is responsible for increasing the accessibility of cleavage sites. However, this is a relatively minor influence when compared to the great reduction in stability resulting from the removal of calcium from the enzyme.

9-4 The binding of metal ions to Caldolysin

Structural analysis of the metal binding sites in thermolysin has shown that each has a high degree of spatial precision (Voorduow & Roche, 1978). To determine the specificy of the calcium binding sites in Caldolysin, the thermostability of the apoenzyme was monitored in the presence of a variety of metal salts. It was assumed that recovery of the normal (calcium-linked) holoenzyme thermostability would indicate occupancy by the metal ions present.

Apocaldolysin (12 μg ml^{-1}, prepared as described in section 9-3), was incubated at 85°C in the presence of 10 mM concentrations of calcium, magnesium, barium, strontium, cobalt, zinc, and copper (chloride or sulphate salts). Enzyme activity was monitored as previously described.

Thermostability profiles and half-life values are presented in Fig. 9-6 and Table 9-5. The data from Fig. 9-6 has been replotted as the reciprocal of active enzyme concentration against time (Fig. 9-7). From the linear portions of these plots, which denote second order reaction kinetics (Voorduow & Roche, 1975a), the rates of autolysis (k_{obsd}) have been calculated (Table 9-5).

Fig. 9-6. The effect of metal ions on the thermostability of apocaldolysin at 85°C. (12μg ml^{-1} enzyme, pH 8.1 Tris acetic acid buffer, 0.1M, 10mM metal ions)

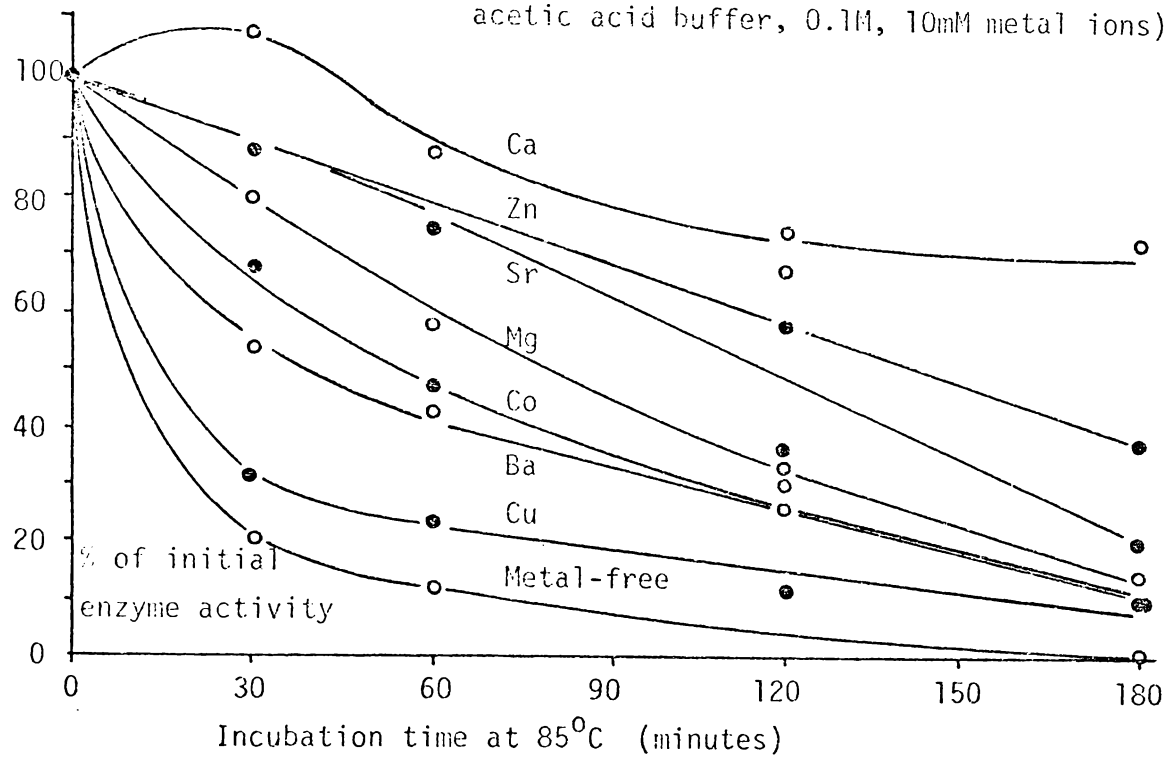


Fig. 9-7. Autolytic degradation of Caldolysin at 85°C in the presence of various metal ions. (conditions as above)

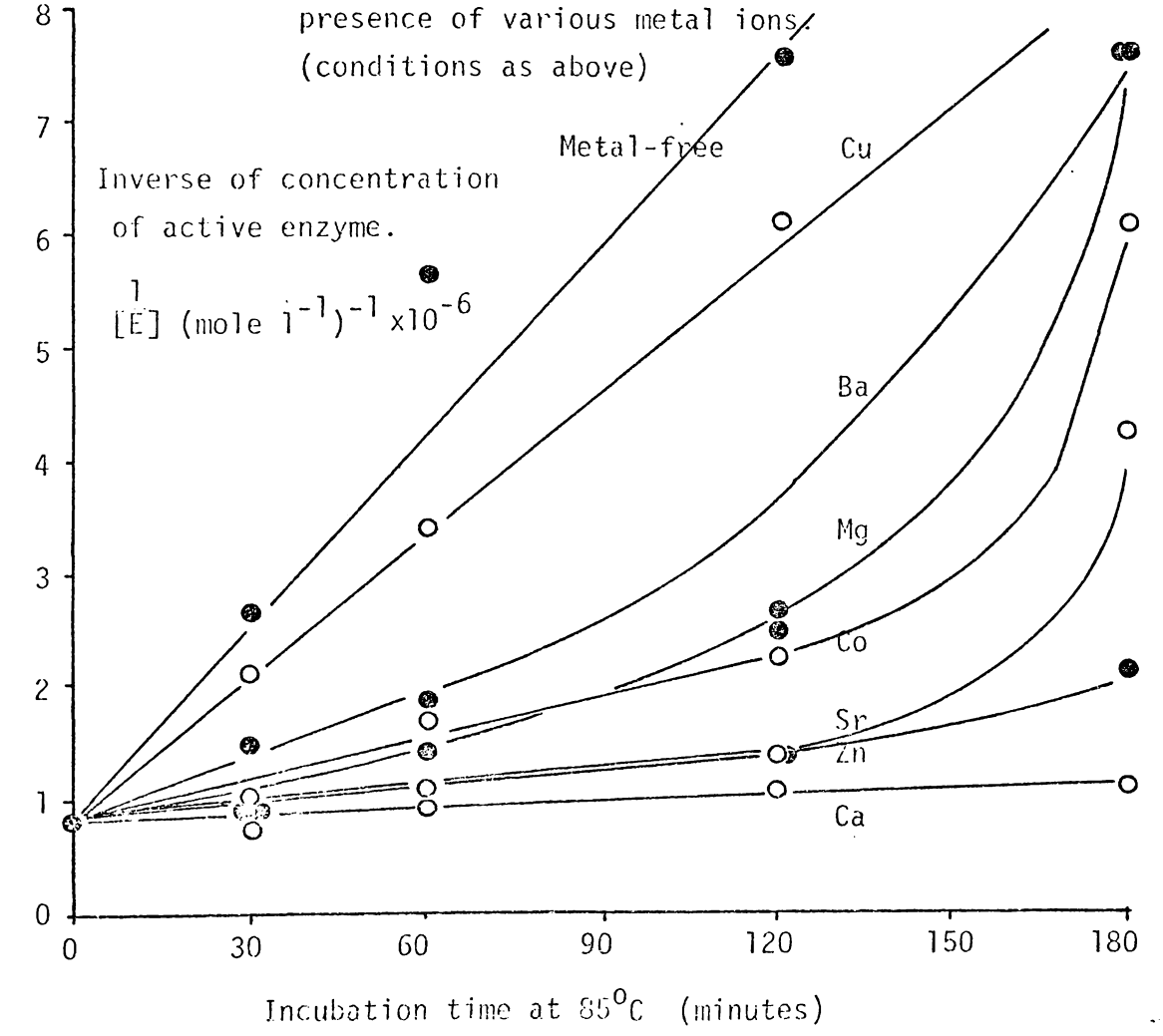


TABLE 9-5 The influence of metal ions on the stability of Caldolysin at 85°C. (12 μg ml^{-1} enzyme, pH 8.1 Tris acetic acid, I = 0.3 M l^{-1}).

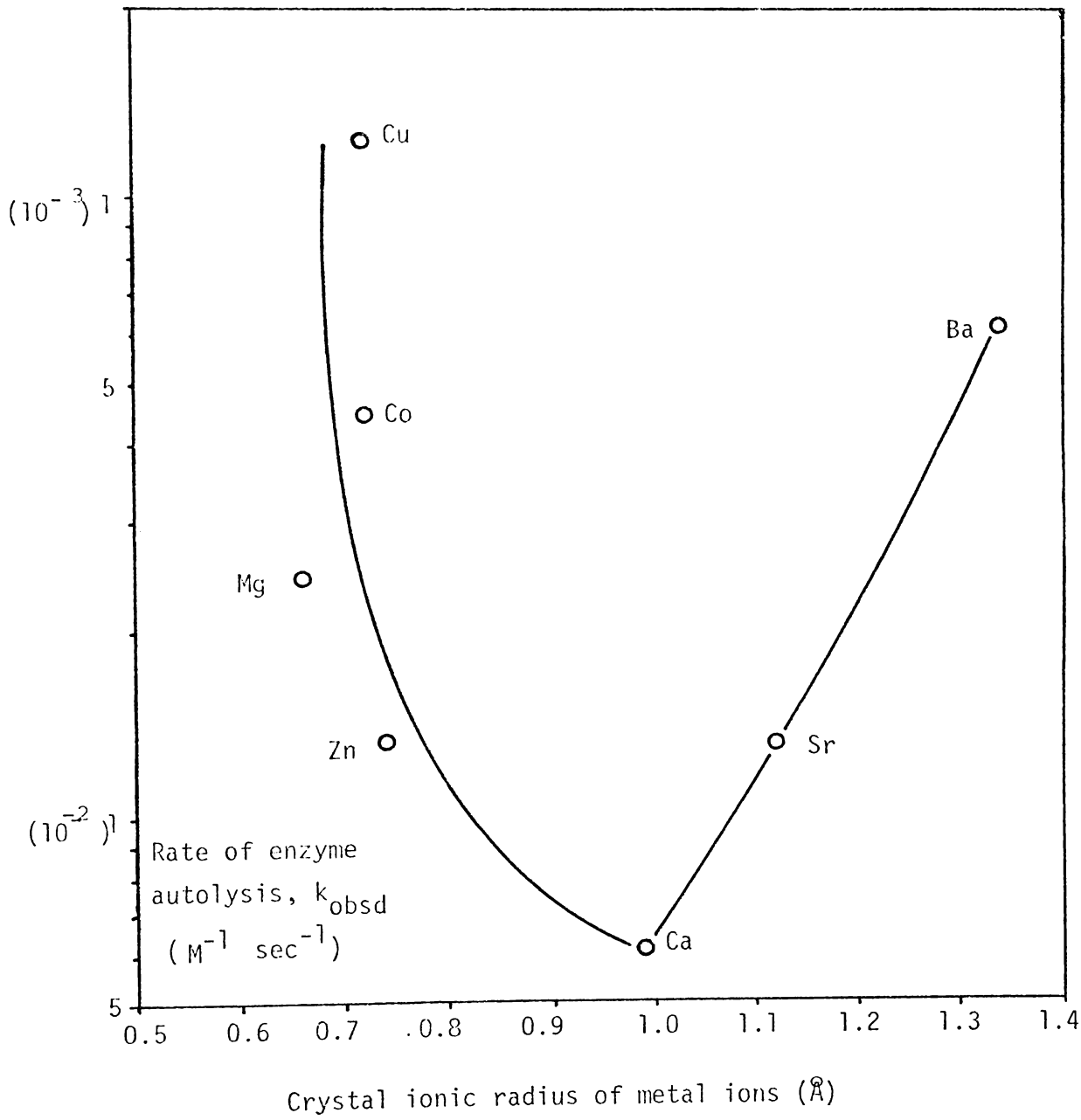
Metal ion	$t_{½}$ (minutes)	k_{obsd} (molar^{-1} sec^{-1})	Crystal ionic radius [a] (Å)
Calcium	est. 340	6.11×10^{1}	0.99
Zinc	144	1.30×10^{2}	0.74
Strontium	115	1.30×10^{2}	1.12
Magnesium	86	2.41×10^{2}	0.66
Cobalt	60	4.26×10^{2}	0.72
Barium	43	6.19×10^{2}	1.34
Copper	21	1.25×10^{3}	0.72
None	est. 5-10	1.89×10^{3}	-

a. Values from CRC Handbook of Chemistry and Physics

Values of k_{obsd} are plotted against the crystal ionic radius of each metal ion (Fig. 9-8). The crystal ionic radius is not an ideal specification for comparison since the size of the hydration shell surrounding each atom is neglected. In solution, all metal ions would normally be fully solvated, although it is not known whether binding to a protein site results in any change in the solvation shell. However, this parameter does at least take account of the divalent ionisation state of the metal ions.

Fig. 9-8 suggests that the ionic radius of a metal ion may bear some relationship to its effectiveness as a substitute for calcium in Caldolysin. If so, this implies that Caldolysin, like Thermolysin, has a precise molecular symmetry for the chelation of calcium ions. However, the inconsistency of the values for cobalt and copper suggests that factors other than size may also be important. To clarify the influence of zinc

Fig. 9-8. Relationship between the rate of enzyme autolysis and the crystal ionic radius of metal ions substituted for calcium.



ions on the thermal stability of Caldolysin, apocaldolysin (4μg ml^{-1} in 0.01 M Tris acetic acid, pH 8.1) was incubated at 90°C in the presence of different concentrations of $ZnSO_4$ (Table 9-6).

TABLE 9-6 The effect of zinc on thermostability of apocaldolysin at 90°C

Metal ion concentration (mM)	$t_{\frac{1}{2}}$ (minutes)
0	4
0.5 Zn	10
1.0 Zn	12
5.0 Zn	8
10.0 Zn	6
20.0 Zn	6
10.0 Zn + 10.0 Ca	24
10.0 Ca	59

It is evident that zinc is incapable of providing the molecular stability generated by the binding of calcium ions. However, it is likely that zinc can occupy the calcium-binding site [a] since the simultaneous addition of calcium and zinc to the apoenzyme provides less than 50% of the thermostability normally resulting from that concentration of calcium.

a. N.B. The crystal ionic radius of zinc in 0.25 Å less than that of calcium.

9-5 The effect of pH on the stability of Caldolysin

It is stated by Chell & Sundaram (1978) that inactivation of enzymes by extremes of pH can be indicative of the presence of external salt-bridges involved in structural stabilisation. However, many residues

will be modified in some way by high or low pH levels, especially if accompanied by elevated temperatures.

The stability of Caldolysin at room temperature (22°C ± 3°C) in buffers ranging from pH 3 to pH 12 was monitored at intervals over a period of 95 days. Assays were carried out at pH 8.1 as described in section 4-4. Results are presented in Figs. 9-8a and 9-8b. (Stability data at pH 7 and 8 are not shown, but are substantially similar to that at pH 9, and 10.9).

Caldolysin is stable in alkaline environments at 22°C for long periods. The small increase in activity during extended incubations is probably a product of evaporative concentration of the enzyme solutions, despite the precaution of sealing incubation tubes between assays.

The stability of Caldolysin in acidic conditions is considerably lower than at high pH levels. The half-life values in buffers of pH 3.6, 4.2, and 6.0 are calculated to be approximately 28 days, 50 days, and 46 days respectively. No explanation for the more rapid loss of enzyme activity at pH 6 than at pH 5 has been found. While the pH-dependent stability of Caldolysin at mesophilic temperatures is very high, this is reduced substantially by increasing the incubation temperature. Results from the incubation of Caldolysin at 75°C and 90°C in high and low pH regimes are presented in Fig. 9-9.

There was no significant loss of enzyme activity at pH 8.1 and 75°C (data not shown) and only 25% in 11 hours at pH 10. These results further enhance the conclusion that Caldolysin is extremely stable in alkaline conditions. At 90°C, half-lives at pH 4 and 10 are estimated to be five minutes and 13 minutes respectively. It must be noted, however, that a combination of extreme pH and high temperature is extremely destructive.

Fig. 9-8a. Loss of enzyme activity on storage of Caldolysin at various pH levels.
($6\mu g\ ml^{-1}$ enzyme, $22^{o}C \pm 3^{o}C$, citric acid-barbitone buffer containing 1.6mM NaN_3 and 5mM calcium).

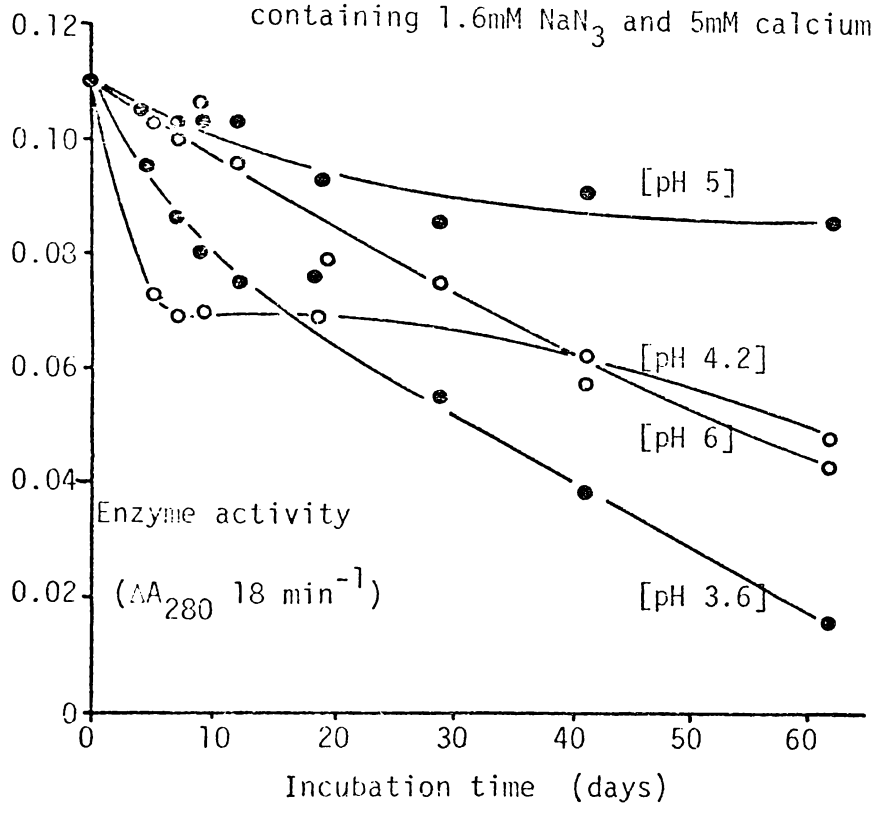


Fig. 9-8b. Loss of enzyme activity on storage of Caldolysin at various pH levels (conditions as above).

