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CALDOLASE: AN ALKALINE SERINE PROTEASE FROM *THERMUS* STRAIN TOK_3

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ABSTRACT

The object of this investigation was the isolation of extreme thermophiles producing extracellular proteases, and the biochemical characterisation of a stable, chelator-insensitive protease.

Two plate assay systems were developed for the initial screening of proteases. The first involved the incorporation of various protease inhibitors (particularly chelating agents) in casein agar plates, the second the inclusion of a variety of native and chromogenic proteins in the agar plates. In conjunction, these methods provided a basis for screening extreme thermophiles for particular proteases, and enabled the identification of fourteen new proteases. Included amongst them was an extracellular serine protease from a *Thermus* strain designated Tok_3, which was selected for further study.

The protease was purified to homogeneity by ammonium-sulphate precipitation followed by ion exchange on DEAE-cellulose and QAE-Sephadex, affinity chromatography on CBZ-phe-TETA-Sepharose-4B, gel filtration chromatography on either Sephadex G-75 or TSK G3000 SW using HPLC system and finally Polyacrylamide gel electrophoresis.

For convenience the protease was assigned a trivial name, and the term Caldolase was chosen. The prefix Caldo- is derived from Latin Caldo (hot) and the suffix 'ase' is a general term for enzymes.

The specific activity of the pure enzyme was estimated to be 38,000 proteolytic units per mg (PU mg ml^{-1}) at 75^{0}C

using casein as the substrate. The purified enzyme had a pH optimum of 9.5 with an isoelectric point of 8.9. Caldolase demonstrated greatest stability between pH 7 and 10. The molecular weight of the protease was estimated by exclusion chromatography on Sephadex G-75 and TSK G3000 SW to be 25,000 daltons.

The enzyme was inhibited by serine inhibitors (DFP, PMSF and di-phenyl carbamyl chloride), partially inhibited by heavy metal ($CUCl_2$), but not inhibited by metal chelators (EDTA, EGTA, O-phenanthroline, and NEPIS), Cysteine inhibitors (PCMB, iodo-acetamide, and N-ethylmaleimide) or trypsin inhibitors. These results indicate that Caldolase is an alkaline serine protease.

Neither Ca^{2+} nor Zn^{2+} ions were detected in the highly purified protease. The presence of four disulphide bonds per molecule of the enzyme was indicated with dithionitrobenzoate. No free sulphydryl groups were found. The purified protease contained approximately 10% carbohydrate. The amino acid composition of Caldolase was determined.

The enzyme exhibited strong substrate inhibition when using casein, azo-casein, and azo-albumin as substrates. No substrate inhibition was observed when low-molecular weight synthetic substrates were used, indicating that substrate inhibition using casein and azo-albumin substrates may be due to steric hindrance rather than binding to the active site of the enzyme.

The Arrhenius plots for both casein and peptide substrates were curved, but without any clearly marked discontinuity. It is concluded that the effect of temperature on the enzyme

conformation is continuous rather than occurring at a particular temperature. No significant differences were observed in K_m values at various temperatures between 45° and 85°C.

Caldolase hydrolysed several protein substrates, including casein, albumin, ovalbumin, haemoglobin, collagen, fibrin and elastin, and a number of synthetic chromogenic peptides. It also possessed esterase activity. The enzyme was not able to hydrolyse peptides possessing fewer than four groups (amino acid residues and terminal blocking group). Caldolase did not hydrolyse Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-phe-Arg). In contrast enzyme hydrolysis of insulin B chain resulted in a very complex pattern, suggesting a low degree of specificity.

The enzyme was very thermostable. Half-life values were: 100°C, 5 min; 90°C, 45 min; and 80°C, 840 min. Caldolase was stable in denaturing agents (GuHCl, urea) at 22°C, but not at 85°C. Exposure of the enzyme to various organic solvents caused no significant loss of catalytic activity.

Ionic strength had a marked effect on enzyme stability. The combination of low salt concentration (below 0.3M NaCl) and low temperatures (under 75°C) results in reversible enzyme denaturation. However, at high temperatures (above 80°C) this phenomenon is rapidly followed by autolysis by the remaining active enzyme.

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LIST OF ABBREVIATIONS

CBZ-D-phe-TETA Sepharose-4B	=	Carbobenzoxy-D-phenylalanyl-triethylene tetraminyl-Sepharose-4B
CBZ-phe-pNE	=	N-CBZ-L-phenylalanine-p-nitrophenyl ester
DFP	=	Di-isopropyl fluorophosphate
DPCC	=	Di-phenyl carbamyl chloride
EDTA	=	Ethylene diaminetetra acetic acid
EGTA	=	Ethylene glycol-bis (β-amino-ethyl ether) N-N′-tetra acetic acid
GuHCl	=	Guanidine-HCl
HEPES	=	N-2-Hydrozy ethyl piperazine-N′-Z-ethane sulphonic acid
HMB	=	4-Hydroxymercuribenzoate
IAA	=	Iodo-acetic acid
NEPIS	=	N-Ethyl-5-phenyl-isoxazolium-3-sulphate
PCMB	=	P-Chloromercuribenxoate
PMSF	=	Phenylmethyl sulphonyl fluoride
SDS	=	Sodium dodecyl sulphate
Suc.ala$_3$-pNA	=	Succinyl-ala-ala-ala-p-nitroanilide
TCA	=	Trichloro acetic acid
TCPK	=	N-α-p-tosyl-L-phenylalanine chloromethyl ketone
TLCK	=	N-α-p-tosyl-L-lysine chloromethyl ketone

CHAPTER 1

INTRODUCTION

1.1 Extremely Thermophilic Bacteria.

1.1.1 Extreme Environment:

In recent years interest has grown in microbial adaptation to extreme environments, resulting in a number of books and reviews being published on the subject. (Kushner, 1978a; Heinrich, 1976; Shilo, 1979; Brock, 1978a; Morita, 1975).

The most commonly considered environmental extremes have been temperature, pH, salinity and nutrient concentration (Brock, 1978a; Kushner, 1978b; Lanyi, 1974; Norberg & Hofsten, 1969; Rao & Argos, 1981; Darland & Brock, 1971; Langworthy, 1978).

Temperature is one of the most important environmental factors, and is one of the easiest variables to measure. There are in existence bacterial species capable of growing at temperatures up to the boiling point of water (extreme thermophilic bacteria) (Heinen and Lauwers, 1981), whilst others can grow at zero or sub-zero temperatures (psychrophiles) (Morita, 1975; Baross & Morita, 1978).

1.1.2 Thermophilic Micro-organisms:

For some years thermophilic micro-organisms have been of general biological interest, having been isolated from a wide variety of sources such as compost, soil and water (Tansey & Brock, 1978). The ability of these organisms to survive at temperatures above denaturation level for most biological macro molecules has been intensively studied in recent years (Friedman, 1978). In particular, attention has been focussed on the spore-forming species *Bacillus stearothermophilus* (Ljungdahl & Sherod, 1976).

The discovery of a new type of extremely thermophilic, aerobic, gram-negative rod isolated from a hot spring in Yellowstone National Park by Brock & Freeze (1969) and called *Thermus aquaticus* gave new impetus to the study of thermophiles.