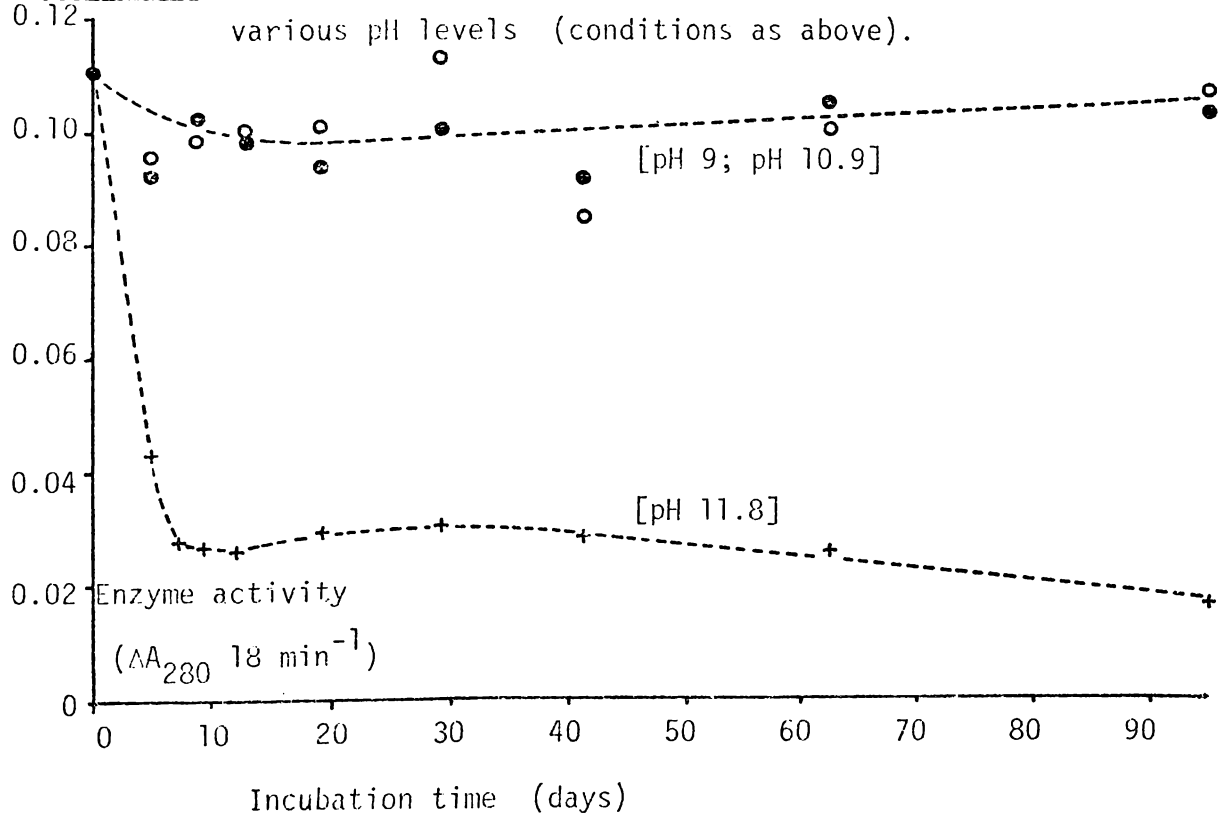
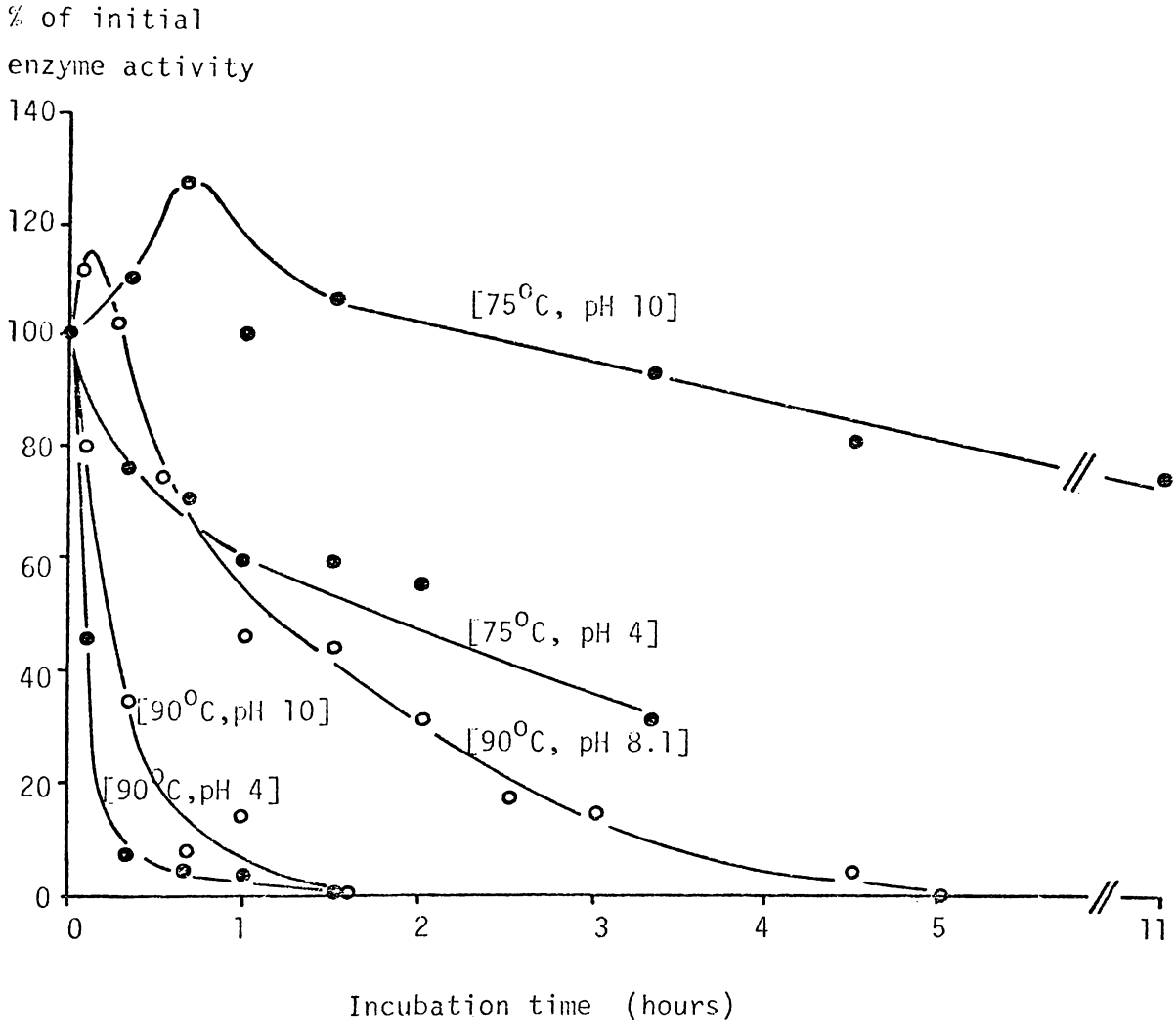


Fig. 9-9. Activity loss of Caldolysin at various levels of pH and temperature.
($5 \mu g\ ml^{-1}$ enzyme: pH 4, citrate-phosphate buffer; pH 8.1, Tris acetic acid buffer; pH 10, diethanolamine:HCl buffer; 10mM calcium present).



9-6 Thermostability and salt concentration

It was noted in section 9-3 that stabilisation of Caldolysin at high calcium concentrations occurred even after the metal-binding sites were apparently saturated. To determine whether this effect might be derived from the non-specific influences of increasing salt concentration (i.e. ionic nature of the environment), the thermostability profiles of calcium-stabilised and calcium-depleted holocaldolysin in the presence of various concentrations of NaCl were determined (Figs. 9-10a and 9-10b).

In the absence of the calcium stabilisation, it is apparent that the presence of high ion concentrations does little to enhance thermostability. The fact that the initial rate of activity loss in both 0.2 M and 2.0 M NaCl is similar, but the level of retention of residual activity is higher with 0.2 M NaCl, suggests that a slight stabilisation by a charged environment is reversed at high concentrations.

Thermal stability of Caldolysin in the presence of 10 mM calcium is not significantly effected by 0.2 M NaCl, but is slightly enhanced by 2 M NaCl. Chell & Sundaram (1978) have suggested that a reduction of thermal stability at high ionic strengths indicates the dispersion of external salt bridges. Despite the fact that analysis of the stability profiles (Fig. 9-10b) is confused by initial activation of the enzyme (discussed in Chapter 10), it is concluded that in Caldolysin, there is no significant contribution to thermostability from external salt-bridging. The destabilisation by 2 M NaCl at low calcium concentrations could be attributed to ionically-induced dissociation of *internal* salt bridges, since these linkages are likely to be exposed by the extensive structural modification of the enzyme which is thought to occur at high temperatures in the absence of calcium.

Fig. 9-10a. The effect of salt concentration on the thermostability of Caldolysin (low calcium level) at 85°C.
(15μg ml^{-1} enzyme, pH 8.1, 0.01M Tris acetic acid buffer, 0.1mM calcium).

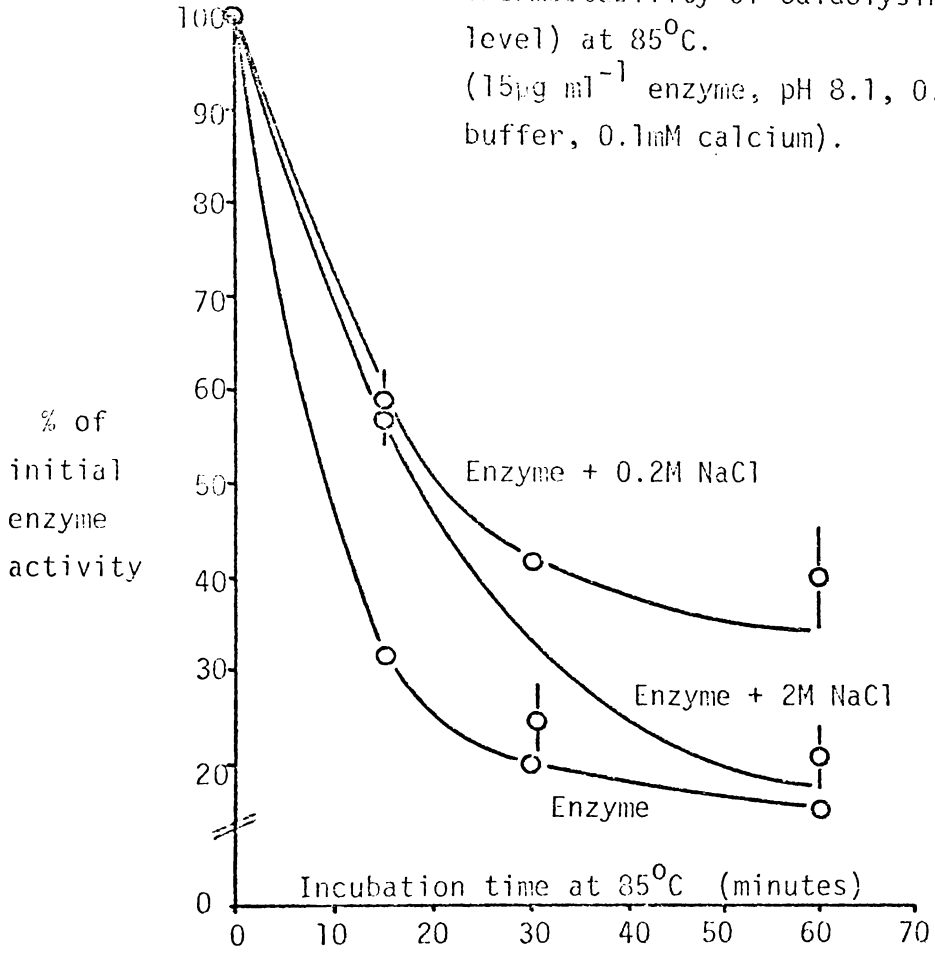
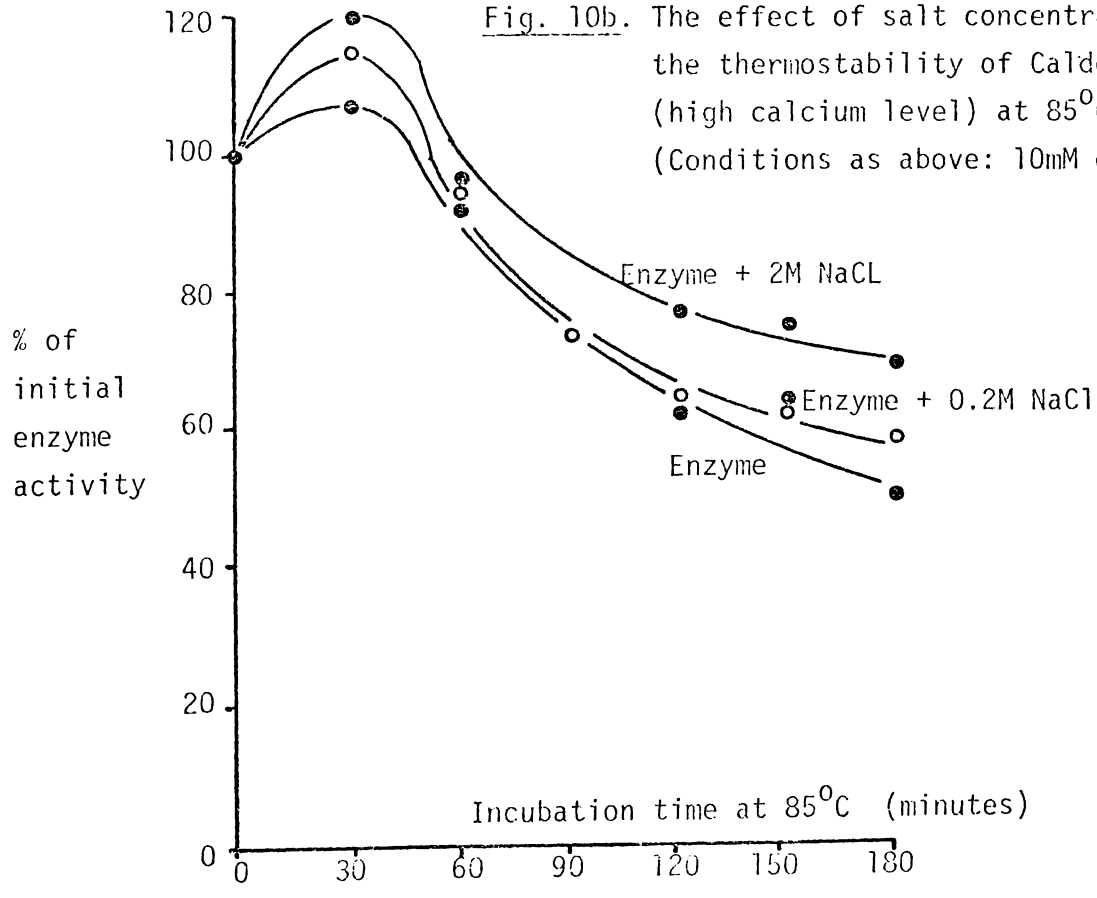


Fig. 10b. The effect of salt concentration on the thermostability of Caldolysin (high calcium level) at 85°C.
(Conditions as above: 10mM calcium)



9-7 The influence of non-enzyme protein on thermostability

Solutions of Caldolysin (2 μg ml^{-1}, pH 8.1, + 10 mM calcium) containing lysozyme (Sigma) at concentrations between 0 and 1 mg ml^{-1} were prepared. These solutions were incubated at 90°C and periodically assayed at 75°C. The resulting thermostability profiles are presented in Fig. 9-11.

Two important features of Fig. 9-11 are firstly, that Caldolysin is inhibited by lysozyme in a manner which is dependent on the concentration of the added protein (substrate inhibition, but see Chapter 10) and secondly, the initial inhibition is reversed by heating for a short period at 90°C. These features are considered to be highly significant in the interpretation of the phenomenon of 'activation' and are discussed in detail in Chapter 10.

Activation apart, it is uncertain whether other definite trends exist. While it is noted that Caldolysin is slightly more stable in the presence of lysozyme than in its absence, there does not appear to be any significant relationship between stability and the amount of lysozyme present.

9-8 The effect of non-ionic solute concentration on the thermostability of Caldolysin

Glycerol was added to solutions of apo- and holocaldolysin to give concentrations of approximately 0.2 M and 2.0 M. During incubation at 85°C, aliquots of enzyme solution were removed periodically for assay at 75°C. Thermostability profiles are presented in Figs. 9-12a and 9-12b.

There is no significant difference in the half-lives of apocaldolysin in the presence or absence of glycerol. However, although the presence of the non-ionic solute does not effect the initial rate of activity loss, it does appear to influence the longevity of the residual enzyme activity

Fig. 9-11. The effect of added protein on the thermostability of Caldolysin at 90°C.
(2µg ml^{-1} enzyme, pH 8.1, 0.1M Tris acetic acid buffer, 10mM calcium)
● —— ●, no lysozyme added
○ —— ○, 0.1 mg ml^{-1} lysozyme added
x —— x, 0.5 mg ml^{-1} lysozyme added
+ —— +, 1.0 mg ml^{-1} lysozyme added

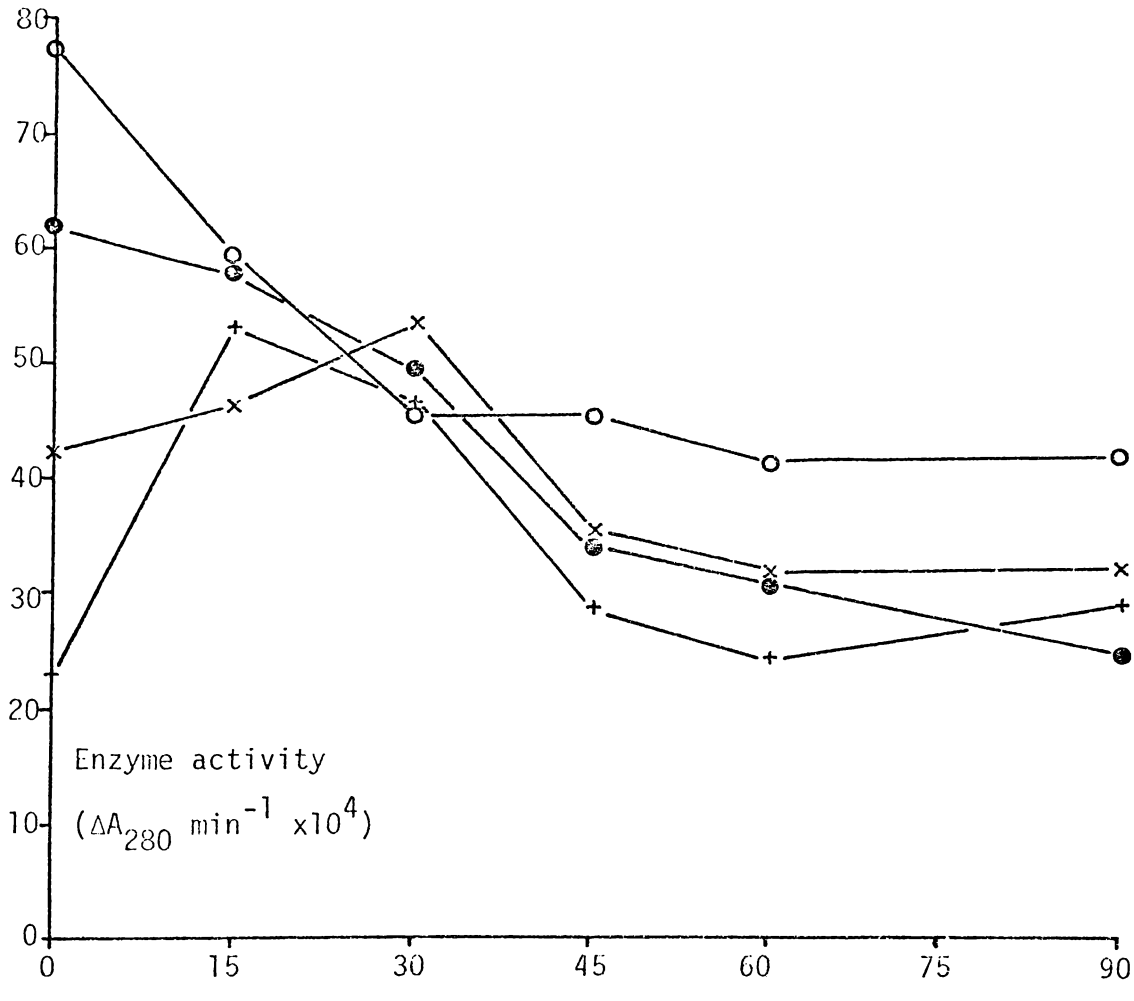


Fig. 9-12a. The effect of glycerol on the thermostability of Caldolysin at 85°C. (20µg ml^{-1} enzyme, pH 8.1, 0.1M Tris acetic acid buffer + 10mM calcium)

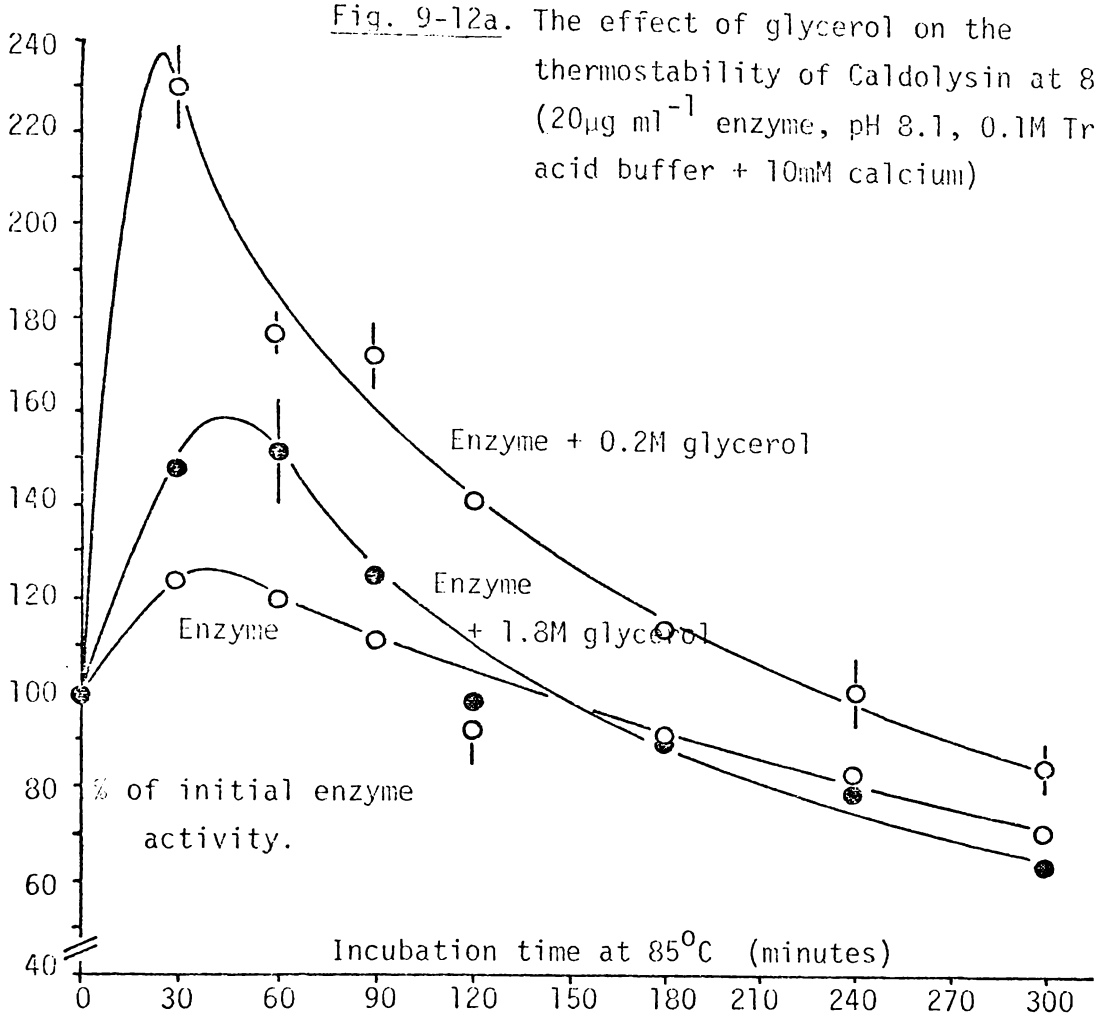
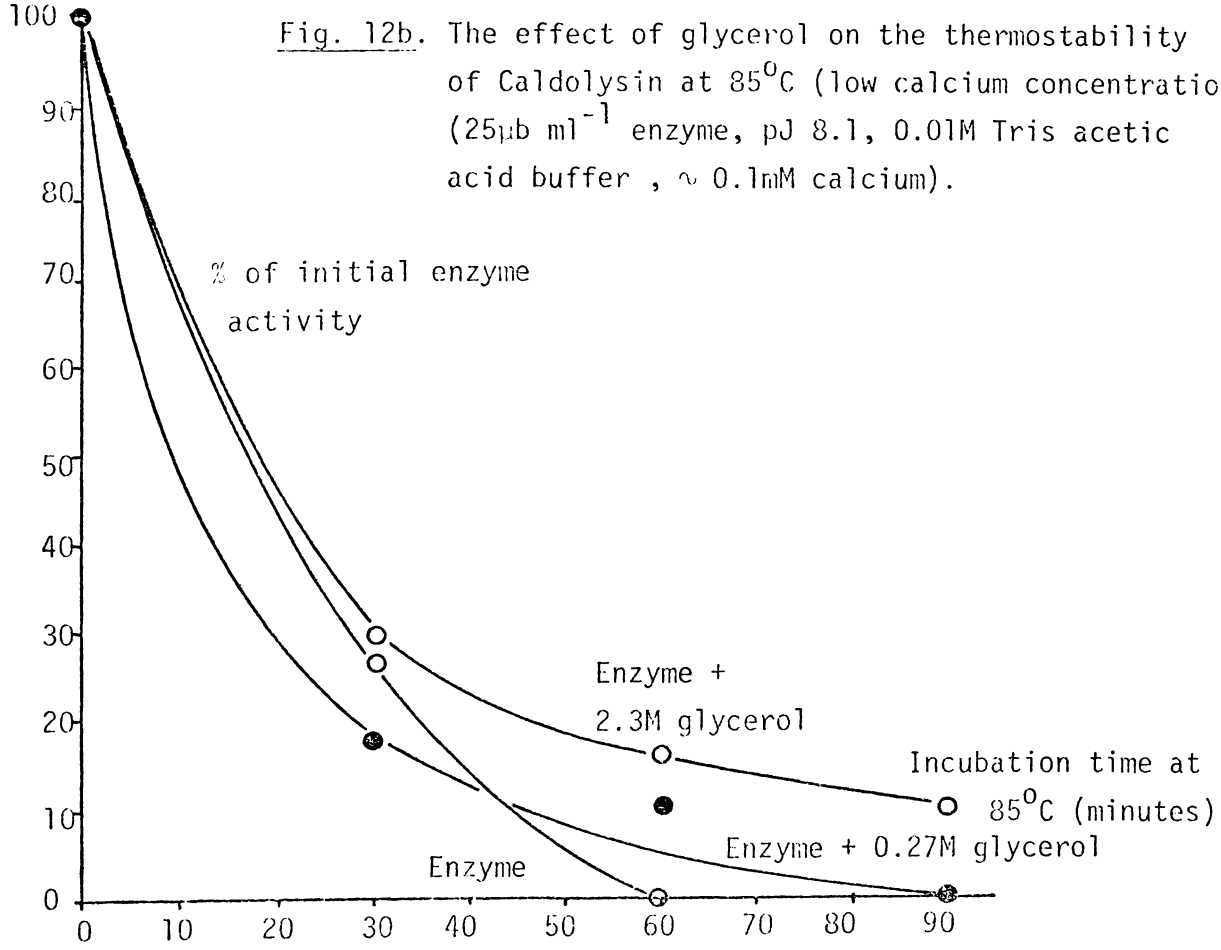


Fig. 12b. The effect of glycerol on the thermostability of Caldolysin at 85°C (low calcium concentration). (25µb ml^{-1} enzyme, pJ 8.1, 0.01M Tris acetic acid buffer , ∿ 0.1mM calcium).



(the final 20%). Autolysis is a concentration-dependent process, and it is assumed that the presence of large numbers of buffering atoms reduces the frequency of enzyme collisions.

In the presence of 10 mM $CaCl_2$, the absolute rate of activity loss is overshadowed by the effects of activation. However, it is concluded that the presence of glycerol at either concentration results in no major change in the thermostability of Caldolysin.

The significance of the activation observed in Fig. 9-12a will be discussed in Chapter 10.

9-9 Temperature - activity relationships in Caldolysin

Many thermophilic (and non-thermophilic) enzymes have shown discontinuities in Arrhenius plots, indicating changes of activation energy, and implying conformational changes in the enzyme structures (Amelunxen & Murdock, 1978a). In most cases a reduction in the slope of the plot at temperatures above the break has been noted, suggesting that the activation energy of the reaction is reduced. Minor gradient changes in Arrhenius plots are distinct from major discontinuities, where an inversion of gradient is indicative of a gross structural change such as thermally-induced protein "melting".

The analysis of structural perturbations in proteases by this method is complicated by the influence of autolysis on the apparent reaction rate. However, corrections for such losses can be made from the thermostability profiles (section 9-1). The activation energy values obtained must be regarded as approximate.

Data for an Arrhenius plot were obtained by the reaction of Caldolysin with 0.5% casein (0.1 M Tris acetic acid, pH 8.1 + 10 mM $CaCl_2$) for short periods at known temperatures. At temperatures above 85°C, brief reaction times were considered desirable to minimise autolysis corrections and

other potential deviations (e.g. activation). Original and corrected data are displayed in Table 9-7.

When replotted as log v against $1/T_{abs}$ (Fig. 9-13), two discontinuities are evident. The first is a minor decrease in gradient at $50^{o}C$ - $55^{o}C$. Calculated activation energies are: E_a ($<50^{o}C$) = 4048 kJ $mole^{-1}$; E_a ($>50^{o}C$) = 2165 kJ $mole^{-1}$. While this decrease in activation energy is likely to be indicative of a conformational change in the enzyme resulting in facilitation of active site binding or reactivity, a structural change in the protein substrate could promote enzyme activity in a similar manner. To determine which of these mechanisms is involved, an Arrhenius plot derived from the hydrolysis of the peptide substrate, Benzoyl-phe-val-arg-pNA, is presented below (Fig. 9-14).

A second discontinuity is observed in Fig. 9-13 at approximately $92^{o}C$. Since it is inevitable that corrections taken from thermostability profiles must also compensate for loss of activity caused by structural melting, the true activity values (compensated for autolysis only) will lie somewhere between the maxima and minima given.

It is suggested that the break at about $92^{o}C$ corresponds to the thermal melting point of Caldolysin. However, the physicochemical analysis of structural parameters (e.g. viscosity changes) would be required to substantiate this contention.

While some proteins have been shown to dissociate by a pH- and temperature-dependent mechanism involving a series of discrete structures (Burgess & Scheraga, 1975), it is likely that few (or possibly only one) of the unfolding stages are necessary for the loss of active site integrity and activity. Thus it is reasoned that while thermal dissociation may span a range of temperatures, loss of enzyme activity may occur during a narrow temperature span. An Arrhenius plot derived from the hydrolysis of Benzoyl-phe-val-arg-pNA by Caldolysin (see section 4-8 for

TABLE 9-7 Temperature-activity relationships

Temperature (°C)	Assay time (minutes)	% Correction [a]	Reaction velocity (v) (ΔA_{280} min^{-1})	Corrected v (ΔA_{280} min^{-1})
20	120	0	0.00062	0.00062
25	60	0	0.00079	0.00079
30	30	0	0.00167	0.00167
35	30	0	0.00267	0.00267
40	20	0	0.0036	0.0036
45	20	0	0.0071	0.0071
50	15	0	0.0104	0.0104
55	10	0	0.0164	0.0164
60	7	0	0.0190	0.0190
65	5	0	0.0268	0.0268
70	5	0	0.0344	0.0344
75	4	0	0.0483	0.0483
80	3	0	0.0583	0.0583
82	4	0	0.0695	0.0695
84	3	0	0.0689	0.0689
86	3	<1	0.0795	0.0796
86	3	<1	0.0742	0.0743
88	3	1	0.0896	0.0904
90	3	2	0.1014	0.1035
92	3	3	0.1098	0.1131
94	3	5	0.1221	0.1282
94	3	5	0.1313	0.1366
96	2	9	0.0975	0.1047
96	3	14	0.0948	0.1081
98	2	20	0.1238	0.1486
98	2	20	0.1120	0.1344
100	2	29	0.1057	0.1364
100	3	43	0.1027	0.1469

a. Data derived from thermostability profiles.

Fig. 2-13. Arrhenius plot for Caldolysin (enzyme (20μg ml^{-1}) incubated at given temperatures with 0.5% casein in 0.1M Tris acetic acid buffer, pH 8.1 (calcium present).

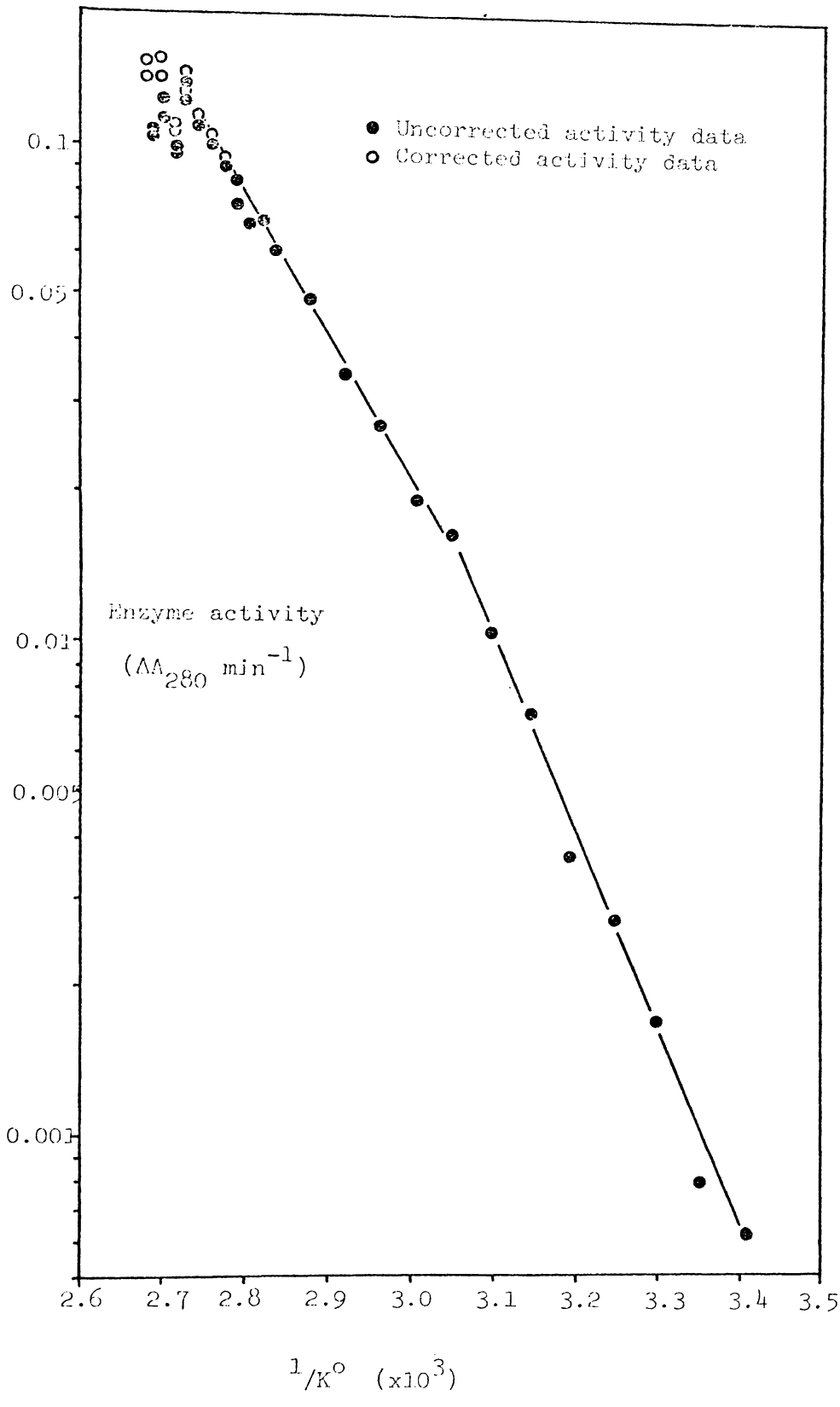
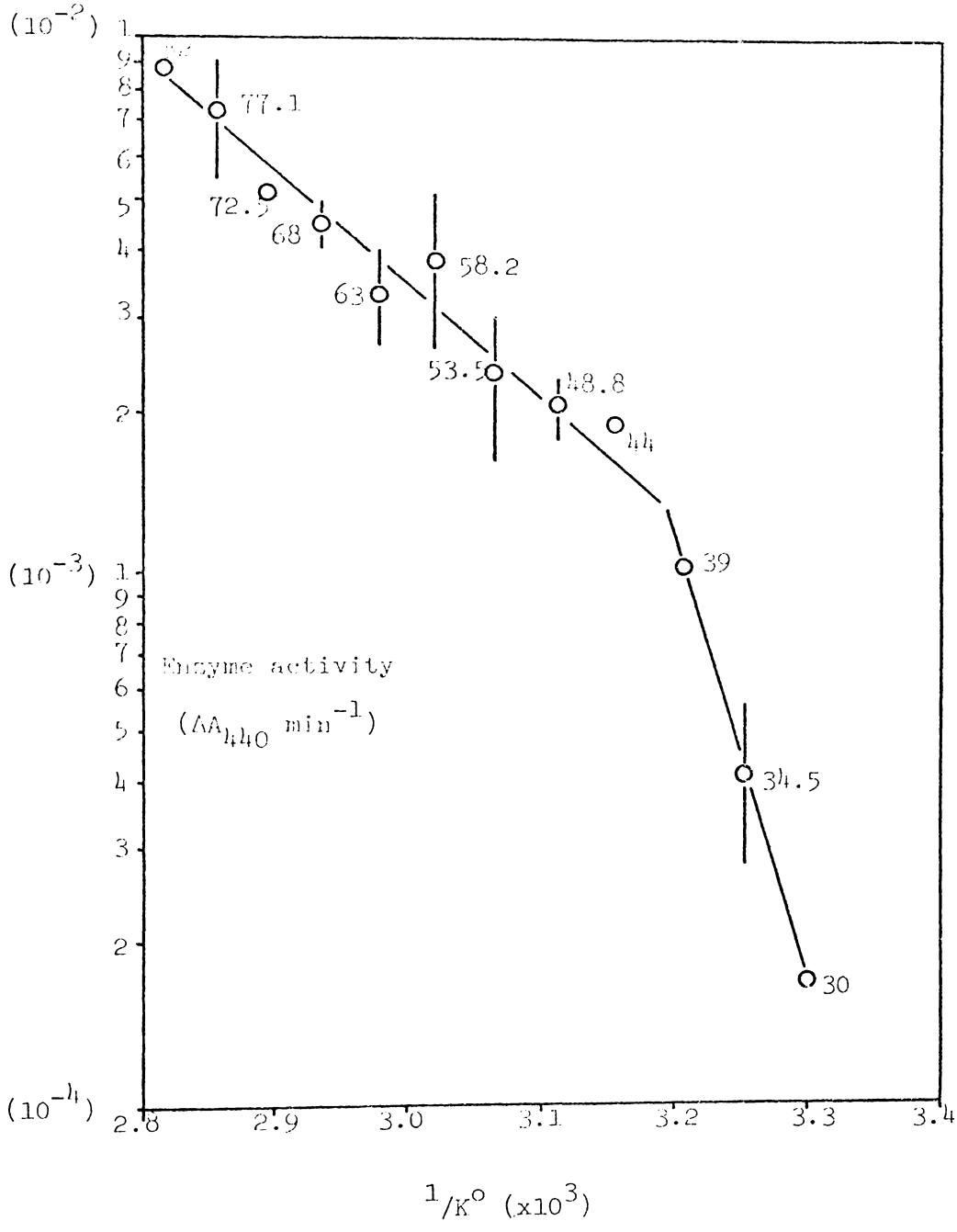


Fig. 9-14. Arrhenius plot of the hydrolysis of Benzoyl-phe-val-arg-p-nitroanilide by Caldolysin. (temperatures (^{o}C) indicated).



methods) is presented in Fig. 9-14, and demonstrates a significant discontinuity between 39°C and 44°C. Since the peptide substrate is most unlikely to structurally alter during changes in temperature, it is concluded that similar 'breaks' in the Arrhenius plots derived from the hydrolysis of both casein and Benzoyl-phe-val-arg-pNA are the product of temperature-induced conformational changes in the Caldolysin molecule.

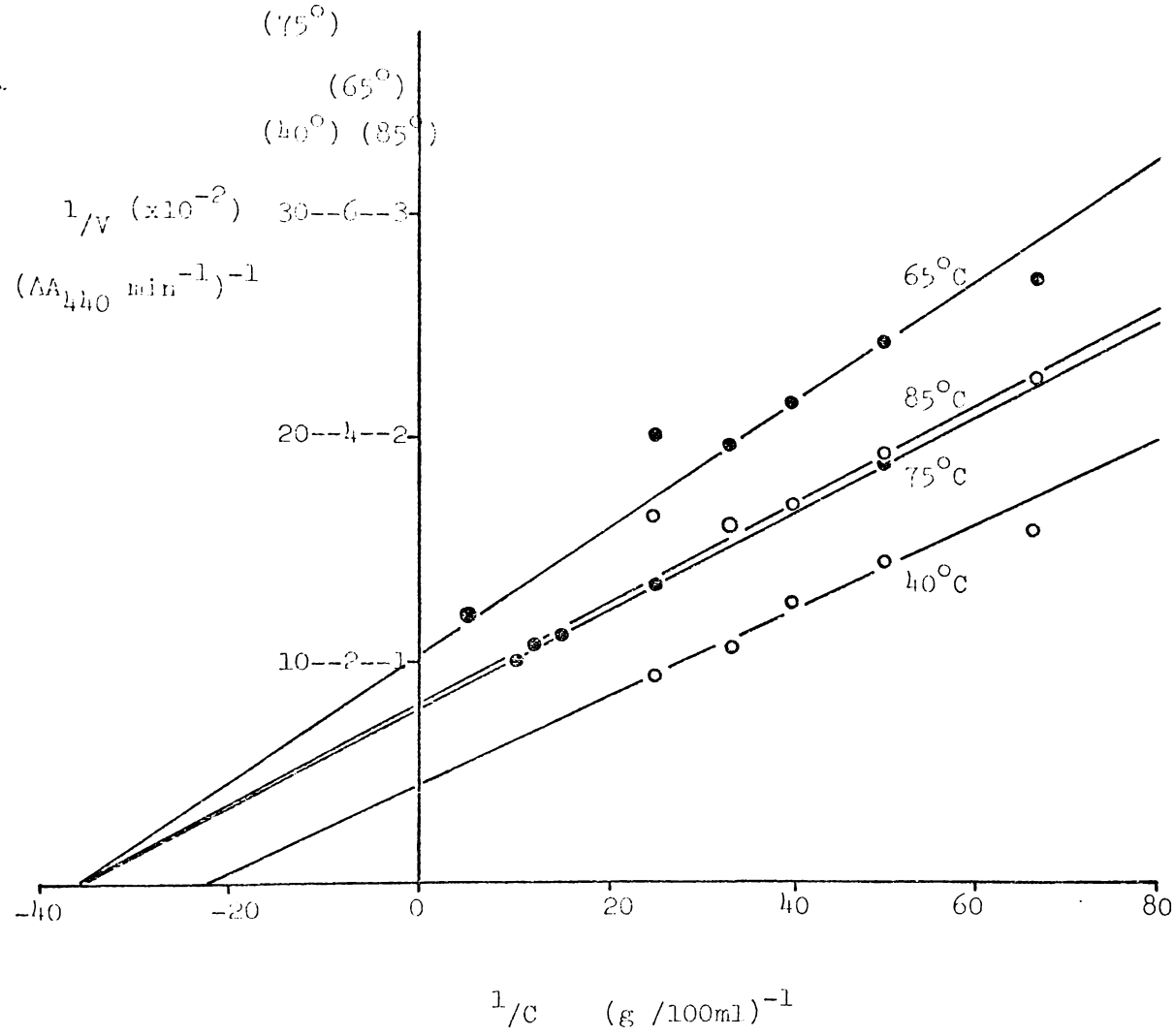
It is noted that the discontinuity in Fig. 9-14 is approximately 10°C lower than that in Fig. 9-13. It is possible that the high molecular weight protein substrate, when bound to the active site (or a non-catalytic site), enhances structural rigidity by multipoint linkages, and hence increases the energetic requirement for the conformational change.

9-10 The influence of temperature on K_m

It was suggested in section 9-9 that the discontinuity in the Arrhenius plot at approximately 50°C was the result of a conformational change in the enzyme structure. Since a change in the activation energy was the criterion for this conclusion, the function of the active site, and hence the K_m, may be different above and below 50°C.

The rate of hydrolysis of various concentrations of casein by Caldolysin (20 μg ml^{-1}, pH 8.1) was determined in the manner described in section 8-7. In the Lineweaver-Burk plots shown in Fig. 9-15, deviations from linearity at high substrate concentrations occur as a result of substrate inhibition. K_m data derived from Fig. 9-15 are presented in Table 9-8. It is clear that the K_m at 40°C is significantly higher than that at 65°C, 75°C, or 85°C, suggesting that a molecular change between 40°C and 65°C results in a facilitation of substrate binding. When compared to activation energy data derived from section 9-9, it is noted that an increase in the affinity of the enzyme for substrate corresponds

Fig. 9-15. The influence of temperature on the K_m for Caldolysin.



to a decrease in activation energy, implying that both substrate binding and catalysis are influenced.

TABLE 9-8 K_m and E_A data for Caldolysin at different temperatures

Temperature	K_m	E_A
(^oC)	(mg ml^{-1})	(kJ $mole^{-1}$)
40	0.043	4048
65	0.027	2165
75	0.026	2165
85	0.027	2165

9-11 The reversibility of thermally-induced enzyme activity loss

It has been shown that treatment of apocaldolysin at 85°C for 30 minutes results in complete loss of enzyme activity. A determination of the reversibility of this state could provide information about the mechanisms by which the enzyme deactivation occurs. If, for instance, autolysis was the sole mechanism, no reactivation could reasonably be expected. However, if denaturation was responsible, reactivation of some protease molecules might occur if suitable conditions for protein refolding were chosen. Since calcium has been shown to play a vital role in the structural stabilisation of Caldolysin, it was considered that the addition of calcium to a denatured sample of apocaldolysin might promote renaturation.

A solution of apocaldolysin (25μg ml^{-1}, 0.01 M Tris acetic acid, pH 8.1, 0.01 mM Ca^{2+}) was incubated at 85°C for 90 minutes. Total loss of activity was observed within 60 minutes. Portions of the inactive enzyme solution were then treated as shown in Table 9-9. Aliquots were removed for assay at the time of storage, and after one and 18 hours treatment.

TABLE 9-9 Recovery of enzyme activity

	Enzyme stored at 20°C	Enzyme stored at 20°C (10 mM Ca^{2+})	Enzyme stored at 85°C (10 mM Ca^{2+})
Initial enzyme activity [a]	4.6	4.6	4.6
Activity after 90 minutes at 85°C	0	0	0
Activity after 1 hour of post-incubative treatment	0	0	0
Activity after 18 hours of post-incubative treatment	0	0	0.5 ± 0.2

a. Expressed as ΔA_{280} $hour^{-1}$ (x 10^2) under the conditions of the Kunitz assay.