Bacteria of this type have been isolated world-wide, (U.S.A.; New Zealand; Japan; Iceland) from neutral or moderately alkaline water between about 50 - 101°C (Oshima & Imahori, 1974; Pask-Hughes & Williams, 1975, 1977; Hickey & Daniel, 1979; Cometta *et al*, 1982).

1.1.3 Classification of Thermophilic Bacteria:

In general, the temperature range of thermophilic organisms is fairly broad, from about 40° to 100°C. Farrell & Campbell (1969) have divided thermophilic organisms into three main groups:

i Strict, or obligate thermophiles demonstrate optimal growth at 65° to 70°C but do not grow below 40° to 42°C

ii Facultative thermophiles have a maximal growth temperature between 50° and 65°C but also are capable of multiplying at room temperature.

iii Thermotolerant organisms have growth maximum at 45° to 50°C. They are also able to grow at room temperature.

Isolation of *Thermus* strains (Brock & Freeze, 1969; Ramaley & Hixson, 1970) resulted in Heinen and Heinen (1972) introducing the new term "Caldoactive" (Caldo from the Latin meaning hot). Based on this new term the thermophiles have been divided into two groups: the thermophilic, including most of the long-known and widely distributed species, and the extremely thermophilic or caldoactive. Even within the caldoactive group, with temperature optima above 70°C, the temperature range is very wide (Sonnleitner & Fiechter, 1983). However, this terminology is likely to be revised in the near future, since organisms capable of growth at temperatures exceeding 100°C have been found in the last few years.

Heinen and Lauwers (1981) were successful in growing an isolate (*Thermus* strain) from the Yellowstone National Park at 105°C. They concluded that 100°C is not the upper barrier for life to exist, provided that water is kept in the liquid phase under high pressure. More recently Baross and Deming (1983) reported that bacterial communities sampled from superheated, deep-sea water (temperatures up to 350°C) could be grown under pressure up to 300°C. Although this has yet to be confirmed, their finding supports the hypothesis that microbial growth is limited not by temperature but by the existence of liquid water, assuming that all other conditions necessary for life are provided. Recent bacterial classification is given in Table 1.1.

Table 1.1 Bacterial Classification

Bacteria	Growth - temperature (°C)		
	Minimum	Optimum	Maximum
Psychrophiles [a]	0->0	15->15	20
(e.g. *Bacillus psychrophilus*, *pseudomas fluorescens*			
Mesophiles [b]	10 - 15	25 - 40	35 - 45
(e.g. *E. Coli*)			
Thermotolerant [c]	15 - 25	> 55	55 - 60
(e.g. *Bacillus coagulans*)			
Thermophiles [c]	30 - 45	50 - 65	65 - 75
(e.g. *Bacillus stearo-thermophilus*)			
Extremely thermophilic (Caldoactive)			
(*Thermus aquaticus*, [d]	40 - 48	75	79
T. Thermophilus, [e]	47	65 - 72	85
Bacillus caldolyticus [f])			105
Ultra thermostable			
Baross & Deming's isolate [g]			300

(a) Morita (1975)

(b) Kogut (1980)

(c) Gibson & Gordon (1974)

(d) Brock & Freeze (1969)

(e) Oshima & Imahori, (1974)

(f) Heinen & Lauwers (1981)

(g) Baross & Deming (1983)

1.1.4 The Genus *Thermus*

Thermus bacteria are now defined as aerobic, gram-negative, non-motile, non-sporulating, rod-shaped organisms with high sensitivity to penicillin (Brock, 1978b).

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 (Brock, 1978b). Cells are generally yellow-pigmented (Williams, 1975) although strain *x*-1 is reported to be non-pigmented (Ramaley & Hixson, 1970).

Electron microscope study of *Thermus aquaticus* revealed that the cell wall of this organism was analogous to gram-negative bacteria and that cell division resembled typical gram-negative bacteria such as *E. coli* (Brock, 1978b). The DNA base composition of *T. aquaticus* was between 65 - 67 moles % GC (Brock & Freeze, 1969).

A comparative DNA analysis of thirteen *Thermus* isolates from Iceland revealed close similarities to *T. aquaticus*, the isolate strains having base composition of about 62 - 65 moles % GC (Pask-Hughes & Williams, 1977).

1.2 PROTEOLYTIC ENZYMES:

1.2.1 The Role of Proteases.

Proteases are known to possess a variety of physiological functions, such as involvement in mammalian fertilisation, sporulation and spore germination, activation of certain viruses important for pathogenicity (Ward, 1983), oncogenic transformation (North, 1982), blood coagulation and fibrinolysis (North, 1982; Kerr, 1979), and regulatory function by limited

proteolysis of proteins, hormones and toxins (Korant, 1980; Kay, 1980).

The existence of proteases within bacterial cells has been known for many years, but only recently have extensive studies been carried out to consolidate and extend the understanding of the physiological functions and biochemical characteristics of these enzymes.

Bacterial proteases are produced both extracellularly and intracellularly and play an important role in the cell's metabolic and regulatory processes (Ward, 1983).

i The role of bacterial extracellular proteases.

The role of these enzymes in nature is normally nutritional, to hydrolyse large polypeptide substrates into smaller molecular entities which can enter the cell (Ward, 1983).

Extracellular proteases are of particular interest in industrial applications since in comparison to intracellular enzymes they are generally more stable and are produced in considerably higher yields (Eveleigh and Montenecourt, 1979).

ii The role of bacterial intracellular proteases

In contrast to extracellular proteases, intracellular protease are multifunctional (Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). Shannon *et al* (1982) have proposed three major roles for intracellular proteases:

a) Processing of post-translational modification of proteins.

b) Degradation of abnormal or defective proteins.

c) Degradation of normal proteins to peptides and amino acids.

1.2.2 Applications of Microbial Proteases

Proteases have proved suitable for use in a variety of processes involving clinical, laboratory and industrial applications. (Aunstrup, 1980; North, 1982). It is now clear that they possess considerable potential for further exploitation (Ward, 1983).

i Laboratory application.

Protein sequencing: Most microbial proteases exhibit broad specificity and are therefore not useful for protein sequence analysis. However, a number of fungal proteases have demonstrated greater specificity e.g. a metalloprotease from *Armillaria mellea* has shown specificity for peptide bonds involving the amino group of lysine and has proved useful for limited cleavage of proteins (Shipolini *et al*, 1974; Barry *et al*, 1981). Protease K, the serine protease from *Tritirachium album* (Ebeling, *et al*, 1974) has been used for selective removal of ribonuclease activity (inactivation of nucleases) from commercially prepared nucleic acids.

ii Clinical application

Digestive-aid preparations are widely used, containing proteases from *Aspergillus* spp. often supplemented by pancreatic enzymes (Aunstrup, 1980; Sizer, 1972).

Treatment of thrombosis: Streptokinase, produced from *Streptococcus* pyogenes is used in the treatment of thrombosis. The enzyme functions by activating plasminogen in the blood (Aunstrup, 1980).