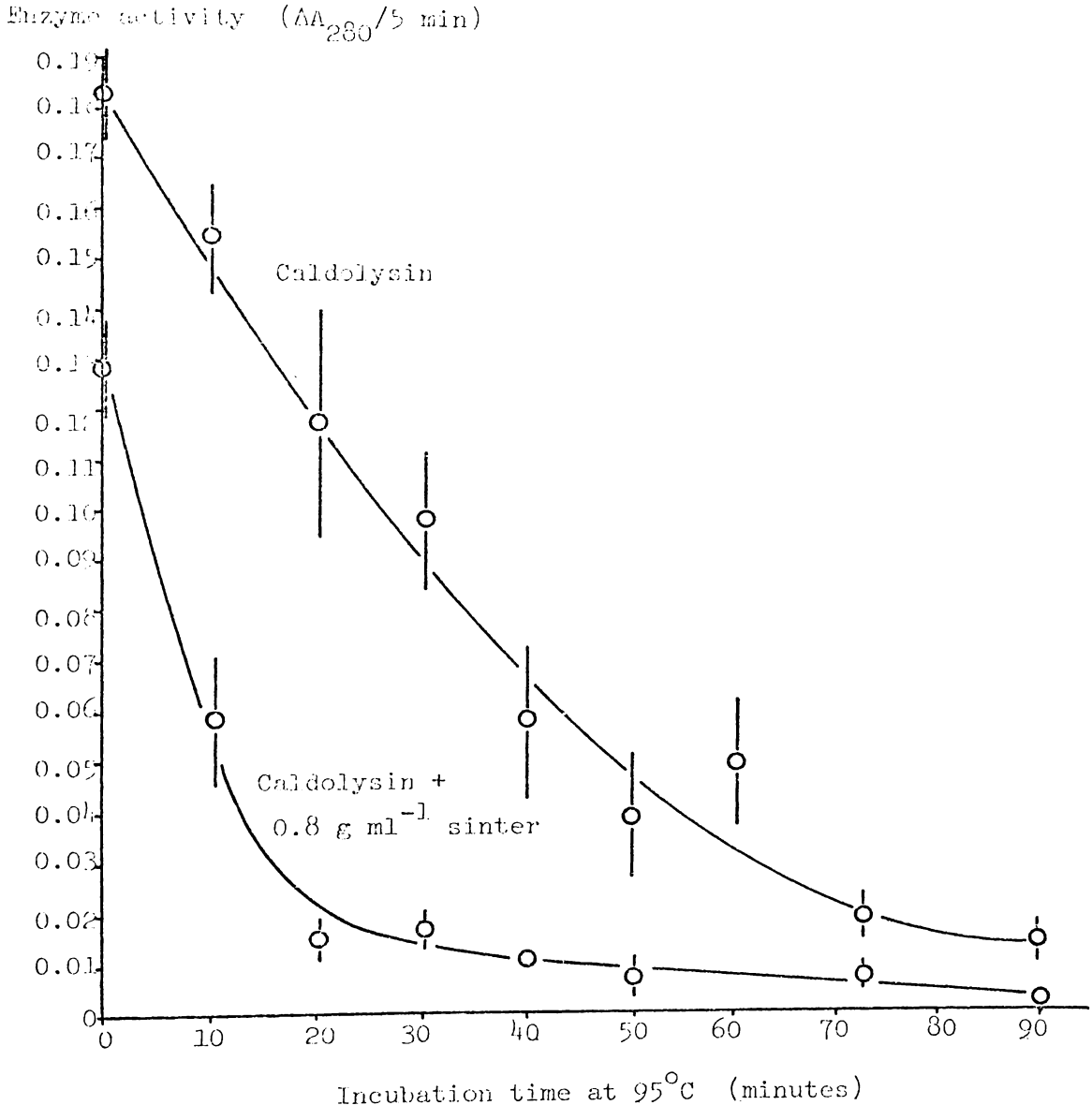
It is concluded that, under the conditions employed, reactivation is not a significant process. However, the range of treatments to which the "inactive" enzyme was subjected is insufficient to permit any further conclusions to be drawn.

9-12 Stability of Caldolysin in the presence of silicate sinter

In the natural thermal pool environment, extracellular enzymes produced by thermophilic miroorganisms are subjected to temperatures of up to 100°C (Brock, 1978). Calcium concentrations in the waters of the Whakarewarewa thermal region range from 20 µmole l^{-1} to 90 µmole l^{-1} (Lloyd, 1975). Under these conditions at 100°C, the half-life of Caldolysin is probably less than one minute, assuming that no other stabilising mechanism is operative (see sections 9-1 and 9-2).

The action of the silicate sinter (with which the alkaline thermal pools are thickly encrusted) on the thermostability of Caldolysin was

Fig. 9-16. The influence of silicate sinter on the thermostability of Caldolysin at 95°C (25µg ml^{-1} Caldolysin in 0.1M Tris acetic acid buffer, pH 8.1, + 10mM calcium).



tested. Silicates are known to have strong absorbtive properties, and the enhancement of thermostability of several proteases on ionic attachment to ceramic (silicate) supports has been documented (see Chapter 12).

Silicate sinter was collected from an alkaline pool at Whakarewarewa (pH 8.1, 86°C), ground, and washed with distilled water. Sinter powder was added (0.8 mg ml^{-1}) to a solution of Caldolysin (25 µg ml^{-1} in 0.1 M Tris acetic acid buffer, + 10 mM $CaCl_2$, pH 8.1). The thermostabilities of the free enzyme and the enzyme sinter suspension were monitored at 95°C (Fig. 9-16).

The two significant features resulting from the presence of the sinter area:

a. An immediate activity loss of about 30% occurs. It is concluded that a proportion of the enzyme is adsorbed to the sinter in a manner preventing enzyme activity.

b. Caldolysin is destabilised in the presence of sinter. While the mechanism for this is not known, it is possible that the high ionic adsorption capacity of the silicate results in the depletion of calcium in solution.

The results of this experiment suggest that the sinter of the thermal pool walls has a detrimental influence on the thermal stability of Caldolysin *in vivo*. However, it has been suggested that at temperatures below the melting point of Caldolysin (about 92°C), autolysis is largely responsible for the loss of enzyme activity. In the very low concentrations of Caldolysin expected to be present *in vivo*, autolysis may be significantly reduced.

9-13 Caldolysin; Stability during storage

Proteolytic enzymes, by nature of their catalytic properties, are subject to loss of enzyme activity by autolysis, a particularly signif-

icant problem in the handling of mesophilic proteases. Caldolysin, however, has demonstrated considerable stability to both high temperatures and extremes of pH (sections 9-1 and 9-5).

To determine the stability of Caldolysin under various common storage conditions, 5 ml volumes of a solution (20 μg ml^{-1} enzyme, 0.1 M CH_3COONa + 10 mM $CaCl_2$, pH 7.1) were placed in sterile preconstricted glass ampoules. These were treated as described in Table 9-10 (six ampoules per category).

TABLE 9-10 Storage conditions of Caldolysin

Category	Treatment	Storage temperature (°C)
1	Lyophilised, sealed under vacuum	-18
2	Lyophilised, sealed under vacuum	Room temperature [a]
3	Lyophilised, sealed at atmospheric pressure	-18
4	Lyophilised, sealed at atmospheric pressure	Room temperature [a]
5	Lyophilised, not sealed [b, c]	-18 [c]
6	Lyophilised, not sealed [b]	Room temperature [a]
7	Frozen, sealed at atmospheric pressure	-18
8	Frozen, sealed at atmospheric pressure	-196
9	Sealed at atmospheric pressure [d]	4
10	Sealed at atmospheric pressure [d]	Room temperature [a]

a. ca. 22°C ± 3°C.

b. Plugged with cotton wool.

c. Stored over desiccant.

d. Solutions sterilised by Tyndallisation (80°C, 20 min ; repeated three times over a 72 hour period).

Lyophilisation was carried out by the use of an Edwards Centrifugal Freeze-drier. Ampoules were sealed under vacuum with a gas-O_2 torch.

Immediately after preparation, one ampoule from each category was opened and the contents assayed (lyophilised samples were resuspended in 1 ml of distilled water). After storage in the given conditions for 1 week, 4 weeks, 10 weeks, and 30 weeks, further samples were assayed (Table 9-11).

TABLE 9-11 Stability of Caldolysin during storage

Category [a]	Percentage of original activity remaining after storage for a given period (weeks).			
	1	4	10	30
1	100	86.5 ± 10.0	80.9 ± 4.3	87.9 ± 4.3
2	100	92.6 3.6	108.3 ± 4.1	105.8 ± 1.7
3	100	-	132.0 ± 1.6	67.7 ± 11.3
4	100	116.9 ± 10.3	103.0 ± 1.5	84.6 ± 6.2
5	89.0 ± 5.5	76.7 ± 4.9	93.2 ± 4.1	84.9 ± 1.4
6	43.0 ± 3.3	166.7 ± 23.3	113.3 ±10.0	70.0 ± 23.3
7	113.0 ± 4.0	79.2 ± 3.4	100	82.1 ± 1.9
8	100	79.2 ± 1.5	108.9 ± 2.3	77.7 ± 3.0
9	100	83.1 ± 8.6	100	70.4 ± 5.0
10	106.7 ± 3.8	82.4 ± 4.6	95.2 ± 1.9	59.5 ± 1.4

a. See Table 9-10.

Despite the considerable variations in the activity data, it is evident that Caldolysin is extremely stable under all conditions applied. Even as a solution at pH 7 and 22°C, only 40% of the initial activity was lost after approximately 7 months. Although the samples in categories 9 and 10 were pre-sterilised, the possibility of bacterial contamination cannot

be completely excluded in either case.

While it has been shown that the proteolysis of casein by Caldolysin is quite significant at 20°C (section 9-9), autolysis at a similar temperature is apparently negligible, presumably a function of the very low protein concentrations present.

9-14. The effect of denaturing agents on the stability of Caldolysin

Caldolysin (approximately 20µg ml^{-1} in 0.1M Tris acetic acid buffer, pH 8.1, + 10mM calcium) was incubated at 18°C and 75°C in the presence of various denaturing agents. Aliquots were removed at intervals for assay by the Kunitz method. The half-life of Caldolysin in the presence of urea, guanidine hydrochloride, and sodium dodecyl sulphate (SDS) is outlined in Table 9-12.

TABLE 9-12 The effect of denaturing agents

Denaturing agent	Half-life of Caldolysin at:	
	18°C	75°C
0.8M urea	>>13 hours	148 minutes
8M urea	>>67 hours	53 minutes
8M urea + 1% mercaptoethanol	>>72 hours	26 minutes
6N guanidine.HCl	>>31 hours	59 minutes
1% SDS	>>13 hours	>5 hours

The data in Table 9-12 indicate that Caldolysin is stable in the presence of high concentrations of denaturing agents at ambient temperatures. At high temperatures, enzymic activity is rapidly lost, particularly in the presence of agents which are thought to disrupt hydrogen-bonding (urea, guanidine.HCl). Stability in the presence

of 1% SDS, a detergent known to dissociate hydrophobic interactions, is high at both low and high temperatures. A decrease in the half-life of Caldolysin (at 75^{o}C in 8M urea) on the addition of 1% mercaptoethanol (a reducing agent active against disulphide bonds) suggests that S-S bonds may contribute significantly to to the molecular stabilisation of the proteolytic enzyme (see section 11-1).

CHAPTER 10

ACTIVATION OF CALDOLYSIN

10-1 Introduction

Enzyme activation can occur by a variety of different mechanisms;

a. Removal of an enzyme-bound inhibitor. Such inhibitors may act directly by active-site binding, or indirectly at another site. For example, Cd^{2+} inhibits the peptidase activity of carboxypeptidase B (Folk & Gladner, 1961), and a number of specific low molecular weight protein inhibitors of proteolytic enzymes have been isolated (Umezawa, 1976).

b. Chemical modification of the active site can facilitate catalysis. Treatment of thermolysin with diethylpyrocarbonate and β-phenylpropionyl-L-phenylalanine increases peptidase and esterase activity by an order of magnitude (Blumberg *et al.*, 1973).

c. Limited hydrolytic cleavage of a portion of the polypeptide chain of an inactive enzyme precursor can result in activation. Proteolytic activation of zymogens is a common phenomenon in eucaryotic cells (Barrett, 1977). Drapeau (1978) isolated a staphylococcal protease, the precursor of which was shown to undergo limited proteolysis during excretion.

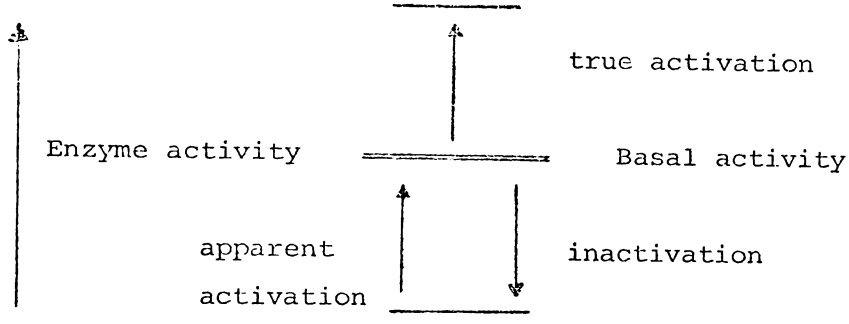
d. Aquisition of a required cofactor. The activity of many enzymes can be impaired by the absence of an essential cofactor. Metal ions are frequently involved as enzyme activators (Dixon & Webb, 1964, p421). Many other protein and non-protein cofactors are known to have essential roles in enzyme catalysis.

e. The susceptibility of the enzyme substrate may alter (see section 5-3).

These mechanisms can be divided into two broad categories, namely:

a. True activation, a process by which catalysis is facilitated. In this category can be included chemical modification and the positive influence of effectors, i.e. b and c above.

b. Apparent activation, usually resulting from the removal of inhibition, i.e. a, d, and e above. The difference in activity levels can be expressed as:



Basal enzyme activity is defined as the rate of catalysis of an enzyme in a state where essential cofactors are present, but without the addition of modifiers of any type.

10-2 Activation of Caldolysin

Previous experiments have shown that under certain conditions, Caldolysin demonstrates transient activation. Corelations have been shown between the degree of activation and the calcium concentration (Fig. 9-2), the NaCl concentration (Fig. 9-10b), the presence of glycerol (Fig. 9-12a), and the concentration of non-enzyme protein (Fig. 9-11). Numerical data from Fig. 9-11 are presented in Table 10-1.

These results suggest the mechanism by which the activation of Caldolysin occurs. The significant features of Table 10-1 are firstly, that increasing concentrations of added lysozyme cause a greater degree of inhibition and secondly, that inhibition is almost totally reversed during incubation at 90°C. This suggests that the activation phenomenon observed in Caldolysin is the reversal of bound-protein inhibition. The responses

to calcium, NaCl and glycerol concentrations can be explained if the protein inhibitor is bound by ionic and/or H-bond interactions, both of which are significantly destabilised by an increase in the charge of the environment. This suggestion is supported by a corelation between the degree activation of Caldolysin at 85°C and the ionic strength of the solvent (Fig. 10-1). Glycerol, although non-ionic, is capable of H-bonding and could selectively interfere with protein-protein H-bonding interactions.

TABLE 10-1 The effect of lysozyme concentration on the activity of Caldolysin

Concentration of lysozyme added (mg ml^{-1})	Activity of Caldolysin after addition of lysozyme	Percentage inactivation	Maximum activity attained during heating at 90°C. [a]
0	62	0	62
0.5	42	32	54 (59) [b]
1.0	23	63	53 (50) [b]

a. Activity = ΔA_{280} min^{-1} (x 10^4) under Kunitz assay conditions.

b. Figures in brackets represent activity of lysozyme-free enzyme after the same period of incubation at 90°C.

Other evidence supports the theory that Caldolysin is inhibited by protein binding. Substrate inhibition observed in Lineweaver-Burk plots from the hydrolysis of casein and azoalbumin (and also azocasein; data not shown) by Caldolysin (section 8-7) indicates that inhibition by high protein concentrations is a general characteristic. It may be significant that inhibition of Caldolysin by casein and lysozyme is observed at concentrations above 0.1 mg ml^{-1}, while the effect is not exhibited by either azoalbumin or azocasein until a concentration of about 1.0 mg ml^{-1} is

Fig. 10-1. Relationship between ionic strength and enzyme activation. (Caldolysin (25µg ml^{-1}, pH 8.1 containing 1 mg ml^{-1} lysozyme) added to ammonium sulphate solutions to give I values below. Solutions incubated at 85°C and assayed at intervals).

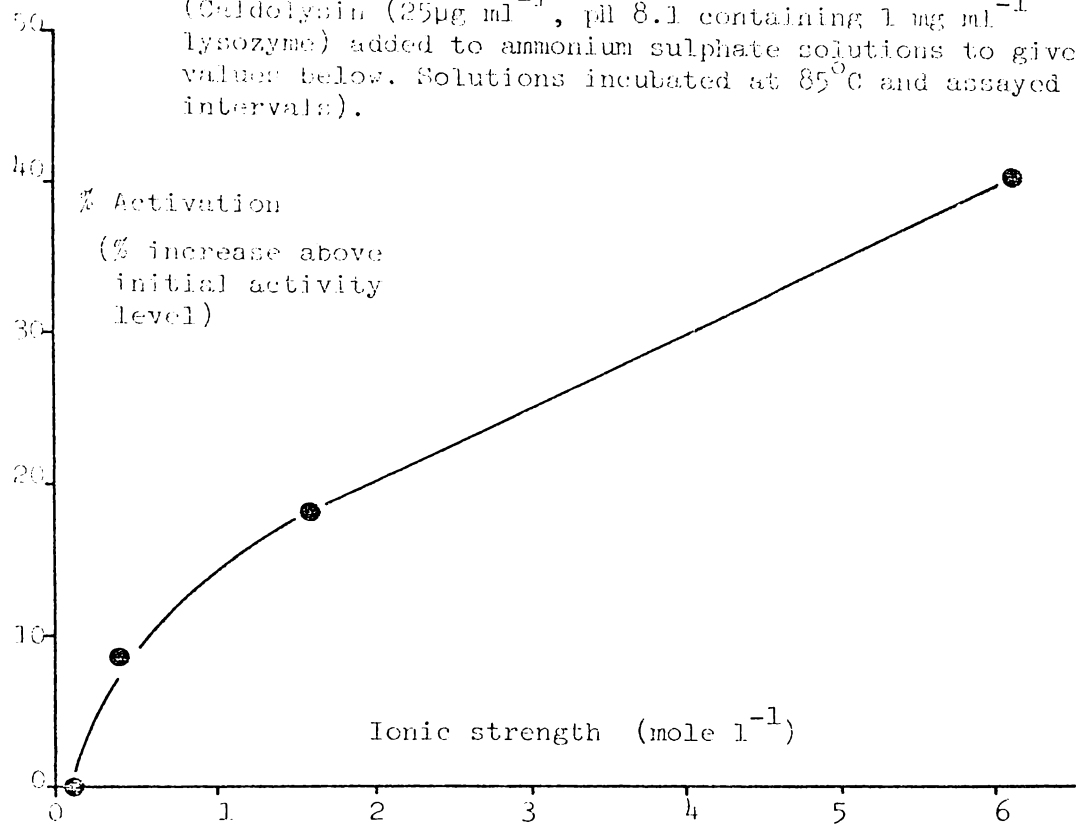
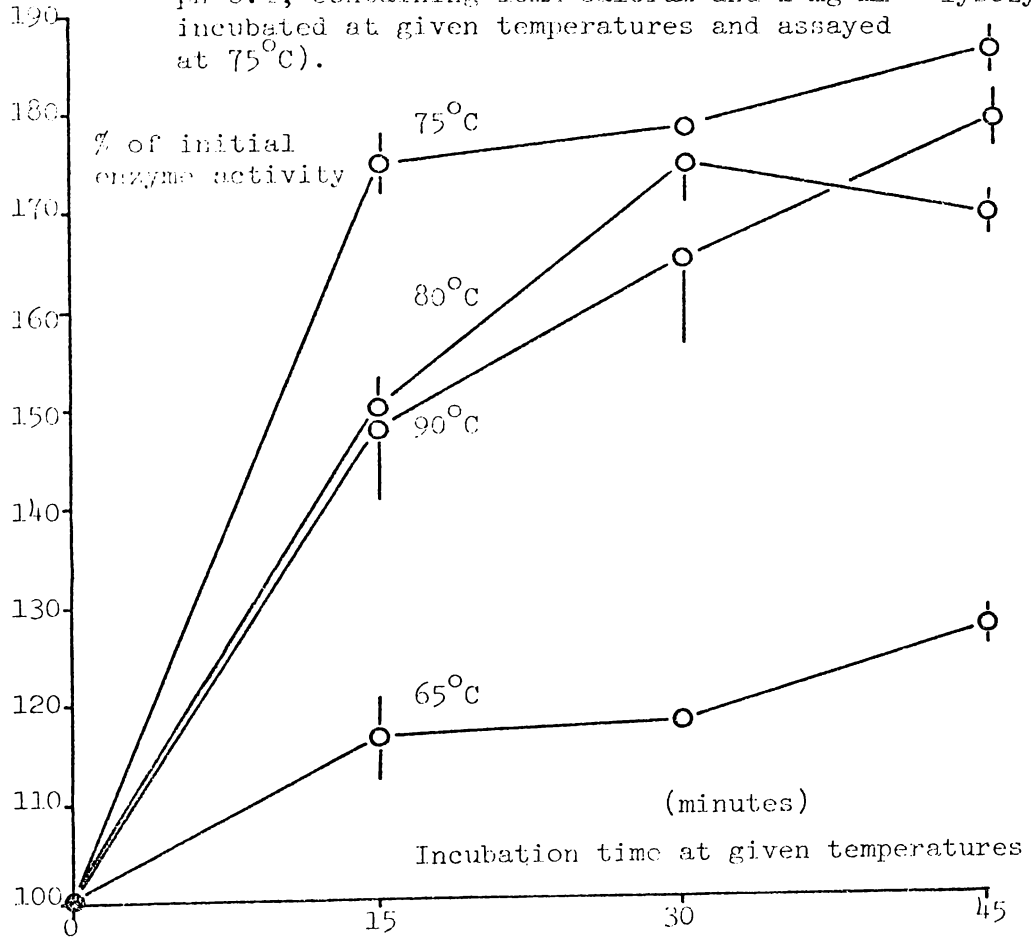


Fig. 10-2. Activation of Caldolysin in relation to temperature. (Caldolysin (20µg ml^{-1} in 0.1M Tris acetic acid buffer, pH 8.1, containing 10mM calcium and 1 mg ml^{-1} lysozyme) incubated at given temperatures and assayed at 75°C).



exceeded. Since the azo-dye moieties are bound to the polypeptide via free amino groups, these amino groups may be the linkage points between enzyme and inhibitor. The reduction in the surface concentration of free $-NH_2$ groups as the result of azo-adduct formation might be responsible for the difference observed.

It was noted that when Caldolysin was immobilised to Sepharose 4B, activity was reduced by no more than 20%, while substrate inhibition was removed altogether (see Chapter 12). This is strong evidence in support of the suggestion that the substrate-inhibition site(s) on the enzyme is not the same as the active site. The size of the protein substrate molecules is such that steric interference could result from binding some distance from the active site. Attachment to an insoluble matrix apparently prevents such binding. In addition, no activation was observed in thermostability profiles of Sepharose-Caldolysin.

The hypothesis that protein-enzyme association is responsible for the activation/inactivation of Caldolysin suggests that inactive enzyme-enzyme complexes might exist. However, gel chromatographic separations showed no activity peaks at elution volumes corresponding to molecular weights of 40 000 or above. To ascertain whether the dissocation of inactive complexes might be dependent on high temperatures, fractions eluted from Sephadex G75 columns were heated to 86°C for 15 minutes and reassayed. No additional activity was generated and it seems unlikely that enzyme dimerisation occurs.

The temperature-dependence of the dissociation of the Caldolysin-lysozyme complex was studies by incubating solutions of Caldolysin containing lysozyme (1.0 mg ml^{-1}) at 65°, 75°, 80°, and 90°C. Aliquots were removed at intervals for assay. The degree of enzyme activation over a 45 minute period is shown in Fig. 10-2. It is concluded that within the bounds of experimental error, the dissociation of the Caldolysin-

lysozyme complex is temperature dependent. The activation observed at 75°, 80°, and 90°C is essentially similar. However, that observed at 65°C is significantly lower, suggesting that the temperature necessary to promote dissociation lies between 65°C and 75°C.

Finally, peptide inhibitors of proteases are numerous, particularly as mechanisms of control in tissue and organelle systems. However, the interactions are often quite specific, while the evidence presented above suggests that the Caldolysin-protein association is non-specific.

10-3 Enzyme activation: Conclusions

It has been reasonably established that the activation phenomonen observed to occur during the heating of solutions of Caldolysin falls into the category of "apparent activation". It is concluded that this is the result of the dissociation of inactive or partially active enzyme-protein complexes. The formation of such complexes appears to be a non-specific process, occurring with a number of different proteins, although possibly influenced by the surface charge or the number of free amino groups.

The absence of substrate inhibition and activation when Caldolysin is immobilised to an insoluble substrate suggests that the inhibitor binding site(s) is/are not necessarily close to the active site, and that the two effects may result from binding to the same site. The dissociation or activation process is apparently temperature dependent. It is also strongly influenced by factors such as increased salt concentration, which could interfere with ionic linkages. Since the non-ionic solute, glycerol, results in the greatest activation observed, it is likely that hydrogen bonding also plays a part in the association of the inhibitor-enzyme complex. The comparative affinities of the substrate and inhibitor sites for protein molecules are indicated in the relative magnitudes of the K_m

and K_S' values (calculated as outlined in section 8-7) for Caldolysin and the substrate azoalbumin:

K_m (azoalbumin) = 0.28 mg ml^{-1}

K_S' (azoalbumin) = $\sim$3 mg ml^{-1}

As shown graphically in Figs. 8-3b and 8-4, little substrate inhibition is observed below 2 mg ml^{-1} substrate concentrations.

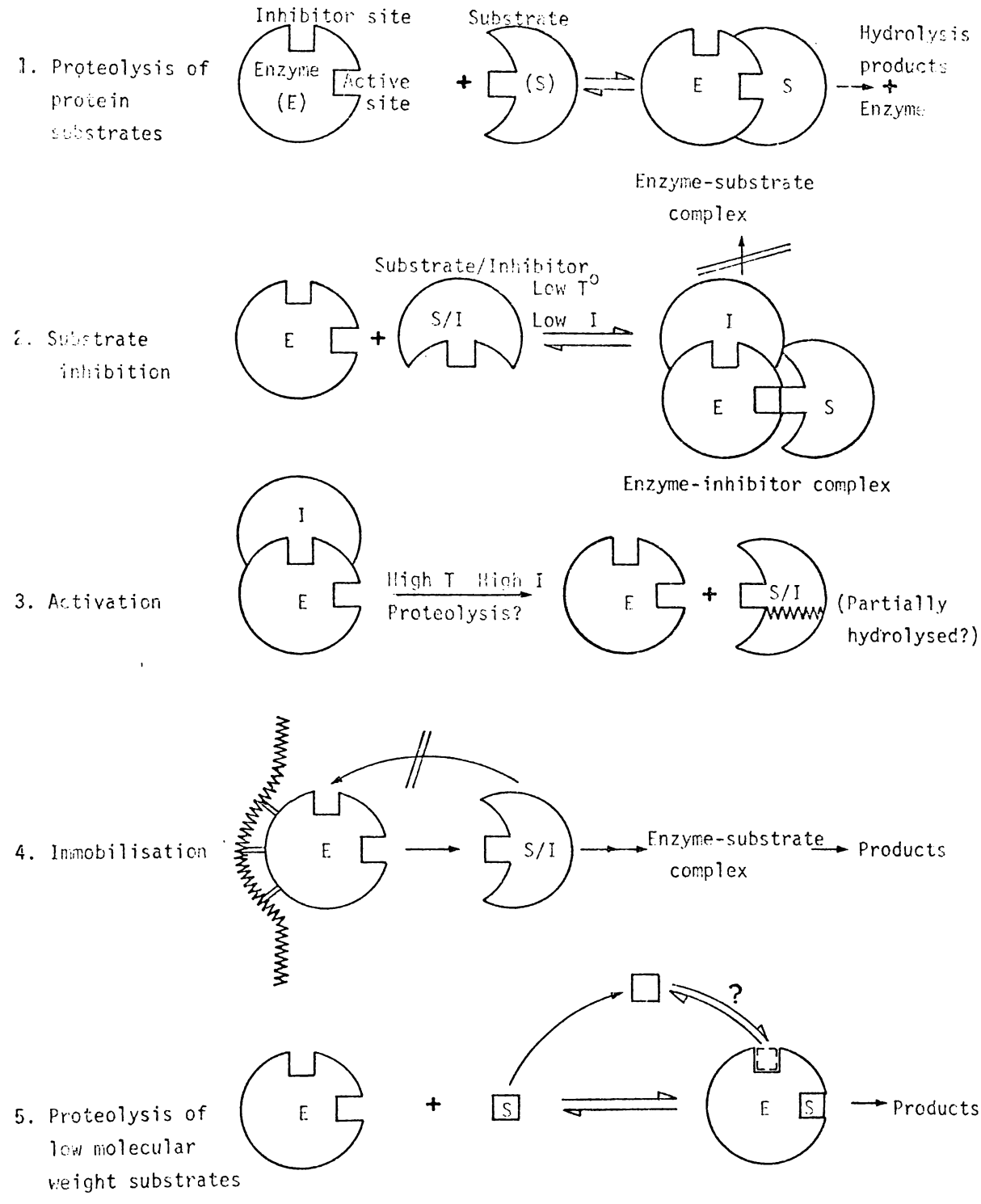
A number of different mechanisms can fulfil some of the above observations. For example, while a state of negative cooperativity between multiple active sites could lead to a 'substrate inhibition' response, such a mechanism could not account for the lack of inhibition when using a low molecular weight substrate. Similarly, while the presence of multiple substrate sites sufficiently close together for steric hindrance to come into effect at high substrate concentrations, or a large active site with the catalytic components localised at one end (thus permitting substrate binding without catalysis, and steric hindrance of further substrate access (Dixon & Webb, 1964)) might explain some of the factors mentioned above, these mechanisms would not be consistent with the observed loss of activation and substrate inhibition on enzyme immobilisation.

Activation is a relatively slow procedure, usually occurring within 15 to 30 minutes. This process is rather slow to be a purely kinetic dissociation, and may involve proteolytic cleavage. By analogy, a number of proteolytic enzymes undergo slow autolytic modification of precursors to attain activity (Dixon & Webb, 1964), although, in the case of Caldolysin, it is likely that cleavage of a bound protein rather than the enzyme itself occurs.

The degree of activation has been shown to vary considerably in different enzyme preparations purified by the same procedures. This observation suggests that autolysis products may also act as enzyme inhibitors, since the level of autolysis fragments present in an enzyme solution will

vary with the age and storage conditions of the preparation. The phenomenon of activation has been observed in other enzyme systems in *Thermus* T-351 (Hickey & Daniel, 1979; Jansen & Daniel, unpublished results). Since activation/inhibition has been shown to be temperature-linked, and preliminary results suggest that it occurs in more than one metabolic system in *Thermus* T-351, such mechanisms may be a factor in the cessation of biochemical activity in thermophiles at non-thermophilic temperatures. However, the conformational transitions known to occur in many 'thermophilic' proteins when cooled to mesophilic temperatures (Amelunxen & Murdock, 1978a) are more likely to be the major factors.

Fig. 10-3. Schematic representation of the substrate-enzyme relationships postulated in coldolysin.



CHAPTER 11

Enzyme Modification and Structural Analysis

11-1 Determination of Sulphydryl and Disulphide groups in Caldolysin

The method of Robyt *et al.*, (1971) was employed to determine the free -SH group content of Caldolysin. 2 ml of Caldolysin (29 μg ml^{-1}) was added to 0.6 ml of 2M 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), incubated at 20°C for 30 minutes, and measured spectrophotometrically at 412 nm against an enzyme-free blank. From the absorbance value (A_{412} = 0.0015) and the molar extinction coefficient of 3-carboxylato-4-nitrothiphenolate (CNT) (ε = 11400 (Robyt *et al.*, 1971); = 14150 (Riddles *et al.*, 1979)), it was calculated that the ratio of CNT produced per mole of enzyme corresponded to considerably less than one sulphydral group (calculated to yield A_{412} values of 0.118 and 0.095 per -SH group, respectively). It is concluded that Caldolysin has no free sulphydryl groups, in agreement with the conclusions derived in section 8-2 from the response of Caldolysin to cysteine-enzyme inhibitors.

To determine the content of cystine in Caldolysin, the method of Anderson & Wetlaufer (1975) was used without alteration. 0.25 ml volumes of Caldolysin solution (29 μg ml^{-1}, pH 8) were incubated with 250 μl of 6N NaOH for periods between 10 and 30 minutes at 20°C. Reactions were terminated by addition of 500 μl of 6N H_3PO_4 containing 2 mM EDTA. The final pH was found to be between 6 and 7. 100 μl of 2 mM DTNB was added and the absorbance monitored for 10 to 30 minutes at 412 nm against an enzyme-free blank.

As a control, the procedure was duplicated using a 2 mg ml^{-1} solution of lysozyme (from egg white, Grade V, Sigma). Data from both determinations is presented in Table 11-1.

TABLE 11-1 Determination of the cystine content of Caldolysin and Lysozyme

Protein	Incubation time (minutes at 20°C)	A_{412}	moles CNT/ moles protein [a]	S-S bonds
Caldolysin	10	0.025	6.65	
	20	0.022	5.58	6
	30	0.024	6.41	
Lysozyme	10	0.069	3.72	4 [b]
	30	0.075	4.12	

a. $\varepsilon_{412} = 11400$

b. Cystine content per lysozyme molecule = 4 (CRC Handbook of Biochemistry, 2nd Edn. (1970)).

From the lysozyme results, a stoichiometry of approximately 1.0 mole of CNT produced per mole of cystine present is indicated. Anderson & Wetlaufer, (1975) derived a stoichiometric relationship of 1.2 ± 0.3 moles CNT per mole S-S. Thus, the presence of six disulphide bonds per molecule of Caldolysin is implied. The presence of disulphide bonds in bacterial extracellular hydrolases is uncommon (Amelunxen & Murdock, 1978b). However, *Sorangium* α-lytic protease contains six half-cystines (Whitaker, 1970), while *Myxobacter* AL-1 protease contains four (Jackson & Matseuda, 1970). In both cases, no response to sulphydryl inhibitors was detected in the native molecule.

Depending on their positioning within the Caldolysin molecule, the disulphide bonds could account for a significant proportion of the intrinsic stability of the tertiary structure. This suggestion is confirmed by results derived from a determination of the influence of 10 mM dithiothreitol (DTT) on the thermostability of Caldolysin. DTT is a strong

reducing agent which readily cleaves disulphide bonds (Konigsberg, 1972: Iyer & Klee, 1973). Thermostability profiles of Caldolysin incubated at 75°C and 20°C with 10 mM DTT are presented in Fig. 11-1.

The destabilisation of Caldolysin at 75°C by DTT is not necessarily the product of the cleavage of disulphide bonds: reduction of side-groups other than cystine could give a similar result if critical non-covalent intramolecular bonds were effected. However, the profile of Fig. 11-1 is consistent with the cleavage of disulphide bonds involved in molecular stabilisation.

The contributions of calcium, intrinsic factors and disulphide bonding to the total free energy of stabilisation of Caldolysin can be calculated using equations derived by Voowduow *et al.*, (1976a) (see below), if it is assumed that the increase in the rate of activity loss in Caldolysin on the addition of DTT is the result of the cleavage of S-S bonds.

TABLE 11-2 Equations for Free Energy contributions

Calcium contribution: $\Delta(\Delta F^{\dagger}_{Ca\ 2+}) = -RT \ln k_o/k_\infty$

Cystine contribution: $\Delta(\Delta F^{\dagger}_{S-S}) = -RT \ln k_{S-S}/k_\infty$

(derived by analogy to the equation above)

Intrinsic contribution: $k_o = \frac{k\ T}{h} \exp(-\Delta F^{\dagger}/RT)$

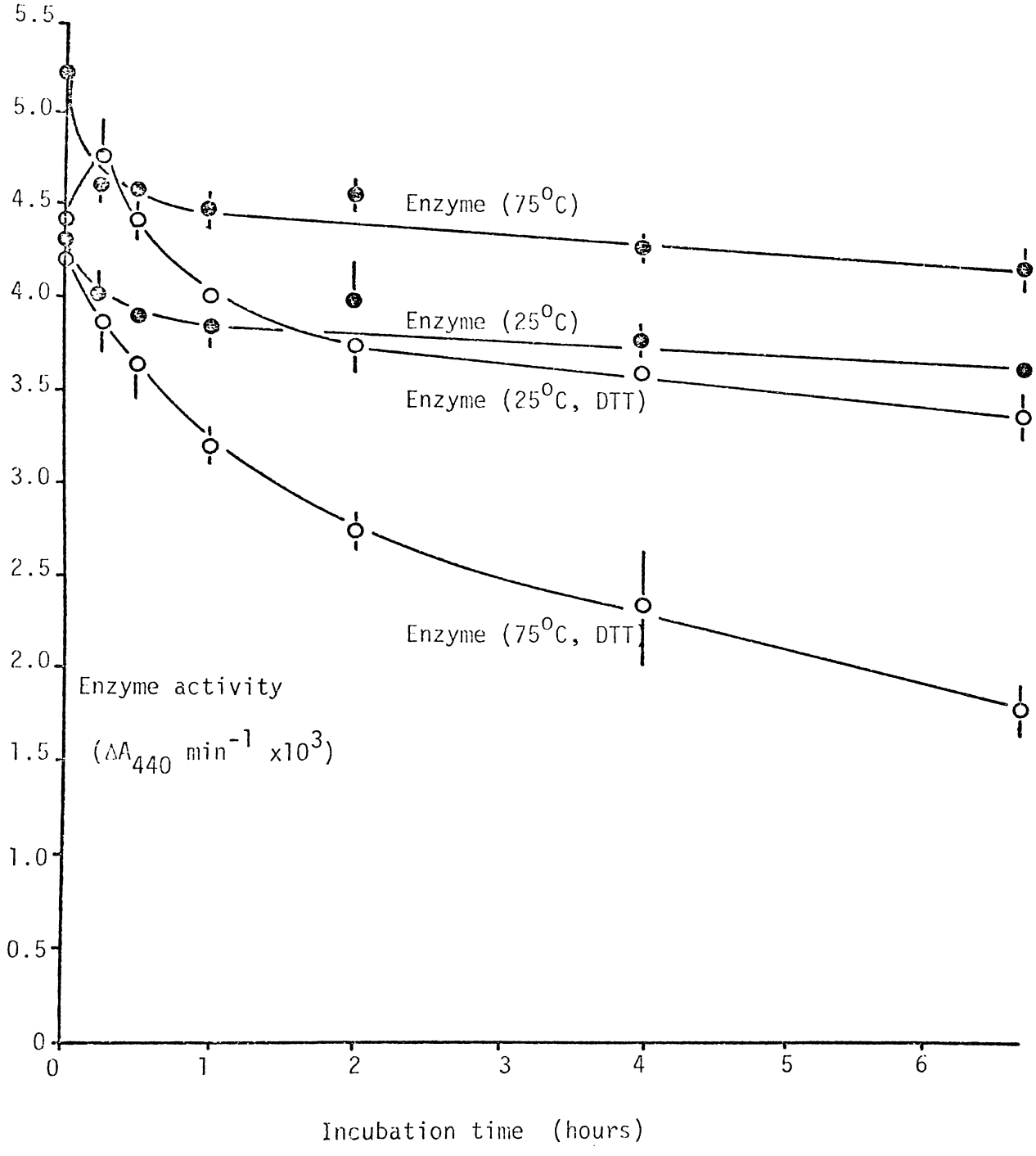
Total free energy of stabilisation: $\Delta F^{\dagger}_T = \Delta F^{\dagger} - (\Delta F^{\dagger}_{Ca}2+)$

where k_o = k_{obsd} at zero calcium concentration

k_∞ = k_{obsd} at infinite calcium concentration. For the purposes of this calculation, k_{obsd} (10 mM) is used.

k_{S-S} = k_{obsd} in the presence of 10 mM DTT.

Fig. 11-1. The effect of dithiothreitol on the stability of Caldolysin at 20°C and 75°C.
(20μg ml^{-1} enzyme, 0.1M CH_3COONa, pH 7.2, containing 10mM calcium and 10mM DTT)



R = gas constant

T = 348°K

k = Boltzman's constant

h = Plank's constant

It is noted that $\Delta(\Delta F^{\dagger}_{S-S})$ is included in $\Delta F^{\dagger}$; i.e., it is part of the intrinsic component of the apoenzyme.

Values for k_o, k_∞, and k_{S-S} were calculated as described in Voorduow & Roche (1975a). Stability profiles of apocaldolysin, Caldolysin + 10 mM $CaCl_2$, and Caldolysin + 10 mM DTT at 75°C were replotted (not shown) as $^1/_{(Enzyme)}$ against time as described in section 9-2, where the slope of a linear plot gives the second order rate constant of autolytic loss of enzyme activity (Table 11-3).

TABLE 11-3 Rate constant for the autolysis of Caldolysin at 75°C.

Enzyme state	Second order rate constant	Value [a]
Holocaldolysin	k_∞	2.67×10^{-13}
Apocaldolysin	k_o	2.04×10^{-10}
Holocaldolysin + DTT	k_{S-S}	2.08×10^{-11}

a. Units are mole min^{-1}.

The free energy contributions calculated from the data in Table 11-3 are given below.

It is concluded that both the calcium and cystine contributions to the stability of Caldolysin are large. They form 47.6% and 31.3% respectively of the total free energy of stabilisation (c.f. a calcium contribution of 28.3% of total stabilisation energy in Thermolysin (Voorduow *et al.*, 1976a)). Assuming that the destabilisation of Caldolysin in the

presence of DTT was entirely due to cleavage of cystine (which is quite possibly not the case), it can be calculated that the cystine contribution is 59.7% of the intrinsic stabilisation of the molecule, implying that disulphide bonds play a major role in the stabilisation of Caldolysin.

TABLE 11-4. Contributions to Free Energy of Stabilisation in Caldolysin [a]

Total free energy $\Delta F_T^{\dagger}$	Intrinsic free energy of stabilisation $\Delta F^{\dagger}$	Calcium contribution [b] $-\Delta(\Delta F^{\dagger}_{Ca}2+)$	Cystine contribution [b] $-\Delta(\Delta F^{\dagger}_{S-S})$
40.3	21.1	19.2	12.6

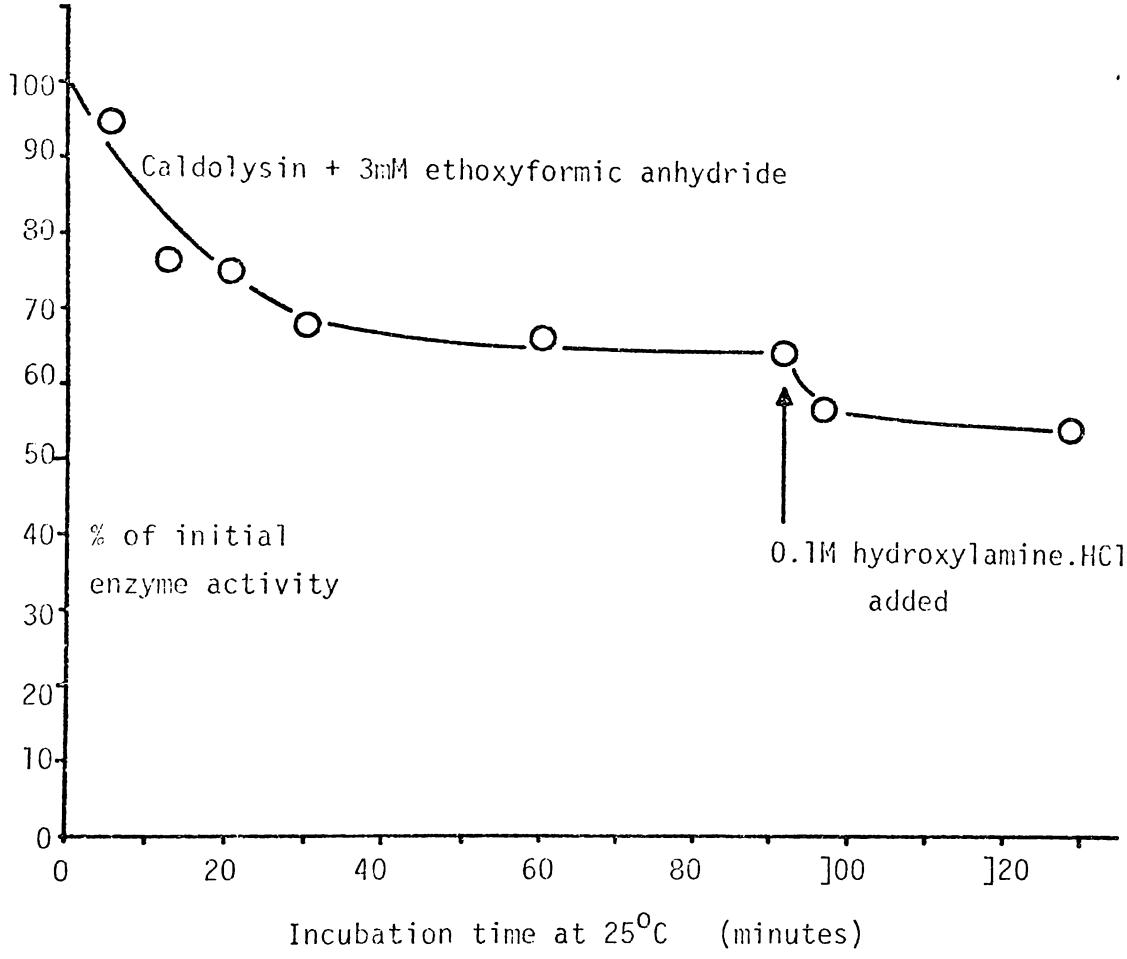
a. Measured at 75°C, pH 7.2. Units are kJ $mole^{-1}$.

b. Values are negative since calculations were derived from data obtained by removal of a stabilising factor.