Serological diagnosis: Extracellular protease from *Candida albicans* has been used as a diagnostic antigen for candidosis (MacDonald & Odds, 1980; North, 1982).

Dental hygiene: a dextranase from *Penicillium funiculosum* is able to remove dental plaque formed by *Streptococcus mutans* (Taylor & Richardson, 1979).

iii Industrial application

~~Commercial exploitation of microbial enzymes considerably~~ preceded the study of their nature and properties, e.g. fungal protease koji from *Aspergillus oryzae* has been used for centuries in the production of soy sauce (Aunstrup, 1980). Sales of microbial proteases were, however, limited until their wide-scale use in detergents was introduced. In 1959, a protease from *B. subtilis* was marketed (Bio 40) followed by the introduction of subtilisin Carlsberg, produced by *B. licheniformis* in 1960 (Aunstrup, 1980). Following these initial developments have come advances in the increased application of enzymes for a diversity of purposes, in the food and textile industry (Kay, 1982; Fox, 1982; Erikson, 1982) for use in detergents and in the production of pharmaceutical products (drugs, hormones, antibiotics) (Solomons, 1977; Rose, 1980; Dunnill, 1983; Hartley & Payton, 1983). Nevertheless, industrial application of enzymes is still in its infancy, and realisation of their full potential is yet to come. Of the two thousand enzymes known today, only about two hundred are commercially available (most of them in milligram and gram quantities) and of these two hundred, a mere sixteen are available in industrial quantities (kilogram) (Klibanov, 1980).

From the economic point of view, proteases rank as the most important industrial enzymes, and the majority of these are from microbial sources. Use of alkaline serine protease of *Bacillus licheniformis* in detergents is the major commercial application of proteases with production of about 500 tonnes

each year (Rose, 1980), followed by the use of *Mucor* protease in cheese manufacture (Ward, 1983; Sardinas, 1972). Application of *Aspergillus oryzae* fungal protease, particularly for modifying the dough in bread and cracker-making, have made this enzyme the third most important of the microbial proteases (Ward, 1983).

In addition to microbial proteases, animal and plant proteases, particularly calf rennet, pancreatic proteases, malt and papain are used commercially in cheese manufacturing, tanning of hides, protein hydrolysis and meat tenderisation. (Ward, 1983).

iv Other applications

Protease uses extend to gelatin hydrolysis on photographic film to aid silver recovery and to the cleaning of delicate biological processing and medical equipment, such as dialysis equipment (Ward, 1983), the degumming of natural silk, and the improvement of rubber recovery by digesting undesired protein in the latex skin (Aunstrup, 1980).

Estimated world sales of proteases in 1981 adopted from Ward (1983) and Godfrey & Reichelt (1983) is given in Table 1.2.

1.2.3 Microbial Protease Classification

Microbial proteases are usually grouped according to their active centres as, serine-, metallo-, cysteine- and acid proteases. A classification system adopted from Enzyme Nomenclature (1978), (Dixon & Webb, 1979a) recommended by the Nomenclature Committee of the International Union of Biochemistry is given in Table 1.3.

Table 1.2 Estimated World Sales of Commercial Enzymes for 1981.[1]

Enzyme	Source	Millions of dollars	Total enzyme (%)
Proteases	Bacterial alkaline protease	90	30
	Microbial rennet	18	6
	Other microbial proteases	10	3.5
	Animal rennet	18	6
	Other animal proteases	8	2.5
	Plant proteases	33	11
Carbohydrases	Pectinases	9	3
	Isomerases	18	6
	Cellulases and lactase	3	1
	α-amylases	15	5
	β-amylases	39	13
Lipases		9	3
Other enzymes		30	10
Total		302	100

1 Ward (1983); Godfrey & Reichelt (1983).

Table 1.3 Classification of Microbial Proteases (Enzyme Nomenclature, 1978)[1]

Protease	Source	Specificity	Type of Active Site
Serine proteinases			Serine and Histidine
e.g. Subtilisin	*B. subtilis*	hydrolysis of proteins and peptide amides	
Thermomycolin	*Malbranchea pulchella var. sulphurea*	ala-, tyr-, phe	
Thiol proteinases			Cysteine
e.g. Clostripain	*Clostridium histolyticum*	arg-, also arg-pro bond	
Streptococcal protease	*Streptococcus* (spp)	hydrolysis of proteins; the amino acid adjacent, on the amino terminal side	
Carboxy (acid) proteinases			Acidic a.a. residue at active site
e.g. *Aspergillus oryzae* carboxyl proteinase	*Aspergillus oryzae*	broad specificity, activates trypsinogen, does not clot milk.	
Mucor pusillus carboxy proteinase	*Mucor pusillus*, *Mucor miehei* }	does not activate trypsinogen, milk clotting activity.	
Metalloproteinase			Metal (e.g. Zn^{2+})
e.g. *B. subtilis* neutral proteinase	*B. subtilis*	preferential cleavage of bonds adjacent to a hydrophobic amino acid residue.	
Thermolysin	*B. thermoprotealyticus*	-Leu > -phe	

[1] Dixon & Webb (1979a)

1.3 MECHANISMS OF THERMOSTABILITY:

The extraordinary thermostability of many proteins from extremely thermophilic organisms has attracted considerable research interest aimed at determining the basis of this phenomenon (Boccu, *et al*, 1976; Frank *et al*, 1976; Zuber, 1979). It has been shown that protein thermostability correlates with resistance to denaturing agents such as detergents, organic solvents, urea and guanidin - HCl (Hocking & Harris, 1980; Cowan & Daniel, 1982a). In addition, thermostable proteins are more resistant to proteolysis than their mesophilic counterparts (Daniel *et al*, 1982).

Contrary to initial expectations, the primary, secondary and tertiary structures of thermophilic proteins are, in general, strikingly similar to analogous mesophilic proteins (Amelunxen & Murdock, 1978; Ljungdahl & Sherod, 1976). This observation is not unexpected since a difference of only a few non-covalent interactions has the potential to alter significantly the free energy of activation for protein denaturation (Ruegg *et al*, 1982). For example, one salt bridge can account for about 8 - 12 kJ mol^{-1} decrease in free energy of a thermophilic protein (Mozhaev & Martinek, 1984). It has been reported that the total extra free energy of stabilisation provided by additional bonds in the thermophilic ferredoxins is less than 10 kJ mol^{-1} (Perutz & Raidt, 1975). Current evidence suggests that no single structural mechanism is responsible for the enhanced thermostability of proteins from thermophilic organisms (Finney *et al*, 1980). The importance of the various mechanisms which contribute to protein thermostability will be discussed in more detail below. The involvement of hydrophobic bonding has attracted attention since these bonds are known to strengthen

in relation to increases in temperature up to 65°C (Brandts, 1967). Attempts have been made to establish a correlation between the stability of proteins and their degree of hydrophobicity (Hasegawa & Imahori, 1976; Nakamura *et al*, 1978; Sato & Nakazawa, 1978; Yutani *et al*, 1979; Gekko, 1982).