11-2 The reaction of Caldolysin with Ethoxyformic anhydride.

Ethoxyformic anhydride (EFA) is known to react with a variety of amino acid moieties, including the imidizole ring of histidine, the guanidino group of arginine, the phenolic hydroxyl of tyrosine, and the sulphydryl group of cysteine (Muhlrad *et al.*, 1967; Melchior & Fahrney, 1970). The imidizole ring is strongly acylated, and the reaction between this side-group and EFA has been used to deduce the active site residency of several proteases (Burnstein *et al.*, 1974; Blumberg *et al.*, 1973). Other definitive indications of a reaction between EFA and histidine are an increase in A_{242} after complexing, and dissociation of the acylated product on addition of hydroxylamine. When these changes are associated with loss and subsequent regeneration of enzyme activity, it is assumed that histidine is a critical component of the active site (Burnstein *et al.*, 1974). A change in A_{278} results from reaction with the phenolic hydroxyl of tyrosine.

Fig. 11-2. Loss of enzyme activity during incubation of Caldolysin with ethoxyformic anhydride and hydroxylamine hydrochloride,
(20µg ml^{-1} enzyme, 0.1M CH_3COONa, pH 7.2 + 10mM calcium + 3mM ethoxyformic anhydride, 25°C)



Caldolysin (20 μg ml^{-1} in Tris acetic acid buffer, 0.1 M, pH 8.1 + 10 mM $CaCl_2$) was mixed with EFA (0.3 M in ethanol) to give a final concentration of 3 mM EFA. During incubation of the reaction mixture at 25°C, aliquots were removed at intervals for assay. After 90 minutes, hydroxylamine.HCl was added to a concentration of 0.1 M, and assays were continued. The changes in enzyme activity during the reaction sequence are shown in Fig. 11-2.

The loss of enzyme activity during the first 30 minutes of incubation suggests that a reaction between Caldolysin and EFA which has not greatly influenced the active site has occurred. The failure of hydroxylamine to reverse the activity loss implies that the reaction involved is definitely not the acylation of an active-site histidine imidizole. This conclusion is supported by the absence of any spectral change at 242 nm and 278 nm during difference spectrometry of a similar reaction mixture. From the extinction coefficients of EFA-modified histidine and tyrosine given by Burnstein *et al.*, (1974), (ε_{242} = 320; ε_{278} = 13101), it can be calculated that the reaction of a single histidine or tyrosine residue in Caldolysin would have resulted in absorbance changes of 0.001 (242 nm) and 0.039 (278 nm), respectively. Burnstein *et al.*, (1974) noted that 13.5 moles of EFA were incorporated per mole of Thermolysin.

It is concluded that while EFA reacts with some residue/s in Caldolysin, it is unlikely that either tyrosine or histidine are critical active site components.

11-3 The reaction of Caldolysin with phenylglyoxal

Phenylglyoxal has been shown to react rapidly with the guanidino moiety of arginine at neutral or alkaline pH's (Takahashi, 1968). Reaction with other residues occurs at a much slower rate (Means & Feeny, 1971). Rapid loss of enzyme activity on incubating Caldolysin with phenylglyoxal would imply the presence of an arginine residue in the protease catalytic site.

Phenylglyoxal was dissolved in 0.2 M ethylmorpholine acetate buffer, pH 8 (Takahashi, 1968) to a concentration of 0.3%. An equal volume of enzyme solution was added (20 μg ml^{-1} Caldolysin, 0.1 M CH_3COONa, pH 7.2 + 10 mM $CaCl_2$) giving a phenylglyoxal concentration of 0.15%. The reaction progress at both 75°C and 20°C was followed by periodic assay (azocasein substrate). A control incubation containing equal volumes of Caldolysin solution and ethyl morpholine buffer was also assayed. Results are presented in Fig. 11-3.

The loss of enzyme activity in the presence of phenylglyoxal suggests that some modification of the protease has occurred. It is possible that loss of catalytic activity is the result of negative influences on the active site (for instance, modification of a residue in, or associated with the active site), rather than loss of enzyme from accelerated autolysis. Although it is noted that arginine residues are considered to play an important role in salt-bridging and stabilisation of some thermophilic proteins (Amelunxen & Murdock, 1978b), the rate of autolysis of Caldolysin has been shown to be negligible at 20°C (section 9-5).

As a result of the slow and incomplete response of Caldolysin to phenylglyoxal, it cannot be assumed that the loss of enzyme activity is derived from modification of arginine residues. Takahashi (1968) shows that when proteins are treated for long periods with an excess of reagent, reaction may occur with the ε-amino group of lysine (20% reaction in 24 hours at 25°C and pH 8) and several other residues.

11-4 The reaction of Caldolysin with Salicylaldehyde

Salicylaldehyde has been used as a specific agent for the chemical modification of lysine residues in proteins (Muhlrad *et al.*, 1970). The formation of azomethine (Schiff's base) linkages can be determined spectrophotometrically by the formation of maxima at 260 nm and 410 nm.

Fig. 11-3. Loss of Caldolysin enzyme activity in the presence of 0.15% phenylglyoxal at 75°C.
(20μg ml^{-1} enzyme, 0.2M ethyl morpholine acetate buffer, pH 8, containing 10mM calcium)

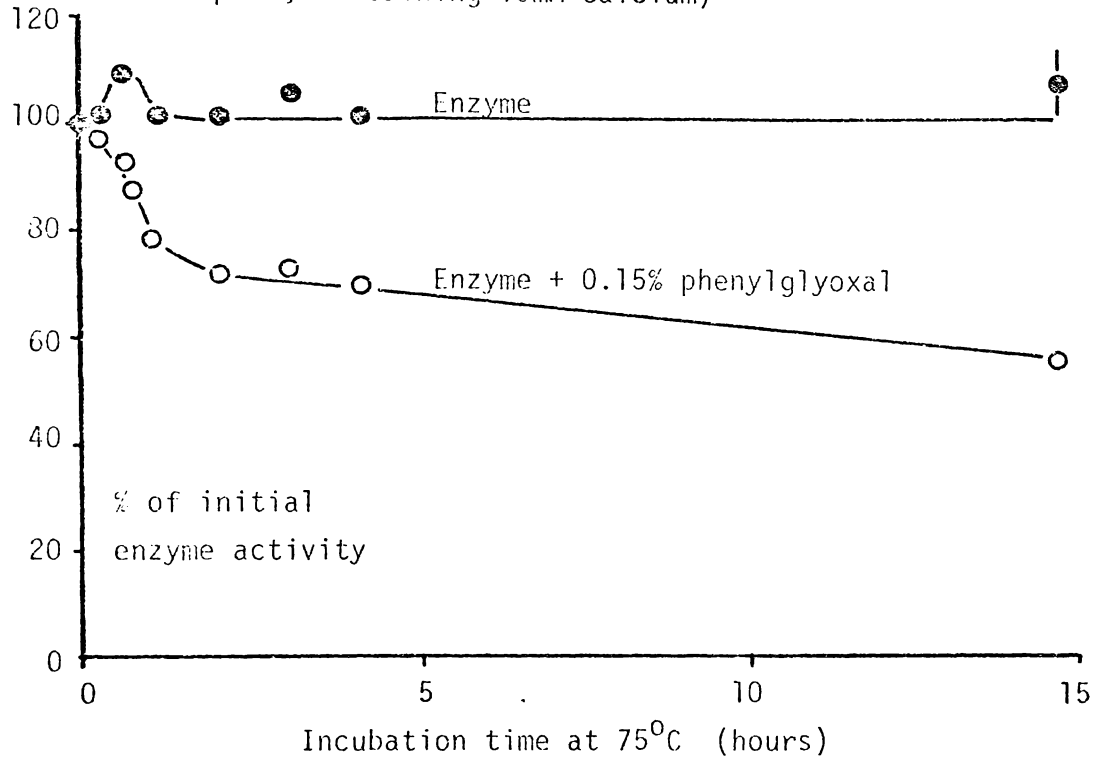
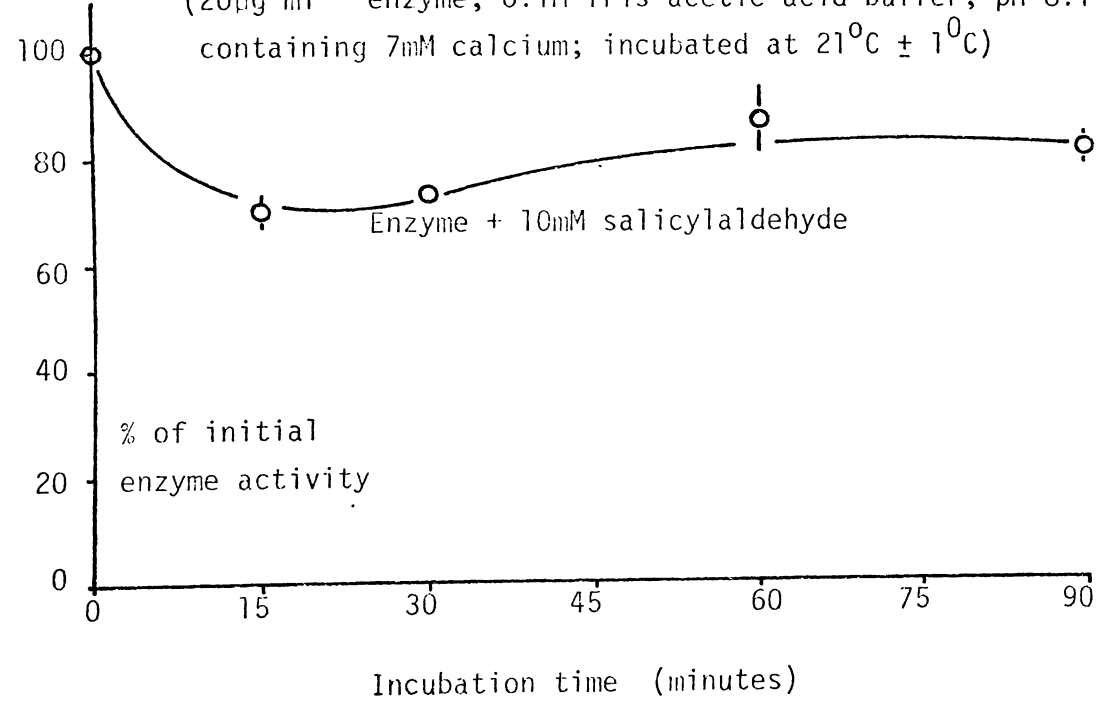


Fig. 11-4. Changes in the enzyme activity of Caldolysin in the presence of 10mM salicylaldehyde.
(20μg ml^{-1} enzyme, 0.1M Tris acetic acid buffer, pH 8.1, containing 7mM calcium; incubated at 21°C ± 1°C)



1 ml of 30 mM salicylaldehyde dissolved in 0.1 M Tris acetic acid buffer, pH 8.1 + 5% ethanol, was added to 2 ml of Caldolysin (20 μg ml^{-1} in 0.1 M CH_3COONa pH 7.2 + 10 mM $CaCl_2$) and incubated at 20°C ± 1°C. Aliquots were removed and assayed at intervals (azocasein substrate). Changes in enzyme activity during an incubation period of 90 minutes are shown in Fig. 11-4.

A rapid loss of approximately 30% of initial enzyme activity implies that salicylaldehyde has effected a small inhibition of catalytic function. At 20°C, there is insignificant loss of enzyme activity by autolysis or denaturation, and it can be reasonably assumed that changes in enzyme activity are the result of active site influences, either direct, or indirectly through a change in protein conformation. It is concluded that chemical modification of one or more lysine residues in non-critical roles has occurred.

The fact that only 30% inhibition has been achieved might be explained by the nature of the active site. It has been shown that Caldolysin, as with *Myxobacter* AL-1 protease, has a large substrate binding site which preferentially binds 3 or 4-unit peptides (section 8-5). Thus, a considerable number of residues are probably involved in substrate binding. It is assumed that the greater the number of residues involved in substrate recognition, the smaller the energetic contribution from each residue. Thus it is suggested that many residues in the active site will probably not be critical in their function, and that the loss of one of these by chemical modification (as in the formation of an azomethine linkage between lysine and salicylaldehyde) would reduce the rate of, but not prevent substrate binding and proteolysis.

This reasoning can be applied as an alternative explanation for the results between Caldolysin and both phenylglyoxal and ethoxyformic anhydride.

CHAPTER 12

IMMOBILISATION OF CALDOLYSIN TO INSOLUBLE SUBSTRATES

12-1 Introduction

The rapid development of immobilisation techniques for enzymes and other biological agents has been motivated by the potential use of such preparations in industrial processes. A vast array of insoluble supports, both organic and inorganic, have been used as substrates for the immobilisation of different enzymes. The general configurations used in the linkage of proteins to insoluble matrices include:

a. Covalent bonding to a solid phase.

b. Covalent bonding to soluble polymers.

c. Physical adsorption to a solid phase.

d. Cross-linking at solid surfaces.

e. Cross-linking with bifunctional reagents.

f. Inclusion in a gel phase.

g. Encapsulation.

Numerous reviews on protein immobilisation have been published recently, including Silman & Katchalski (1966); Chibata (1970); Goldman *et al.* (1971); Melrose (1971); Zaborski (1973); Falb (1974); Mosbach (1976), and Kent *et al.* (1978). The immobilisation of proteolytic enzymes has received much attention in attempts to extend and increase the viability of already established industrial, commercial, and scientific uses of the free enzymes. Recent reviews on the immobilisation of proteases include those by Goldstein (1970), Crook *et al.* (1970), and Mosbach (1976).

Substantial alterations in the properties of proteases when in the immobilised state have been reported. These include loss of specific activity (Ohmiya *et al.*, 1978), both positive and negative changes in

K_m (Chapman & Hultin, 1975; Mason *et al.*, 1975), and increases in both pH stability and thermostability (Ohmiya *et al.*, 1978; Chapman & Hultin, 1975; Mason *et al.*, 1975).

The immobilisation of proteases has been used as a means of studying the reversible conformational transitions of the enzymes. In the bound state, interference normally resulting from aggregation and autolysis is supressed, since intermolecular interactions are minimised (Gable & Kasche, 1976; Horton & Swaisgood, 1976).

12-2 Immobilisation of Caldolysin to Glass Beads

Porous ceramic materials are frequently used as substrates for enzyme immobilisation (Weetall, 1976). Such substrates have the advantages of resistance to attack by microorganisms, and high structural rigidity which permits good flow characteristics in column reactors (Chapman & Hultin, 1975). Attachment of proteins to ceramic particles can be achieved by a number of different processes, such as the isothiocyanate method of Weetall (1970), and the silanization and cyanogen bromide coupling methods described by Weetall (1976).

Caldolysin was immobilised on non-porous glass beads by the silane glutaraldehyde-coupling method described by Stolzenbach & Kaplan (1976). 10 g of glass beads (Corning glass, 100 mesh) was washed in an excess of 5% HNO_3 at 100°C for 30 minutes. The acid-washed glass was filtered and rinsed, then added to a 10% aqueous solution of γ-aminopropyl triethoxysilane (adjusted to pH 3.5 with HNO_3). The suspension was incubated at 75°C for approximately three hours to permit silanization to occur. After filtering, the silanized glass was added to a 20 ml volume of 5% glutaraldehyde in 0.01 M, pH 7, phosphate buffer. This was reacted *in vacuo* for two hours at room temperature, and finally washed exhaustively with distilled water.

17 ml of a solution of Caldolysin (25 μg ml^{-1}) of known activity was added to the prepared ceramic substrate. The suspension was stirred at room temperature for 18 hours to complete glutaraldehyde cross-linking. The immobilised enzyme was subsequently filtered, washed with 100 ml H_2O, 100 ml 1 M NaCl, and a further 500 ml H_2O. The filtrate and washings were assayed by the Kunitz method.

The immobilised complex was assayed by a modification of the Kunitz method. 14 mg samples of the enzyme-bead complex were placed in reaction tubes, mixed with 2 ml of 0.5% casein substrate, and incubated at 75°C with continual shaking. The remainder of the assay was performed as previously described (section 4-4). The proteolytic activities of the original enzyme solution, the immobilised preparation, and the washings (non-immobilised enzyme) were calculated (Table 12-1).

TABLE 12-1 Activity of Glass-bead-immobilised Caldolysin

	Enzyme activity (PU)
Total enzyme activity of original solution	25.4
Total enzyme activity not bound to glass beads	0.6
Total activity of ceramic-bound enzyme	0.2
Recovery of activity in immobilised state =	1%

It is concluded that Caldolysin is either inactivated during the attempt to cross-link it to the silanized glass, or is bound in such an orientation that steric hindrance prevents access of the protein substrate to the catalytic site.

12-3 Immobilisation of Caldolysin to Sepharose 4B

Agarose and beaded agarose (Sepharose) gels have been frequently used as substrates for enzyme immobilisation (e.g. Porath & Axen, 1976). There are a number of reaction mechanisms by which coupling can be achieved, but one of the most common is the linkage of protein to cyanogen bromide - activated polysaccharide.

Sepharose 4B (Pharmacia) was activated with cyanogen bromide as described by Fujiwara & Tsuru (1977). During activation, the Sepharose suspension was maintained at 25°C, and at pH 10 to 11 by dropwise addition of 4N NaOH. The activated gel was washed and stored at 4°C.

15 ml of a Caldolysin solution (25 μg ml^{-1} in 0.1 M CH_3COONa, pH 7.2) was adjusted to pH 9.7 and added to 40 ml of settled activated Sepharose 4B. The mixture was incubated at 4°C for 72 hours. Subsequently, the Caldolysin-Sepharose complex was filtered and washed with distilled water. Assay results for the free enzyme, immobilised enzyme (assayed in a manner similar to that described in section 12-2), and gel washings are presented in Table 12-2.

TABLE 12-2 Activity of Sepharose 4B-immobilised Caldolysin

	Enzyme activity (PU)
Total activity of free enzyme solution	17.0
Total activity not bound to Sepharose	0.7
Total activity of Sepharose-bound enzyme	12.0
Recovery of activity in immobilised state =	73%

12-4 Immobilisation of Caldolysin to Carboxymethyl-cellulose

The Curtius azide method, first described by Michael & Ewers (1949), modified by Mitz & Summaria (1961), and detailed by Crook *et al.* (1970) and Lilly (1976), was used to immobilise Caldolysin to CM-cellulose. 5 g of CM-cellulose (Pharmacia) was treated with methanol in acid, hydrazine hydrochloride, and sodium nitrite in acid, as described in the papers cited above.

To the activated cellulose was added 77 ml of Caldolysin (61.5 μg ml^{-1} in pH 9.2 buffer). The substrate-enzyme coupling reaction was accompanied by a decrease in pH, which was readjusted to 8.7 by addition of saturated sodium borate solution during the 60 minute duration of reaction. The complex was subsequently washed with aliquots of distilled water, NaCl, acetic acid, and sodium bicarbonate solutions. The immobilised complex and all solutions were assayed as previously described. Activity data are presented in Table 12-3.

TABLE 12-3 Activity of Caldolysin immobilised to CM-cellulose

	Enzyme activity (PU)
Total acitvity of free enzyme solution	239
Total activity not bound to CM-cellulose (washings)	29
Total activity of CM-cellulose-immobilised Caldolysin	66
Recovery of activity in immobilised state =	31%

12-5 Comparative data for free and immobilised Caldolysin

It has been demonstrated in sections 12-2 to 12-4 that the immobilisation of Caldolysin to various insoluble substrates occurs with considerable differences in the recovery of active immobilised enzyme (i.e. 1%

for glass beads, 31% for CM-cellulose, and 73% for Sepharose 4B). This may be due to loss of activity by denaturation, or differences in inhibition due to the site of the enzyme-matrix covalent linkage. Although free amino groups on the protein can react with the active moiety of each of the above insoluble matrices (Quiocho, 1976; Syere & Uyeda, 1976; Crook *et al.*, 1970), the acyl azides of activated glass can react with any exposed nucleophilic group on the enzyme. Thus, sulphydryl, amino, or hydroxyl groups would yield thioester, amide, and ester linkages respectively (Syere & Uyeda, 1976). In comparison, the reaction of proteins with the imidocarbonates of CNBr-activated Sepharose is thought to occur primarily via the ε-amino groups of lysines, and the α-amino groups of N-terminal amino acids (Syere & Uyeda, 1976).

The activity retained after immobilisation of Caldolysin to Sepharose (73%) is high when compared to other published data. In binding a range of proteases to Dowex MWA-1 anion exchange resin, Ohmiya *et al.* (1978) found activity yields ranging from 3% to 39%. Mason *et al.*(1975) obtained activity yields of 41.4% and 57.7% on coupling *B. subtilis* neutral protease to glass by the azo- and glutaraldehyde methods, respectively.

A range of characteristics of the immobilised Caldolysin preparations, including thermostabilities, pH activity profiles, and Michaelis-Menten kinetics, were compared with those of the free enzyme. Since the residual activity of the glass-bead immobilised enzyme was extremely low, no further study of this complex was carried out.

The thermostabilities of the immobilised Caldolysin preparations were determined at different temperatures and calcium concentrations. Volumes of immobilised enzyme were suspended in 0.1 M Tris acetic acid buffer, pH 8.1, containing known concentrations of calcium. The suspensions were incubated at the desired temperature, and aliquots removed at intervals for assay after agitation of the suspension to ensure homogeneity. Immobilised apoenzyme suspensions were obtained by eluting the insoluble complex

(held in a Pharmacia K12 glass column) with 10 mm EDTA for several hours, and finally washing with distilled water. (The term 'apoenzyme' is subject to the conditions discussed previously: it is possible that in the immobilised state, tightly bound calcium ions might not be removed by such treatment). Thermostability data is presented in Table 12-5.

TABLE 12-5 A comparison of the thermostabilities of free and immobilised Caldolysin

Enzyme status	Calcium status	Ca^{2+} (mM)	Half-life (minutes) at T°C		
			85	90	95
Free	Holo	10	360	60	28
Sepharose-bound	Holo	10	1060	165	125
CM-cellulose-bound	Holo	10	-	110	-
Free	Apo	0	-	<6	-
Sepharose-bound	Apo	0	-	28	-
Free	Holo	0	-	approx. 15	-
Sepharose-bound	Holo	0	-	64	-

The immobilisation of Caldolysin on Sepharose results in an increase in thermostability of 3 to 4-fold over a number of different temperatures and conditions, while a thermostability increase of approximately 2-fold results from covalent linkage to CM-cellulose. The decrease in stability of the holo-enzyme Sepharose complex when incubated in a calcium-free buffer suggests that the stabilisation by high calcium concentrations is as significant a factor in the immobilised state as in the free enzyme, while the decreased stability of the Sepharose-immobilised enzyme after EDTA treatment ("apoenzyme") indicates that immobilisation does not prevent the removal of at least some of the calcium-conferred stabilisation.

The pH activity profiles for Caldolysin and two immobilised Caldolysin preparations are presented in Fig. 12-1. The pH optimum of the free enzyme (approximately 8) is unchanged by covalent linkage to cellulose, but reduced to 6.8 on binding to Sepharose. There is little other alteration in the profiles at high pH levels. Activity at pH's between 4.5 and 7.5 is diminished in comparison to that of the free enzyme. Chapman & Hultin (1975) demonstrated an increase in the pH optimum in covalently linking subtilisin BPN' to porous glass beads, an effect which they attributed to attraction of hydrogen ions to the negative surface charges of the glass. Despite the presence of negative charges on the surface of cellulose particles, the reduction in pH optimum suggests that the mechanism of Chapman & Hultin (1975) is probably not applicable in this case.

Lineweaver-Burk data for free Caldolysin, and the cellulose and Sepharose-bound enzymes were obtained using azo-casein as a substrate (Fig. 12-2). Although the determination of an accurate gradient from the CM-cellulose data is difficult, immobilisation to Sepharose or cellulose probably has little or no effect on the K_m of Caldolysin. This implies that covalent linkage has no effect on the binding groups of Caldolysin and thus probably little effect on the conformation of the molecule. Interestingly, substrate inhibition is relieved when Caldolysin is immobilised. This suggests that the enzyme is attached to the matrix either through the inhibitor sites (see Chapter 10), or in such a manner so as to block the inhibitor sites. This is further evidence that the inhibitor sites are not located near the active site.

Arrhenius plots for Caldolysin and Sepharose-caldolysin are presented in Fig. 12-3. Temperature-related discontinuities are observed in both plots. As discussed in section 9-9, Caldolysin undergoes a minor conformational change at about 50°C. Fig. 12-3 indicates that in the immobilised state, no conformational change occurs until a temperature of 55°C

Fig. 12-1. pH-activity profiles of Caldolysin (A), Sepharose-Caldolysin (B), and CM-cellulose-Caldolysin (C). (1.6ml of universal buffer at given pH added to 0.4ml of 0.5% azocuscin in 0.01M Tris acetic acid buffer, pH 8.1, 10mM calcium; reaction initiated by the addition of 100µl of enzyme, incubated at 75°C).

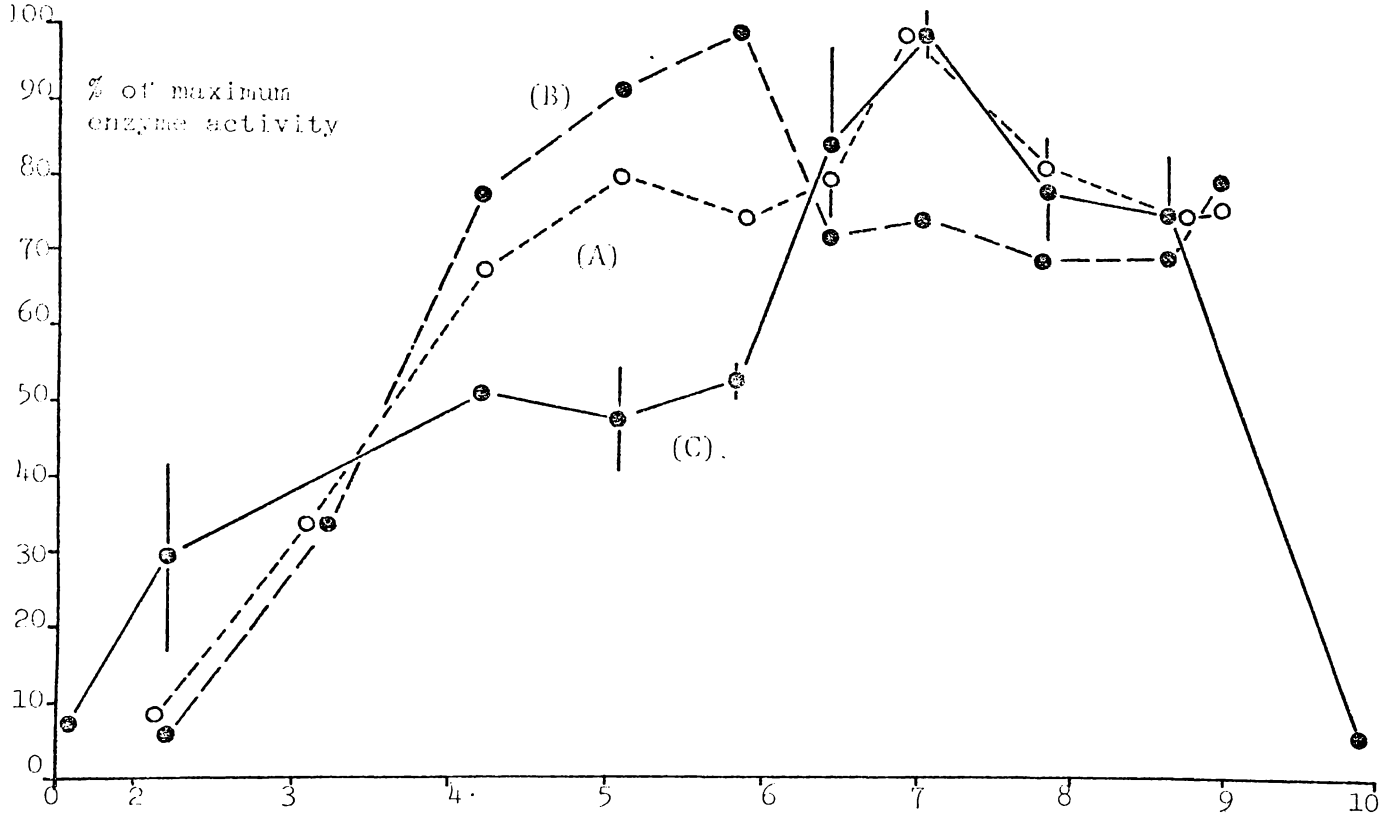


Fig. 12-2. Lineweaver-Burk plots for free and immobilised Caldolysin.

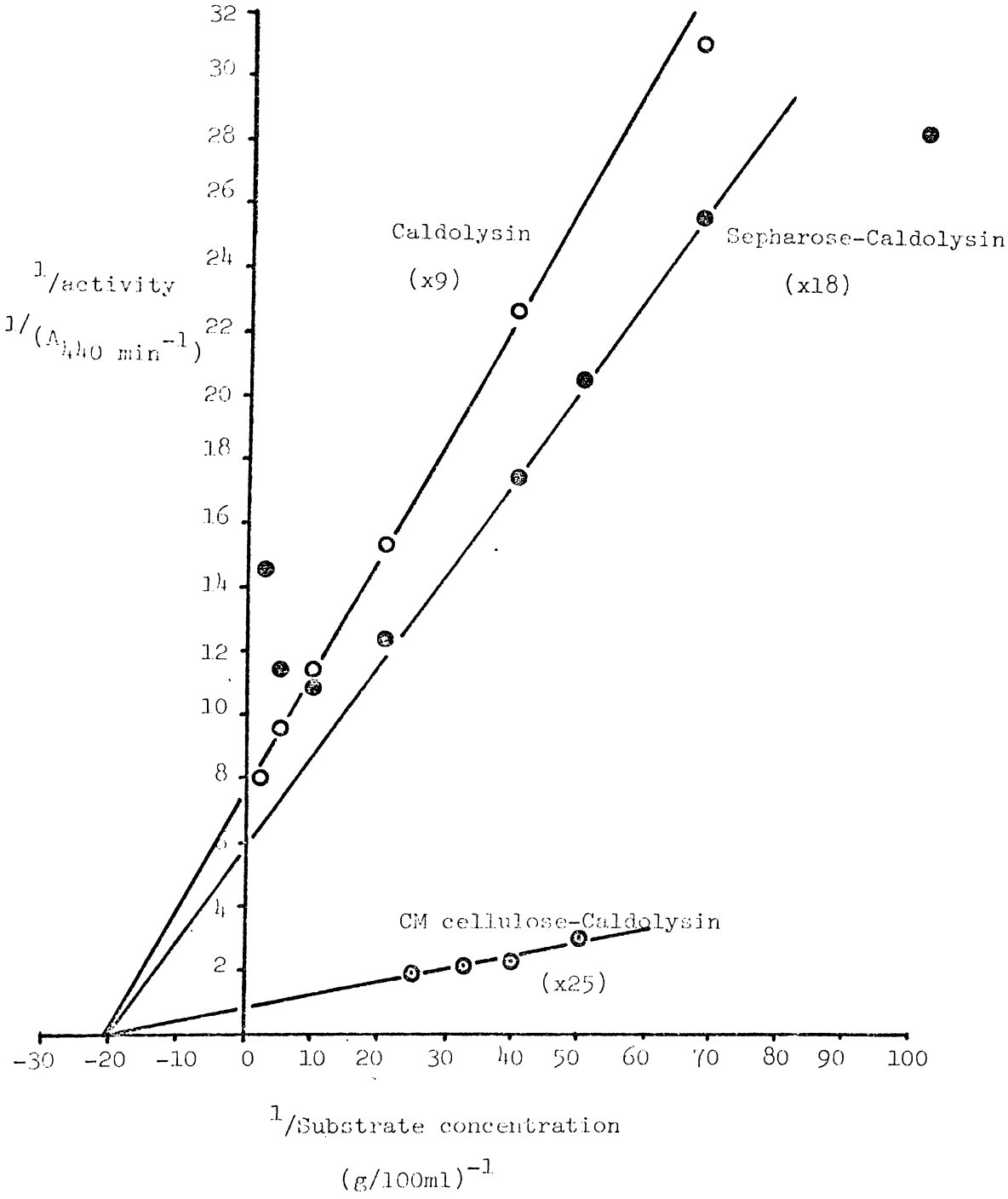
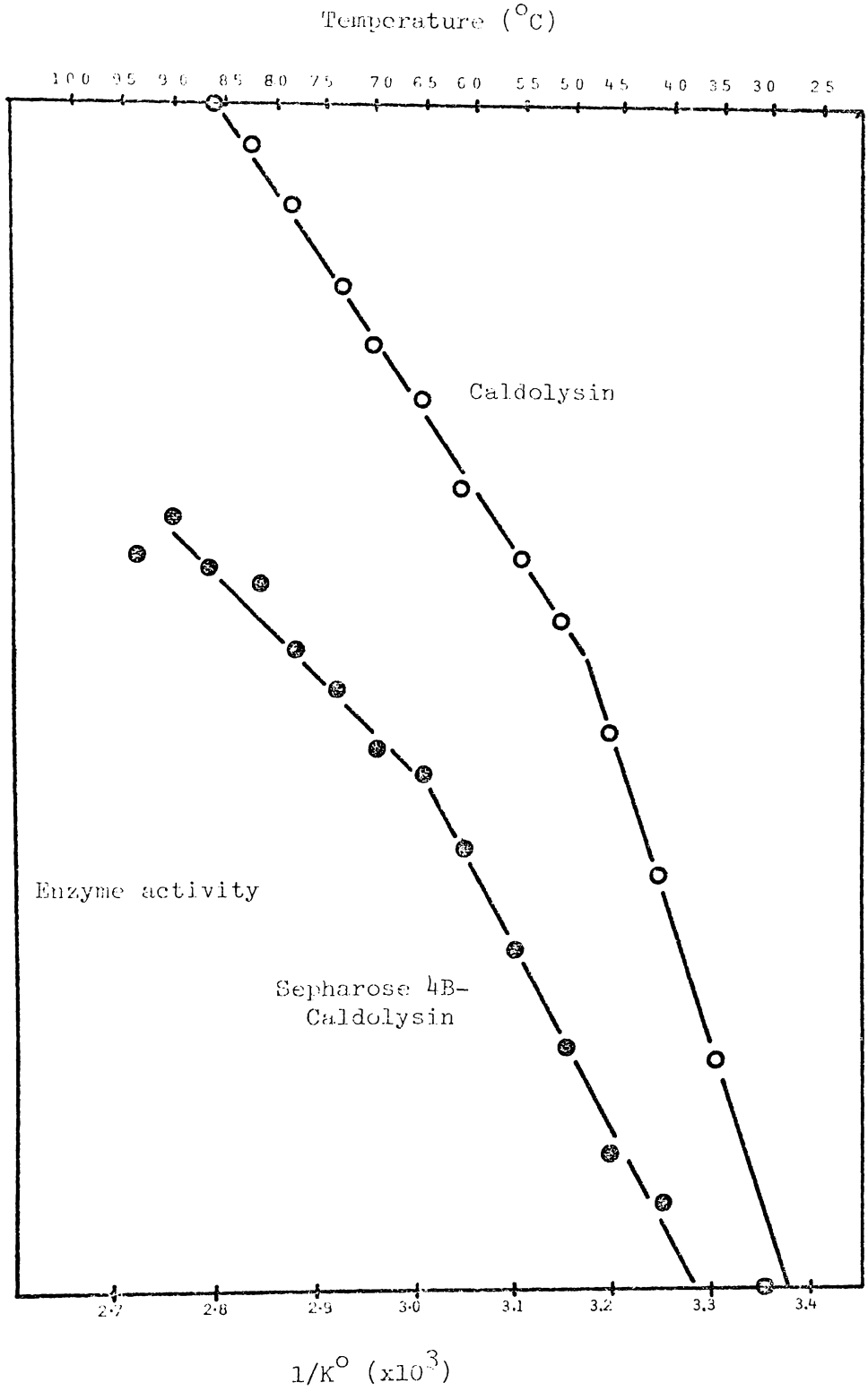


Fig. 10-3. Arrhenius plots for Caldolysin and Sepharose 4B-Caldolysin.



to 60°C is reached. This could be explained in terms of increased molecular rigidity derived from a multi-point covalent linkage to an insoluble matrix.

12-6 Continuous-flow proteolysis by immobilised Caldolysin

A Pharmacia K9 column containing approximately 1 g of Sepharose-caldolysin was immersed in a 75°C water bath. Protease substrate (0.1% azo-casein in 0.1 M Tris acetic acid buffer, pH 8.1) was passed through the column, the flow rate being governed by the head height of the eluent vessel and changes in the flow resistence of the gel. Eluted material was collected in 2 ml aliquots in a Pharmacia Redirac fraction collector. Prior to elution, 3 ml of 5% trichloroacetic acid was pipetted into each fraction-collector tube. Thus, unhydrolysed azo-casein was precipitated immediately on collection. After subsequent centrifugation, the absorbance (at 440 nm) of each supernatant was determined. The rate of hydrolysis was calculated as follows:

> For a volume of substrate (v) passing through (x) grams of gel in (t) minutes, the residence time (r) (i.e. the time 1 ml of substrate is in contact with the enzyme) will be;
>
> $$r = t/vx \ (\text{min ml}^{-1}\ \text{g}^{-1})$$
>
> Therefore the rate of hydrolysis = A_{440}/r

A typical continuous-flow proteolysis sequence is shown in Table 12-6.

The decrease in A_{440} values throughout the progression of the experiment is the result of flow-rate increases (derived from a reduction in the gel resistence), not the loss of enzyme activity.

The stability of the immobilised enzyme preparation during storage at 25°C and 75°C was tested by repeated continuous proteolysis determination (Fig. 12-4a and Fig. 12-4b).

Fig. 12-4a. The stability of Sepharose-immobilised Caldolysin at 25°C (0.1M Tris acetic acid, pH 8.1, + 10mM calcium).

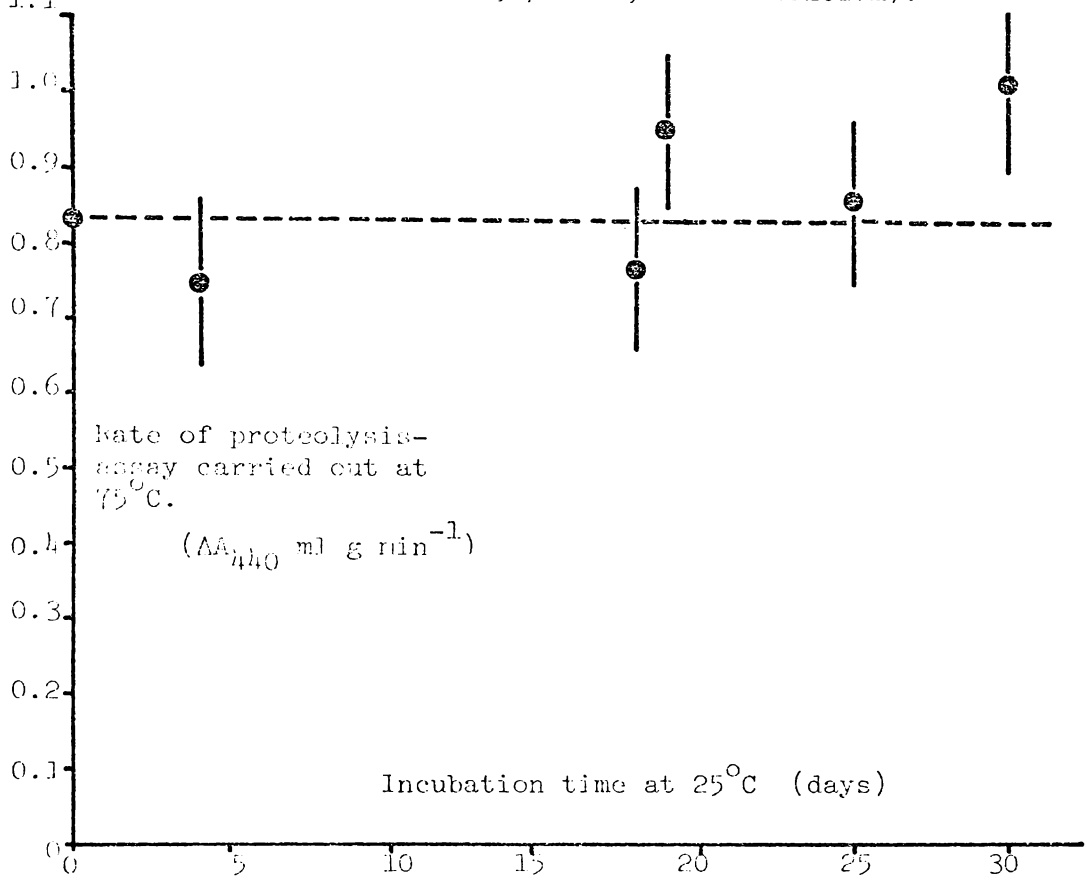


Fig. 12-4b. The stability of Sepharose-immobilised Caldolysin at 75°C (conditions as above).

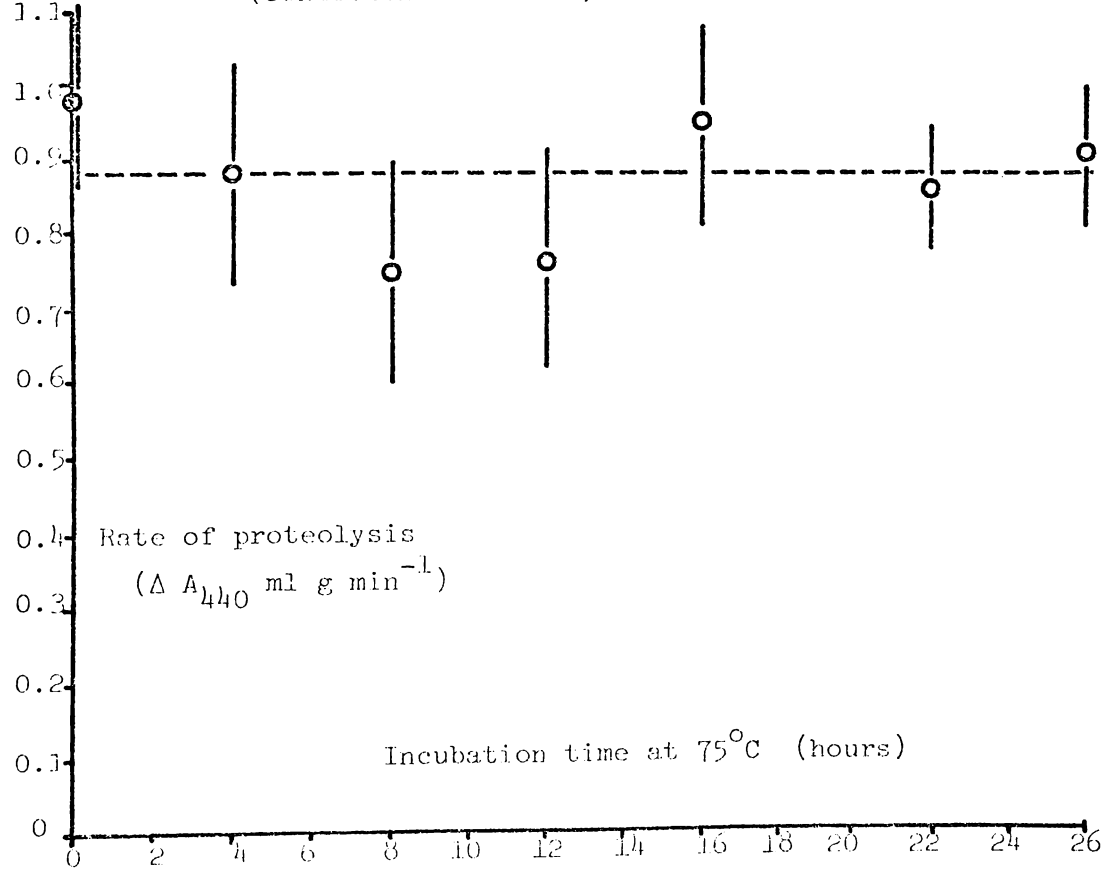


TABLE 12-6 Continuous-flow proteolysis of azo-casein by Sepharose-caldolysin

Fraction	A_{440}	Fraction	A_{440}
1	0.180	8	0.118
2	0.176	9	0.113
3	0.143	10	0.119
4	0.125	11	0.124
5	0.121	12	0.112
6	0.123	13	0.109
7	0.118	14	0.111

Average A_{440} = 0.129; t = 21 minutes; x = 28 ml;

r = 0.75 ml min^{-1}

Therefore, average rate of hydrolysis = 0.129/0.75

= 0.086 g ml min^{-1}

It is evident that Sepharose-caldolysin is completely stable with respect to both denaturation and dissociation of the enzyme-matrix complex, at both 25°C and 75°C. It is concluded that immobilised Caldolysin preparations are suitable for continuous proteolysis of soluble substrates. However, it was found impossible to use casein as a substrate for continuous-flow proteolysis, since the precipitation of paracasein during proteolysis rapidly clogged the gel and prevented further substrate passage.