Attention has been focussed upon the possibility that an increase of hydrogen bonds in thermophilic proteins are responsible for their thermostability (Colman *et al*, 1972; Barnes & Stellwagen, 1973; Matthews *et al*, 1974). The contribution of disulphide bonds in the enhanced thermostability of Caldolysin from *Thermus aquaticus* 351 (Cowan, 1980) and glutamine synthetase from *B. Caldolyticus* (Wedler *et al*, 1976) has been considered. Since thermolysin consists of a single polypeptide chain without disulphide bridges, it is possible that calcium ions might perform this bridging function within the molecule (Feder *et al*, 1971; Matsubara, 1970).

The involvement of salt bridging (ionic interactions) in the thermostability of some proteins has been reported (Perutz & Raidt, 1975; Perutz, 1978; Bieseker *et al*, 1977; Ruegg *et al*, 1982).

The role of metal ions in protease stability has been the subject of extensive investigation (Khoo *et al*, 1984; Roche and Voordouw, 1978; Shimizu & Hatano, 1982; Nonokawa & McDonald, 1968). Voordouw *et al* (1976) have found that the stabilising effect of calcium ions on thermophilic proteases (thermolysin, thermomycolase) and mesophilic proteases (subtilisin Carlsberg BPN' and neutral protease A) was almost equal, and therefore concluded that the extra thermal stability of thermophilic enzymes was due to their relatively high intrinsic stability.

The importance of high ionic strength in maintaining both the activity and stability of various enzymes has been reported (Hengartner & Zuber, 1973; Crabb *et al*, 1977; Shannon *et al*, 1982; Griffiths & Sundaram, 1973). Many enzymes from obligate and extreme halophiles require 3 - 5 M NaCl for maximum stability (Keradjopoulos & Wulff, 1974).

It has been suggested that carbohydrate moieties stabilise the three-dimensional structure of glycoproteins, since extensive oxidation of the carbohydrate residue of glucoamylase I from *Aspergillus niger* (Pazur *et al*, 1970) and glucose oxidase from *Aspergillus niger* (Nakamura & Hayashi, 1974) by periodate markedly affected the stability of the enzymes. It has also been proposed that carbohydrate moieties protect glycoproteins against proteolysis by covering potential proteolytic cleavage sites (Barker & Gray, 1983).

Many attempts have been made to relate the thermostability of proteins to a particular amino acid, or group of amino acids such as hydrophobic, aliphatic, or charged residues (Frank, *et al*, 1976; Zuber, 1978; Ikai, 1980; Biffen & Williams, 1976; Ljungdahl *et al*, 1976; Amelunxen & Singleton Jr., 1976). It is now clear that the amino acid composition of mesophilic and thermophilic proteins is not sufficiently precise to explain the relation of protein structure and thermal stability. Several investigators have reported that the stability of a protein may be greatly enhanced by an increase in hydrophobicity resulting from the substitution of a few suitably located amino acids (Yutani *et al*, 1978; Ogasahara *et al*, 1980; Yutani *et al*, 1980). By comparing the amino acid sequence of thermophilic and mesophilic ferredoxins and glyceraldehyde-3-phosphate dehydrogenases, Argos *et al* (1979)

have shown that gly-ser, ser, lys and asp, in mesophiles were substituted by ala, ala, thr, arg and glu, respectively in thermophiles. They suggested that the overall effect of these exchanges was to increase the internal and decrease the external hydrophobicity and enhance the compactness of the molecule through incorporation of helix-forming amino acid residues in proteins. Tanaka *et al*, (1973) showed that the amino acid sequence of the ferredoxin of *C. tartarivorum* was different from that of the ferredoxin of *C. thermosaccharolyticum* at only two positions. The first enzyme lost 50% of its activity after 2 hours at 70°C while the second retained 90% of its activity after similar treatment (Devanathan *et al*, 1969). Grutter *et al*, (1979) showed that a decrease of approximately 14°C in the melting temperature of bacteriophage T_4 lysozyme resulted from the substitution of a single arginine residue by histidine.

1.3.1 Conclusion:

In summary, it can be said that stabilisation of proteins against thermal denaturation is conferred by small changes in the amino acid sequence (Langridge, 1968; Yutani, 1977) resulting in a relatively small number of additional intra-molecular interactions of several possible types (hydrophobic interactions, hydrogen bonds, salt bridging). Such changes can affect stability without any obvious protein structural alteration (Grutter *et al*, 1979). The binding of metal ions may also be important in some cases (Khoo *et al*, 1984; Roche & Voordouw, 1978).

2. GENERAL METHODS.

2.1 SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS

Gel electrophoresis was used for:

i Molecular weight determination

ii Test of homogeneity (purity)

2.1.1 Assay method

Electrophoresis in a 10% polyacrylamide gel slab was carried out by a modification of the discontinuous buffer system of Laemmli (1970) using the glass plate sandwich technique. Using a vertical electrophoresis unit (LKB model 2001), a 10% slab gel was prepared (see below) between glass plates (16 x 18cm) separated by 1.5mm spacers. After pouring, the gel was immediately overlayered with distilled water to give a flat surface. After the lower gel had polymerised, the distilled water was removed and a 5% gel solution was added on top. Immediately after the addition of the upper gel, wells were made in the gel by insertion of a special sample applicator comb. After the upper gel had polymerised, the comb was gently removed. The samples in the sample buffer were loaded onto the gel in 50 µl amounts.

Standard proteins in the sample buffer (2 part sample buffer and 1 part standard proteins) were heated at 100°C for 1 - 2 minutes. Longer periods and higher temperatures were found to be necessary for the complete dissociation of Caldolase.

Consequently, the enzyme solution in the sample buffer was heated at 110°C for 5 minutes. Bromophenol blue was included in one of the samples as tracking dye. The gel was

electrophoresed for about 4 - 5 hours at 100 - 120 volts. It was then stained for one hour with 1% (w/v) Coomassie brilliant blue G250 (Sigma). After destaining overnight, the gel was allowed to rehydrate in 7% (v/v) glacial acetic acid, and was then stored in this solution and photographed.

Molecular weight determinations were carried out by comparison with the migration of protein markers of known molecular weights. The markers used were obtained from Sigma Chemical Company, St. Louis, U.S.A., as follows:

Bovine serum albumin (66,000 daltons), ovalbumin (45,000 daltons), α-chymotrypsinogen (25,000 daltons) and cytochrome C (12,000 daltons). In a second preparation the following protein standards were used: α-lactalbumin (from bovine milk) (14,200 daltons), carbonic anhydrase (from bovine erythrocytes) (29,000 daltons), albumin (from chicken egg) (45,000 daltons) and albumin serum (monomer, 66,000 daltons and dimer 132,000 daltons).

SDS - PAGE : Tris-glycine buffering system (Laemmli, 1970).

Solution:

1. Stock Lower Gel Buffer:

   18.2g Tris (hydroxymethyl) methylamine
   0.4g Sodium dodecyl sulphate (SDS)
   50 ml Distilled water

   Adjust pH to 8.8 with 1 M HCl
   Add distilled water to make final volume 100 ml.

2. Stock Upper Gel Buffer:

   6.4g Tris (hydroxymethyl) methylamine
   0.4g Sodium dodecyl sulphate (SDS)
   50 ml Distilled water

   Adjust pH to 6.8 with 1 M HCl
   Add distilled water to make final volume 100 ml.