CHAPTER 13

OTHER PROTEASES

13-1 *Thermus* T-351 intracellular proteases

25 g of wet *Thermus* T-351 cells (grown on 0.3% standard medium as described in section 4-2) were disrupted by repeated passage through a French press. A cell-free extract was obtained by centrifugation at 10 000 x g for 20 minutes. Further centrifugation at 25 000 x g for 60 minutes resulted in the removal of remaining cell fragments, and provided a high-speed supernatant as described by Hickey & Daniel (1979). Kunitz protease assays and Bradford protein assays were carried out on both fractions (Table 13-1).

TABLE 13-1 Preliminary extraction of *Thermus* T-351 intracellular proteases

Sample	Volume (ml)	Activity (PU ml^{-1})	Total activity (PU)	Protein (mg ml^{-1})	Total protein (mg)	Specific activity (PU mg^{-1})
Cell-free extract	15	1530	22950	4.1	61.5	373.2
High-speed supernatant	12	590	7080	3.7	44.4	159.5

As might be expected, the levels of protease present in the cell-free extract were about 12 times higher than those found in the culture medium; intracellular cell-free extract = 1530 PU ml^{-1}, c.f. extracellular cell-free supernatant = 118 PU ml^{-1}. However, on a production basis, a 20 litre volume of media would yield about 2.36×10^6 PU of crude extracellular protease, but only 4.6×10^4 PU of crude intracellular protease (20 litres of culture fluid yields about 40 g of *Thermus* cells). The

90% decrease in activity resulting from the high-speed centrifugation step suggests that much of the intracellular protease is membrane-bound, and is lost when cell organelles and membrane fragments are sedimented.

No further purification of characterisation of the *Thermus* intracellular proteases was carried out.

13-2 Other extracellular proteases from *Thermus* T-351

As outlined in sections 7-5 and 7-9, two minor extracellular proteases were separated from Caldolysin during SP-Sephadex C-25 ion exchange chromatography. These proteases comprised between 10% and 30% of the extracellular proteolytic activity in *Thermus* culture fluid. Two significant and distinguishing differences between Caldolysin and the minor extracellular proteases were noted:

1. When electrophoresed at pH 8.1, both minor proteases demonstrated anionic mobility (Fig. 7-5). The respective mobilities of the two proteases suggested pI values of approximatly 7.5 and 6 (c.f. pI (Caldolysin) = 8.5 $\pm$ 0.5).

2. When applied to casein-agar assay plates, the minor protease fractions produced a precipitation of para-caseins within a few minutes at room temperatures. These enzymes may be capable of substantial proteolysis at mesophilic temperatures, unlike Caldolysin.

No further characterisation of the minor extracellular proteases was carried out.

13-3 Yeast Extracellular Proteases

Cultures from the Te Kauwhata Oenological and Viticultural Research Station culture collection (Numbers 1-110, 115, 132) were screened for extracellular protease production (as discussed in the preface) by the techniques described below:

a. Cultures were screened by stab inoculation of casein-agar gel slabs (composition: see section 4-7). Gels were then incubated at 37°C for 48 hours. Although most inocula showed signs of active cell growth after this period, no para-casein precipitation (indicating excretion of extracellular proteases) was observed.

b. 20 ml volumes of culture medium (1% yeast extract, 1% trypticase peptone, 1% glucose, pH 7) were inoculated with yeast, and incubated at 37°C. At intervals, throughout the subsequent 48-hour growth period, aliquots of each culture were removed and assayed by the casein-agar plate method (section 4-7) at 37°C and 55°C. No proteolysis was detected.

c. Selected yeast cultures (10, 92, 92-1, 93, 107, and 113) were grown as described in b. above. Aliquots were removed during the incubation period and assayed by the Kunitz method at 37°C. No proteolysis was detected.

It is concluded that any production of extracellular proteolytic enzymes by the strains of *Saccharomyces cerevisiae* tested was below the limit of detection of the assay methods used.

CHAPTER 14

GENERAL DISCUSSION AND CONCLUSIONS

Caldolysin, the major extracellular proteolytic enzyme from *Thermus* T-351 has, on the basis of its specificity and structural characteristics been classified as a metal-chelator-sensitive lytic protease. Structural and functional homology with the most fully characterised lytic protease (*Myxobacter* AL-1 protease (Jackson & Matseuda, 1970); and *Sorangium* α-lytic and β-lytic proteases (Whitaker, 1970)) and other metalloproteases such as Thermolysin, has been noted (Table 14-1). The strict requirement of calcium for thermostability initially suggested that Caldolysin might be closely related to Thermolysin. However, failure to detect any zinc ligand in Caldolysin, and the subsequent demonstration that the presence of zinc ions had little effect on either enzyme stability or activity, indicated that the two enzymes were not closely related. Other significant dissimilarities include the fact that Caldolysin possesses no hydrolytic activity against low molecular weight peptide analogues, and does not contain an active site histidine residue. Conversely, Thermolysin has not been reported to possess lytic activity.

It can be observed in Table 14-1 that Caldolysin does not show a very close resemblance to any of the other lytic proteases. The unusual characteristic of an active-site requirement for a minimum of four bulky groups (demonstrated by the hydrolysis of tetraglycine and pentaglycine but not triglycine, and precluding the use of most low molecular weight assay substrates) is also possessed by *Myxobacter* AL-1 protease. However, specificity is notably different, since Caldolysin lyses Gram-negative but few Gram-positive microorganisms, while the exact reverse is reported for the Al-1 protease (Ensign & Wolfe, 1965).

TABLE 14-1 Comparison of the properties of some proteases

Property	Enzyme				
	Myxobacter AL-1	Sorangium		Thermolysin	Caldolysin
		α	β		
Molecular weight	13500 [a]	19778 [b]	19000 [e]	37500 [g]	20000
pI	10 [e]	>9 [b]	~10 [e]	<8 [f]	8.5 ± 0.5
Cystine residues	2 [a]	3 [b]	2 [e]	0 [g]	6
Active site residues	?	Serine [b]	?	Histidine [h]	possibly Lysine
Esterase activity	No [a]	Yes [b]	?	No [g]	No
Lytic activity	Predominantly Gram-positive [c]	Gram-negative [b]	Yes, specificity unknown	No	Gram-negative
Elastase activity	?	Yes [b]	?	Yes [g]	Yes
Specificity	Small a.a. on either side of splitting point	Small aliphatic a.a. on C side of splitting point	Lysine on amino side of splitting point	Hydrophobic or bulky a.a. on amino side of splitting point	Small aliphatic a.a. on either side of splitting point
Size limit in active site	4 residues [a]	No [b]	?	No	4 residues
Carbohydrate present	Hexose [e]	?	?	No	?
pH optimum	9.0 [a]	alkaline [e]	alkaline	7.5	7.5 - 8.0
Metal ion requirement	possibly Zn [a]	No [e]	1 Zn [e]	1 Zn, 4 Ca [g]	Ca
Inhibitors	EDTA, citrate, phosphate ($>10^{-2}$M) [e]	DIFP [b]	?	EDTA, o-phenanthroline [g]	None found
Exopeptidase activity	No	No [e]	No [e]	No	No
Thermostability	Moderate	Low	?	High	Very high

a. Jackson & Matseuda (1970)
b. Whitaker (1970)
c. Ensign & Wolfe (1965)
d. Ensign & Wolfe (1966)
e. Matsubara & Feder (1971)
f. personal data
g. Matsubara (1970)
h. Morihara (1974)

Many other lytic proteases have been isolated: e.g. MR, ML, and SL endopeptidases from *Streptomyces albus* G (Petit *et al.*, 1965); L_3 enzyme from *Streptomyces* (Mori *et al.*, 1960); Lysostaphin (Browder *et al.*, 1965); L_{11} enzyme from *Flavobacterium* (Kato *et al.*, 1962); Autolysin from *B. subtilis* (Young *et al.*, 1964); *E. coli* enzyme (Weidel & Pelzer, 1964); Autolysin from *Listeria monocytogenes* (Strominger & Ghuysen, 1967); Autolysin from *Staphylococcus aureus* (Strominger & Ghuysen, 1967); lytic enzyme from the thermophile *Micromonospora vulgaris* (Golinova *et al.*, 1973); peptidases from *Bdellovibrio bacteriovorus* (Fackrell & Robinson, 1973); lytic enzyme from *Streptomyces globisporus* 1829 (Yokagawa *et al.*, 1973); lytic enzyme from *Pseudomonas aeroginosa* (Lache *et al.*, 1969), and from thermophilic Actinomycetes (Desai & Dhala, 1969). However, most have been insufficiently characterised to provide adequate comparative data, and none demonstrates a degree of thermostability equal to that of Caldolysin.

The characterisation of Caldolysin has yielded a broad impression of the structure of the molecule. It is concluded that a globular structure of 20 000 daltons contains three functionally dissimilar types of site. The first is the active site, and since this requires more than three amino acid residues to achieve substrate binding or catalysis, the binding groups are presumably widely spaced (cf. Morihara *et al.*, 1969). The requirement for a large substrate molecule may be derived from the weak nature of the individual side chain-enzyme binding interactions, so that at least four such interactions are required to achieve a stable complex.

Attempts to identify critical active-site amino acid residues in Caldolysin have been largely unsuccessful. A number of common active site components (e.g. histidine, as in Thermolysin (Burnstein *et al.*, 1974); serine, as in Thermomycolin (Ong & Gaucher, 1976); and cysteine,

as in *Streptomyces rectus* protease (Mizusawa & Yoshida, 1976)), have been categorically excluded. Furthermore, the limited influence of phenyl glyoxal on Caldolysin suggests that arginine does not play a critical active site role. Some evidence does suggest that a lysine residue is involved in either substrate binding or catalytic activity. The technique of reversibly inhibiting the enzyme is often used in the study of proteases. However, no means of obtaining complete and reversible inhibition of Caldolysin has been found.

The specificity of Caldolysin has not yet been fully determined. However, current results suggest a preference for small neutral aliphatic amino acids on either side of the cleavage point. This specificity is similar to that quoted for *Myxobacter* Al-1 protease and *Sorangium* α-lytic protease (Morihara, 1974). Caldolysin hydrolyses a number of proteins, including the insoluble fibrous proteins, collagen and elastin. This specificity, coupled with an inability to hydrolyse ester substrates, is characteristic of the microbial neutral proteases, since most show no esterase activity (Matsubara & Feder, 1971), and will cleave one or more of the fibrous proteins.

From the discontinuities observed in the Arrhenius plots of Caldolysin, it is clear that the active site undergoes some alteration facilitating enzyme activity at approximately 50°C. This is substantiated by the difference in K_m values above and below this temperature. The most likely cause is a temperature-induced conformational change in the molecular structure.

The second type of binding site present in Caldolysin is the calcium-binding site. It was shown that molecular stabilisation by metal ions was probably related to the ionic radius of the ion, which suggests that precise molecular symmetry is involved in the binding of calcium. The moieties with which calcium interacts within the sites are possibly

carboxylate groups, as has been found in Thermolysin (Roche & Voorduow, 1978). This could possibly be experimentally verified by esterification of the molecule, and a subsequent determination of the calcium-binding capabilities and thermostability of the enzyme. Structural analysis by X-ray crystallography would be required for an absolute determination.

It has been clearly shown that the binding of calcium ions in Caldolysin is the major contributing factor to the thermostability of the molecule. However, it is considered that in the presence of calcium, at temperatures below about 92°C the loss of enzyme activity is primarily the result of autolysis, since plots of 1/[E] vs time ([E] = concentration of active enzyme remaining) have been shown to display second order kinetics, indicative of autolytic degradation (e.g. Voorduow & Roche, 1975a). By analogy to the theoretical derivations of Voorduow & Roche, (1975a), the most susceptible sites for autolytic attack may be the same as, or closely related to the calcium-binding sites. When unoccupied, these sites either act as loci for autolytic cleavage, or permit structural changes which render other susceptible peptide bonds accessible. Drucker & Borchers (1971) concluded that the susceptibility of Thermolysin to autolysis was directly related to a minor calcium-concentration-dependent conformational change. In reviewing metal ion relationships in Thermolysin, Voorduow & Roche (1978) have concluded that each of the bound calcium ions fulfills a different stabilising function. A single site (Ca_3) is the dominant stabilising influence against purely thermal unfolding, while other sites are responsible for autolytic protection (see also Dahlquist *et al.*, 1976). While analysis in this detail has not been possible in the current studies on Caldolysin, it may be that similar calcium-site differentiation is responsible for the relationships between autolysis and denaturation at different temperatures.

A third type of site considered to be present on the surface of the Caldolysin molecule is a non-catalytic protein binding site, involved in the activation/inhibition phenomena discussed in Chapter 10. Evidence bearing on this site has been difficult to interpret unequivocally: there are fundametal difficulties in investigations of the effect of a protein inhibitor on the action of a protein (the enzyme), when the inhibitor may also act as a substrate for the enzyme. The effect is complicated by the complex interactions of the enzyme and its substrate (substrate inhibition) and by the fact that the enzyme may itself act as a substrate (autolysis). Not only does the latter characteristic render complete purification of such an enzyme impossible, but an inhibitor-free enzyme preparation can be obtained only transiently since autolysis products and/or the normal products of substrate breakdown may subsequently act as inhibitors.

Although the exact relationship between enzyme activation/inhibition and substrate inhibition is unclear, the following conclusions have been derived:

1. Substrate inhibition results from the binding of protein to sites other than the active site (evidence: the absence of substrate inhibition when using peptide substrates; absence of substrate inhibition when in the immobilised state).

2. The activation/inhibition site/s is/are located at some distance from the active site (evidence: the absence of activation when assaying very pure samples with peptide substrates; the absence of activation in the immobilised state).

3. The binding of protein to the activation/inhibition site/s is non-specific (evidence: inhibition and activation has been observed with lysozyme, and activation of both purified and partly purified Caldolysin (inhibited by protein or proteolysis fragments) has been observed).

4. Substrate inhibition is non-specific (evidence: substrate inhibition has been observed with albumin, azoalbumin, casein, and azocasein).

5. Immobilisation causes simultaneous loss of substrate inhibition and the activation/inhibition effect.

6. Inhibitor binding is of an electrostatic nature (evidence: correlation between the extent of activation and ionic strength).

7. The release of inhibitors from the activation/inhibition site/s is temperature dependent (evidence: relationhip between the rate of activation and temperature). However, the release of inhibitors is evidently more complex than would be expected for a purely kinetic dissociation, since activation has been shown to occur over a 15 to 30 minute period. It is possible that proteolysis plays a part in the removal of inhibitor proteins.

The simplest explanation for the evidence above is that the third type of site is involved in both substrate inhibition and the activation/inhibition effect.

The inhibitory influence of the binding of protein to non-catalytic sites might result from any of a number of mechanisms, of which the most likely are:

1. Steric hindrance of the substrate-active site interaction, or;

2. Induction of a conformational change in the molecule, and associated distortion of the active site.

That no activation effect or substrate inhibition was shown with the peptide substrate (Benzoyl-phe-val-arg-pNa) does not assist in choosing between these alternatives, since, although no steric effects would be expected from a molecule of such size, it cannot be assumed that this substrate would be bound to the inhibitor sites. In fact, since the

interaction is likely to be electrostatic, it is possible that the singly charged BPVApNa molecule would be incapable of binding.

While it would be convenient to suggest that the Arrhenius plot discontinuities at 40°C to 50°C were related to the release of inhibitor proteins, it has been shown that the rate of thermally induced release of inhibition (apparent activation) is maximal above 65°C.

The presence of non-catalytic protein binding site(s) on Caldolysin may explain a number of anomalies noted during the period of this research, in particular the massive loss of protein observed during Millipore Ultrafiltration, and the anomalous mobility of Caldolysin during gel filtration chromatography. In both cases, the binding of Caldolysin to protein impurities (denatured or native) embedded in the filtration matrix would result in retardation and loss of enzyme. This suggestion is supported by the following observations:

1. The mobility of Caldolysin on Sephadex gels was highest when using fresh gel. During continued use, progressively increasing retardation was observed.

2. The addition of 1 M NaCl to gel chromatography buffers significantly reduced enzyme retardation.

3. Treatment of filters with 1 M aqueous NaCl after ultrafiltration of protease solutions resulted in the release of considerable quantities of activity.

It is appreciated that these may also be the result of simple non-specific absorption. Retardation of proteases during gel chromatography has been observed previously (Voorduow *et al.*, 1974a), and it was suggested that the basicity of the proteins might be responsible (Arvidson *et al.*, 1973; Ensign & Wolfe, 1966). However, in one of the examples above (Voorduow & Roche, 1974a), and in the case of Caldolysin, basicity can be excluded as a potential cause.

As stated previously, the factor primarily responsible for the thermostability of Caldolysin is metal ion stabilisation. However, the presence of disulphide bonding (calculated as 31% and 60% of the total and intrinsic energies of stabilsation of Caldolysin, respectively - for a discussion of assumptions, see section 11-1) appears to contribute a significant proportion of the protease thermostability. As discussed in section 2-7, extracellular bacterial hydrolases have been generally found to be cystine-free. However, Caldolysin and a number of other extracellular bacterial lytic proteases are exceptions to this rule. Hsui *et al.*, (1964) noted that cystine was absent from extracellular bacterial amylases, and suggested that metal ions might form functionally similar intramolecular cross-linkages instead. It is apparent that Caldolysin possesses both mechanisms of structural stabilisation.

The stability of Caldolysin in the presence of denaturing agents and to other extreme conditions is high. Its thermostability has been exceeded only by that reported from an extracellular protease isolated from the mesophile *Streptococcus lactis* (Williamson *et al.*, 1964).

In the presence of chaotrophic agents and at extremes of pH, Caldolysin is very stable at ambient temperatures. However, at higher temperatures, activity loss is accelerated by either of the above conditions. Similar responses were found for Thermolysin, which was stable at room temperature in the presence of 8 M urea and 0.12% cetyltrimethylammonium bromide (a cationic detergent), but rapidly denatured at 80°C under similar conditions (Ohta, 1967). Caldolysin exhibited considerable stability in the presence of 1% sodium dodecyl sulphate (an anionic detergent), losing only 50% activity after incubation for more than five hours at 75°C. This suggests that either hydrophobic bonding is a less significant intramolecular stabilising feature in Caldolysin than in Thermolysin, or that the surface structural characteristics of the former prohibit access of the SDS to the molecular interior, thus preventing

hydrophobic disruption. Chaotrophic agents thought to disrupt hydrogen-bonding (e.g. urea. guanidine.HCl) reduce the stability of Caldolysin considerably (e.g. at 75°C: $t_{\frac{1}{2}}$ (8 M urea) = 53 minutes; $t_{\frac{1}{2}}$ (6 M guanidine.HCl) = 59 minutes. These results may indicate that hydrogen-bonding provides a greater relative contribution to the intrinsic energy of stabilisation of the Caldolysin molecule than hydrophobic bonding.

It is concluded from an analysis of the structural and functional characteristics of Caldolysin, that this enzyme may fulfil a dual role *in vivo*. Although experimental data suggest that the existance of Caldolysin *in vivo* at high temperatures in the low calcium environments of the thermal pool waters may be transient, the high specific activity may permit a significant proteolytic contribution. Furthermore, at temperatures below 92°C, where activity losses are primarily derived from autolysis, the longevity of Caldolysin *in vivo* may be considerably extended as a result of the extremely low protease concentrations excreted into the pool waters. A decrease in the thermostability of Caldolysin in the presence of silicate sinter *in vitro* suggests that surface stabilisation of the protease *in vivo* is unlikely to be a contributing factor. The broad specificity of Caldolysin implies a primarily nutritional function. Since peptides with fewer than four residues cannot be hydrolysed, it may be that extracellular peptidases are also produced by *Thermus* T-351.

The significance of the lytic activity of Caldolysin is uncertain. However, it is possible that the Gram-negative antibacterial specificity has evolved as a measure for the prevention or supression of competitive bacterial growth. It has been shown that the majority of aquatic bacteria are of Gram-negative type (Costerton *et al.*, 1974).

Caldolysin has been successfully immobilised to several insoluble matrices. While yields were very matrix-dependent, a high recovery of

activity after immobilisation to CNBr-activated Sepharose 4B was obtained. Immobilisation to both Sepharose and CM-cellulose resulted in an increase in thermostability, although no significant broadening of the pH/activity profiles was noted. An Arrhenius plot for Sepharose-caldolysin demonstrated a discontinuity similar to that of the free enzyme, although raised by approximately 12°C. This is consistent with an increase in molecular rigidity resulting from a multi-point covalent linkage to a massive matrix. However, no significant active site changes appear to result from immobilisation, as no change in the K_m could be detected.

That the already high stability of the native enzyme can be facilitated by immobilisation suggests that Caldolysin may be of potential value in some industrial and commercial processes. Thermophilic enzymes present a number of advantages for industrial application (Doig, 1974).

a. Heat stable enzymes allow enzyme reactors to be operated at higher temperatures. Resulting advantages include decreased fluid viscosity, increased solute solubility, and possibly accelerated reaction rates.

b. Such enzymes exhibit very high stability in the presence of detergents and aqueous-organic solvent solutions.

c. The heat-stability and solvent resistance of these enzymes may prevent denaturation during chemical coupling procedures, thus improving yields of immobilised enzyme.

d. The common problem of microbial contamination in "mesophilic" enzyme reactors is eliminated by operating at high temperatures, especially above 75°C.

e. Many protein substrates may be denatured at the optimum temperature for thermophilic enzymes, thus facilitating hydrolysis.

This research has shown that the properties of Caldolysin are applicable to each of the categories above.

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