3. Stock Acrylamide:

   30 g Acrylamide
   0.8g Bisacrylamide (NN^1-methylenebisacrylamide)
   100 ml Distilled water

   N.B. Acrylamide is neurotoxic.
   The solution was filtered then stored at 4°C in a tinfoil covered bottle.

4. Ammonium persulphate:

   10% (w/v) ammonium persulphate in distilled water

5. Sample Buffer:

   30 ml Stock upper gel buffer
   15 ml Glycerol
   3 g Sodium dodecyl sulphate (SDS)

   Bring to 100 ml with distilled water, and add β-mercaptoethanol to a final concentration of 1%.

6. Lower Gel 10%:

10 ml Stock lower gel buffer
13.4 ml Stock acrylamide
16.6 ml Distilled water
0.3 ml Ammonium persulphate (10%)
30 μl TEMED (NNN^1N^1- tetramethyl ethylene-diamine)

7. Upper Gel 5%:

2.5 ml Stock upper gel buffer
1.67 ml Stock acrylamide
5.87 ml Distilled water
90 μl Ammonium persulphate (10%)
30 μl TEMED

8. Electrophoresis Buffer:

5.1 g Tris
15.4 g Glycine
1 g SDS
1 litre Distilled Water

9. Staining Solution:

50 ml Methanol
10 ml Glacial acetic acid
50 ml Distilled water
1.1 g Coomassie Brilliant Blue (G250) (filter solution)

10. Destaining Solution:

50 ml Methanol
10 ml Glacial acetic acid
50 ml Distilled Water

11. Hydrating Solution:

7% (v/v) Glacial acetic acid in distilled water.

2.1.2 Silver Staining for the Detection of Proteins in Polyacrylamide gels.

In addition to the Coomassie brilliant blue stain, the more sensitive silver stain was also used (Merril *et al*, 1981). The proteins were fixed overnight in a solution of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in double distilled water. The gel was then rinsed twice, using a 10% (v/v) ethanol and 5% (v/v) acetic acid solution (15 minute rinses) to remove excess sodium dodecyl sulphate (SDS). The gel was then soaked for 15 min. in 200 ml of oxidiser (0.0034M potassium dichromate and 0.0032M nitric acid). This was followed by two washes (5 min. each) in double distilled water prior to soaking in 200 ml of 0.012M silver nitrate for 20 min. under a bright light (about 30cm distance). The gel was then washed with 400ml double distilled water for 1 min. followed by rapid rinsing with 200 ml developer (0.25M sodium carbonate and 0.5% commercial formalin per litre). The gel was gently agitated in a second 200 ml of the developer solution until the image had reached the desired intensity. Development was terminated by discarding the developer and adding 100 ml of 5% (v/v) acetic acid. The gel was washed twice with 200 ml of water and photographed.

2.2 ISOELECTRIC FOCUSSING.

Isoelectric focussing (IEF) was used for:

i Determination of isoelectric point of Caldolase (pI)
ii Test of homogeneity (purity) of the enzyme.

Isoelectric focussing was carried out on commercially prepared Servalyt Precotes (Serva, pH 3 - 10). A salt-free protease sample was used. After application of the enzyme

sample and standard proteins the plate was electrophoresed on a flat-bed electrophoresis unit (Pharmacia) connected to a constant wattage power supply (ISCO) and a temperature control unit. The voltage gradually increased from 250 to 1200 volts during the 110 minutes focussing period, while the current dropped from 23 MA to below 10 MA. The temperature of circulated cooling water was 4°C. The gel was fixed with 20% (w/v) Trichloroacetic acid (TCA) for 10 min. and subsequently washed with tap water. The gel was then stained for 10 min. with Coomassie brilliant blue G250 (100 mg dye dissolved in 250 ml of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in distilled water) for 10 min. The destaining was carried out twice for 5 min. each, using a solution of 40% (v/v) methanol and 10% (v/v) acetic acid in double distilled water. Some gels were silver stained for greater sensitivity. It was also possible to destain the Coomassie brilliant blue stained gels (with destaining solution overnight) and then silver stain them. Finally the stained gel was dried in an incubator at 75°C for 30 min. and then photographed. A mixture of standard proteins (10 mg ml^{-1}) with known isoelectric points (Pharmacia Fine Chemicals) was run in parallel in the same gel. These were as follows: trypsinogen (pI - 9.30), lentil lectin-basic (pI - 8.65), lentil lectin-middle (pI - 8.45), lentil lectin-acidic (pI - 8.15), horse myogloblin-basic (pI - 6.85), human carbonic anhydrase B (pI - 6.55), bovine carbonic anhydrase B (pI - 5.85), β-lactoglobulin A (pI - 5.20), soybean trypsin inhibitor (pI - 4.55), amyloglucosidase (pI - 3.50).

The pI value of Caldolase was determined by comparison with the pI value of the standards.

2.3 PROTEIN ASSAY METHODS

Due to the high specific activity of Caldolase, the enzyme samples which were used for protease assay contained a very low protein concentration (5 - 25 μg ml^{-1}). A sensitive protein assay method was found to be essential. The following protein assay methods were evaluated.

2.3.1 Bradford Protein Assay

The binding of Coomassie brilliant blue G.250 to protein is the basis of the Bradford method (Bradford, 1976). In this assay the binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm.

This method is rapid, simple and has the advantage of experiencing little interference from many common laboratory reagents. It was found that obtaining accurate results depended upon the cleanliness of the test tubes. Glass cuvettes were found to be the best, since the dye binds to quartz cuvettes, and slight dye binding was also observed in plastic cuvettes.

The dye solution was prepared by dissolving 100 mg of Coomassie brilliant blue G.250 (Sigma) in 50 ml 95% (v/v) ethanol and 100 ml 85% (v/v) phosphoric acid, diluted to a final volume of one litre with distilled water.

The protein assay was carried out by the addition of 100 μl of enzyme solution to 5 ml of the dye solution. The solutions were mixed and after 5 min. the absorbance at 595 nm was measured. Bovine serum albumin (Sigma) was used as a standard and all protein determinations were carried out in triplicate.

2.3.2 Modified method of Lowry (Peterson, 1977).

The Lowry method is sensitive, but it is subject to interference by many reagents. To counteract this problem, Peterson (1977) showed that precipitation of protein by a mixture of sodium deoxycholate and trichloroacetic acid prior to the Lowry assay, greatly reduced interference by various reagents, (e.g. Tris). In this work the method of Peterson (1977) was found to be more reliable and accurate than other techniques, and was used throughout except where stated otherwise.

2.4 PROTEASE ASSAY METHODS

2.4.1 Casein-agar plate.

The casein-agar plate assay is a very sensitive, rapid, and inexpensive technique for the detection and semi-quantitative assay of proteases. This method is particularly convenient for preliminary screening of large numbers of bacterial cultures and was used during the screening of *Thermus* strains. In the casein-agar plate the presence of protease is inferred by a ring of precipitated p-casein around the protease sample well (Cowan and Daniel, 1982b; Montville, 1983).

The plate diffusion method has been used for the screening of novel bacterial phenotypes (Kiyohara, *et al*, 1982) and the screening and detection of bacteriocin producer strains, (Tagg, *et al*, 1976). Recently, Cowan and Daniel (1982b) have compared the sensitivity of the casein-agar plate assay for mesophilic and thermophilic proteases at high temperatures (55° - 75°C). They found that sensitivity of the assay is significantly increased at high temperatures.

By comparing the results with those from inhibitor-free plates, this method enabled the rapid and efficient identification of different types of protease.

A mixture of 0.2% (w/v) casein and 2% (w/v) agar in distilled water was prepared. It was then heated to just below boiling point with constant stirring. The pH was adjusted to 7.5 at about 75°C and 0.01% (w/v) sodium azide was added to prevent bacterial growth. Portions of 20 ml were poured into plastic petri-plates. The gel was allowed to solidify at room temperature before the plates were inverted and stored at 4°C.

Various concentrations of different protease inhibitors (EDTA, EGTA, etc.) were also incorporated in the casein-agar plates. As an alternative to the casein-agar plate, a variety of other protein substrates (collagen, fibrin elastin, etc.) and chromogenic protein substrates (Azocoll, Azo-casein, Azo-albumin and Elastin-Congo Red) were incorporated into agar-plates (e.g. 0.2% (w/v) Azocoll and 2% w/v) agar).

The assay method for protease detection was carried out by cutting 6 - 8 wells (6mm diameter) in each plate and filling them with 50 µl of each protease sample. The plates were left at room temperature for about 30 minutes during which period the enzyme diffused into the gel. The plates were then wrapped in Gladwrap (R) and incubated at 70 - 75°C for 8 - 12 hours. When protease standards (trypsin, chymotrypsin, etc.) were used, the plates were incubated at 50°C overnight. During incubation a milky ring composed of partially hydrolysed casein was formed around each well. The diameter of each ring was measured and compared with the control.

2.4.2 Quantitative Assay Condition for Caldolase (Kunitz method)

The Kunitz method (1947) in which casein is used as a substrate, has been widely employed for protease determination. In this assay the degree of hydrolysis is determined by the measurement of trichloroacetic acid (TCA) soluble material at 280nm following removal of precipitated protein.

The Kunitz method adopted by Cowan and Daniel (1982b) for proteases from extreme thermophiles was used with slight modifications. The substrate was prepared by dissolving 0.2% casein (Hammersten) in 0.1 M Tris acetate pH8.0; the suspension was continually stirred in a 75°C water bath until dissolved.

2ml volumes of substrate were pre-incubated in a 15ml glass centrifuge tube for 3 - 5 min. in a thermostatted 75°C water bath. The reaction was initiated by the addition of 100µl of enzyme to the substrate and continued for an appropriate period of time. Proteolysis was terminated by the addition of 3ml of 5% TCA. The samples were left at room temperature or on ice for at least 10 minutes, after which all tubes were centrifuged at 4000 rpm (using MSE centrifuge) for 10 minutes. All protease assays were carried out in duplicate or triplicate. The absorbance of each supernatant was measured at 280 nm against a distilled water blank.

The $\Delta A280min^{-1}$ was converted into µg tyrosine released per minute by comparison with a standard tyrosine curve, assuming that tyrosine is responsible for all absorption.

Proteolytic activity was expressed as proteolytic units (PU), where: 1 PU = 1 µg tyrosin released $minute^{-1}$ from 0.2% casein at 75°C. Specific activity was defined as PU per mg protein (PU/mg^{-1} enzyme).

3. SCREENING FOR PROTEASE ISOLATION AND SELECTION OF *THERMUS* STRAIN TOK_3

3.1 EXTRACELLULAR ENZYME PRODUCTION

The production of extracellular enzymes has recently been reviewed by several authors. (Glenn, 1976; Ramaley, 1979; Davis and Tai, 1980; and Priest, 1983).

Pollack (quoted by Ramaley, 1979) has defined extracellular enzymes as "those enzymes which are present in the medium around the cell, having originated from the cell without any alteration to the cell's normal processes of growth and production", and distinguished extracellular from both cytoplasmic enzymes (released by cell lysis) and surface-bound enzymes.

Although some gram-negative bacteria secrete enzymes, the majority of the wild-type strains retain these enzymes in the periplasmic space. Gram-positive bacteria on the other hand, release a large proportion of their extracellular enzymes into the medium (Costerton *et al*, 1974). This is partly due to the cell-wall complexity of gram-negative bacteria compared to gram-positive bacteria (Ramaley, 1979). Conversion of the gram-negative bacteria to sepharoplasts or osmotic-shock treatment of the cells releases these periplasmic enzymes into the medium without release of cytoplasmic enzyme. Release of gram-negative periplasmic enzymes has been observed in a mutant of *Salmonella typhimurium* which is defective in its membrane lipopolysaccharide (Ramaley, 1979).

Since both periplasmic and extracellular enzymes are located outside the bacterial cell's cytoplasmic membrane, Glenn (1976) has suggested that the term "extracellular"

should be applied to both.

Extracellular enzymes are generally considered to be synthesised on membrane-bound ribosomes. Davis and Tai (1980) have suggested the signal sequence theory in which the proteins are synthesised as a large precursor with an additional hydrophobic N-terminal signal sequence. It is this signal sequence that is responsible for the interaction between the nascent polypeptide chains and the cytoplasmic membrane. Following this process, cleavage of the signal sequence by a membrane-bound enzyme occurs. This permits release of the proteins to their final active form.

3.2 SCREENING FOR PROTEASE ISOLATION

3.2.1 Introduction.

To date, a limited number of proteases from extremely thermophilic bacteria have been reported and investigated, (Heinen and Heinen, 1972; Matsuzawa *et al*, 1983; Taguchi *et al*, 1983). Only Caldolysin the extracellular protease from *Thermus aquaticus* strain T351 (Cowan and Daniel, 1982a), has been subjected to detailed characterisation.

The initial object of this research project was to isolate and characterise an extracellular serine protease from an extremely thermophilic bacterium. It was intended that such a protease should be different from existing thermophilic proteases worked on in this laboratory and to this end chelator insensitivity was sought in addition to thermostability and high enzyme productivity.

A screening programme was devised and conducted to

analyse the laboratory stock cultures and hot spring samples from the Rotorua thermal region (New Zealand).

The initial study consisted of three basic steps:

(i) isolation of a pure bacterial culture

~~(ii) detection of protease~~

(iii) determination of the type of protease.

3.2.2 Methods

3.2.2.1 Screening of Laboratory Stock Cultures

3.2.2.1.1 Aerobic culture

Each laboratory stock culture was initially grown in 200 ml Erlenmeyer flasks containing 50 ml of *Thermus* medium (Hickey and Daniel, 1979) (Table 3.1) or Oshima medium (Oshima and Imahora, 1974) (Table 3.2). The flasks were incubated in a Gallenkamp orbital incubator set at 70° or 75°C and 200 revolutions per minute for 24 - 48 hours. Each sample was then subcultured onto agar plates (agar plates were prepared by adding 2% agar to the *Thermus* medium or Oshima medium). A single colony from each plate was then transferred into a 200 ml flask containing 50 ml *Thermus* medium and incubated at 70° or 75°C overnight.

3.2.2.1.2 Detection of protease

A 10 ml culture sample was centrifuged at 4000 rpm for 10 minutes using a MSE centrifuge, and the cell-free supernatant assayed for proteolytic activity by the Kunitz method and casein-agar plate method. (See general methods, Chapter 2, for further description).

3.2.2.1.3 Determination of the type of protease

This was carried out as described in the general methods (Chapter 2) using both the casein-agar plate containing various protease inhibitors (Fig. 3.1) and the agar-plate incorporating a variety of native and chromogenic proteins (Refer Chapter 6, Fig. 6.1 and 6.2).

3.2.2.1.4 Anaerobic cultures

Thermophilic anaerobes were grown in Zeikus medium (Zeikus, 1979). Each culture was inoculated into the medium in a McCartney bottle under nitrogen gas, sealed with a rubber lined metal screw cap and incubated at 70° or 75°C for 24 to 48 hours. Protease activity detection and determination of the cell-free supernatant of each sample was carried out as described in the general methods (Chapter 2).

3.2.2.2 Screening of Hot Spring Samples

To increase the possibility of locating a protease which fulfilled the specified requirements, a number of water samples from thermal pools were screened. Samples were collected from pools in the Rotorua thermal region (New Zealand). Pool temperatures and pH values ranged from 60° to 102°C, and pH 6.57 - 8.6, respectively.

In the laboratory, 10 ml from each hot pool sample were added to 100 ml of either *Thermus* medium or Oshima medium. The inoculated media were then incubated at 75°C for 24 to 48 hours and a loopful of the turbid culture streaked on solidified medium. Single colonies were selected and grown,

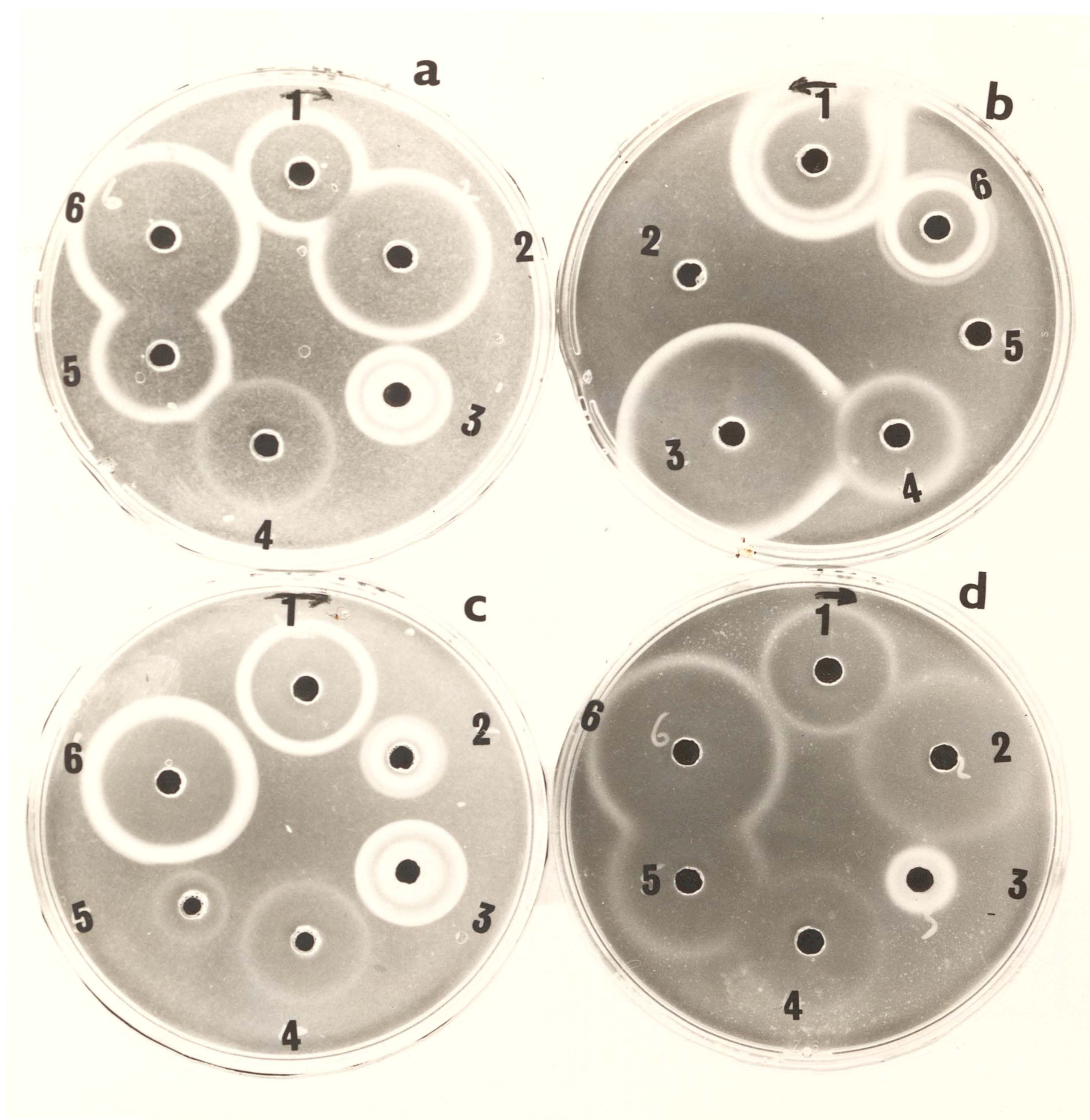


Fig. 3.1 Zones formed on casein-agar plates containing, no inhibitor (A), EDTA (B), EGTA (C) and Iodo acetic acid (D) after 18 hours of incubation at 50^{0}C with Caldolase at 12 μgml^{-1} (1), Thermolysin at 40 μgml^{-1} (2), Papain (3), Trypsin (4), a metal protease from *B. polymyxa* (5), and (6) subtilisin BPN′ each at 80 μgml^{-1}.

extracellular protease was determined using methods previously described (Section 3.2.2.1).

3.2.3 Results

Over one hundred aerobic and anaerobic laboratory stock cultures and hot spring samples were investigated for protease production. When the screening process was completed fourteen promising protease-producing strains had been isolated (Table 3.4), including isolate Tok_3. For the growth and protease production of both laboratory stock cultures and direct hot pool cultures, *Thermus* medium proved to be more suitable than Oshima medium. No significant protease producer was found in the anaerobes. Extracellular protease from isolate Tok_3 showed a greater resistance to chelating agents (EDTA and EGTA) than either Thermolysin (Voordouw and Roche, 1975b) or Caldolysin (Cowan and Daniel, 1982a) and for this reason it was selected for further study.

It was found that the following methods in conjunction with each other form an excellent technique for the screening and isolation of a new protease when dealing with a large number of micro-organism strains:

i Plate assay diffusion using various protein substrates

ii The casein-agar plate incorporating various protease inhibitors.

Table 3.1 The Standard *Thermus* Medium

(From Hickey & Daniel, 1979)

Component	Gram/litre
Yeast extract (Difco)	3.0
Trypticase peptone (B.B.L.)	3.0
Ammonium sulphate	1.30
Magnesium sulphate	0.25
Calcium chloride	0.074
Sodium dihydrogen phosphate	0.078
Trace elements (x1000 Concentrate)	**1 ml /litre**
$FeCl_3.6H_2O$	0.019
$MnCl_2.4H_2O$	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

The medium was heated to 75°C while adjusting the pH to 7.5 with 10N NaOH before autoclaving at 121°C for 15 minutes. The medium was then cooled to 75°C and inoculated with the bacterial sample.

Agar Plate:

Preparation of agar plates involved the dissolution by heating of 2 percent agar in the *Thermus* or Oshima medium. The pH was adjusted to 7.5 and then the mixture was autoclaved. After cooling to about 60°C, 20 ml aliquots were dispersed into plastic petri dishes. After solidification of the agar, the plates were inverted and stored at 4°C.

Table 3.2 Oshima Medium (Oshima and Imahori, 1974)

Component	Gram/litre
Yeast extract (Difco)	4.0
Trypticase peptone (B.B.L.)	8.0
Sodium chloride	3.0

The mixture was heated while adjusting the pH to 7.4 with 10N NaOH. The medium was then autoclaved at 121°C for 15 minutes before cooled to 75°C and inoculated with the bacterial sample.

Table 3.3 Zeikus Medium (Zeikus, 1979)

Component	gram/litre
Yeast extract	3.0
Trypticase peptone	10.0
NH_4Cl	1.0
$MgCl_2.6H_2O$	0.2
KH_2PO_4	0.3
$Na_2HPO_4.7H_2O$	2.0
Sodium thioglycollate	1.0
2.5% $FeSO_4$	0.03ml
2% Resazurin	1.0 ml
Trace elements (X1000)	**1ml/litre**
$FeCl_3.6H_2O$	0.019
$MnCl_2.4H_2O$	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
Vitamin Solution	5 mls
Biotin	2 mg/ml
Folic acid	2 "
Pyridoxine	10 "
Riboflavin	5 "
Thiamine	5 "
Nicotinic acid	5 "
Pantothenic acid	5 "
Vitamin B_{12}	0.1 "
P-aminobenzoic acid	5 "
Thioctic acid	5 "

The medium was heated to 75 °C while adjusting the pH to 7.4 with 10NaOH before autoclaving. The vitamin solution was filtered and added after the medium had been autoclaved at 121 °C for 15 minutes.

Table 3.4 Protease Producing Isolates.

Isolate	Protease activity	Milk clotting activity *	E. coli lysis *
OK_6	+ + +	+	+ + + +
OK_7	+ + +	-	+ + +
Tok_3	+ + +	-	+ + + +
OK_8	+ + +	+	+ + +
L	+ + +	+ +	+ + +
N	+ + +	+	+
E	+ + +	+ + + +	+ + + +
RT_6	+ +	-	+ + +
Tok_8	+ +	-	+ + +
Tok_4	+ +	-	+ + +
OK_5	+	+	+ +
OK_9	+	-	+
TP	+	+ +	+
OK_2	+	+	-

* = data from D. Cowan (unpublished results)

mm = The diameter of casein hydrolysis or E.coli lysis in the plate assay.

+ + + + = 18 mm

+ + + = 15 mm

+ + = 10 mm

+ = 7 mm

- = No casein hydrolysis, E.coli lysis or milk clotting activity.

3.3 STRAIN TOK_3 GROWTH AND MAINTENANCE

Strain Tok_3 was isolated from a hot pool (designated Pool 6) in the Tokaanu thermal region (New Zealand) and purified as described previously.

A pure culture of the bacterium was grown for 24 hours at 75°C on *Thermus* agar plates. Approximately 1.0 ml of suspending medium (see below for composition) was pipetted onto the surface of the plate and mixed with the bacterial cells. This cell suspension was pipetted into the base of previously sterilised 0.5 ml glass ampoules containing an identifying strip of filter paper typed with the date and number of the culture. The tubes were immersed in liquid nitrogen to freeze the contents then attached to adaptors on a virtis freeze-drying machine (Freeze mobile 3). The tubes were then sealed under vacuum and stored both at room temperature and at 4°C.

Reconstitution was carried out by inoculating 50 ml of *Thermus* medium with the contents of an ampoule and incubating at 75°C.

Table 3.5 Suspending Medium

Component	g/100 ml H_2O
Meso-inositol	5.0
Beef extract	10.0
Peptone	10.0
NaCl	1.0

The pH of the above mixture was adjusted to 7.5 and autoclaved at 121°C for 20 minutes.

3.4 MORPHOLOGY OF TOK_3 ISOLATE

Tok_3 isolate was an aerobic, gram-negative, non-motile, non-sporulating, yellow-pigmented, rod-shaped bacterium. Filamentous morphology was also observed (Fig. 3.3). The size of rods was about 0.5 x 0.7 - 8 µm with filaments up to 40 µm long. These properties are typical of the genus *Thermus*.

3.5 *THERMUS* STRAIN TOK_3 GROWTH RATE AND PROTEASE PRODUCTION

3.5.1 Method:

Cultures of *Thermus* strain Tok_3 were grown aerobically in *Thermus* medium at 75°C for 50 hours. At two hourly intervals, samples were aseptically removed and the absorbance measured at 650 nm. The protease activity of the cell-free supernatants was measured using the Kunitz method.

3.5.2 Results:

The maximum enzyme production was at the end of the logarithmic growth phase of the bacteria while at the stationary phase enzyme production diminished significantly and protease activity gradually declined (Fig. 3.2).

3.6 EFFECT OF AERATION ON GROWTH RATE AND PROTEASE SECRETION

That aeration has a significant effect upon both the growth rate and the enzyme production of *Thermus* strain Tok_3 was demonstrated by the results obtained when varying quantities of the bacteria were grown in 2 litre flasks. When batches of 500 ml and 1000 ml of the bacteria were grown separately in 2 litre flasks, the cell growth and protease

secretation of the 500 ml batch were more rapid than those of the 1000 ml batch. (Fig. 3.2) Similar results were obtained when *Thermus* strain Tok_3 was grown at 80°C. (Table 3.6).

Table 3.6 *Thermus* Strain Tok_3 Growth Rate at 80°C.

Culture Volume (ml)	A650 (0 time)	A650 (24h)
500	0.102	0.844
1000	0.105	0.476

A very low level of protease activity was detected in the cell-free supernatant of the *Thermus* strain Tok_3 culture grown at 80°C. This is presumably due to autolysis.

Attempts to grow the bacteria above 80°C were unsuccessful.

3.7 ANAEROBIC GROWTH OF *THERMUS* STRAIN TOK_3

To test whether *Thermus* strain Tok_3 is a facultative aerobe, attempts were made to grow it anaerobically (see section 3.2.2.1.4). There was no growth under these conditions.

The above results support that *Thermus* strain Tok_3 is an aerobic bacterium.

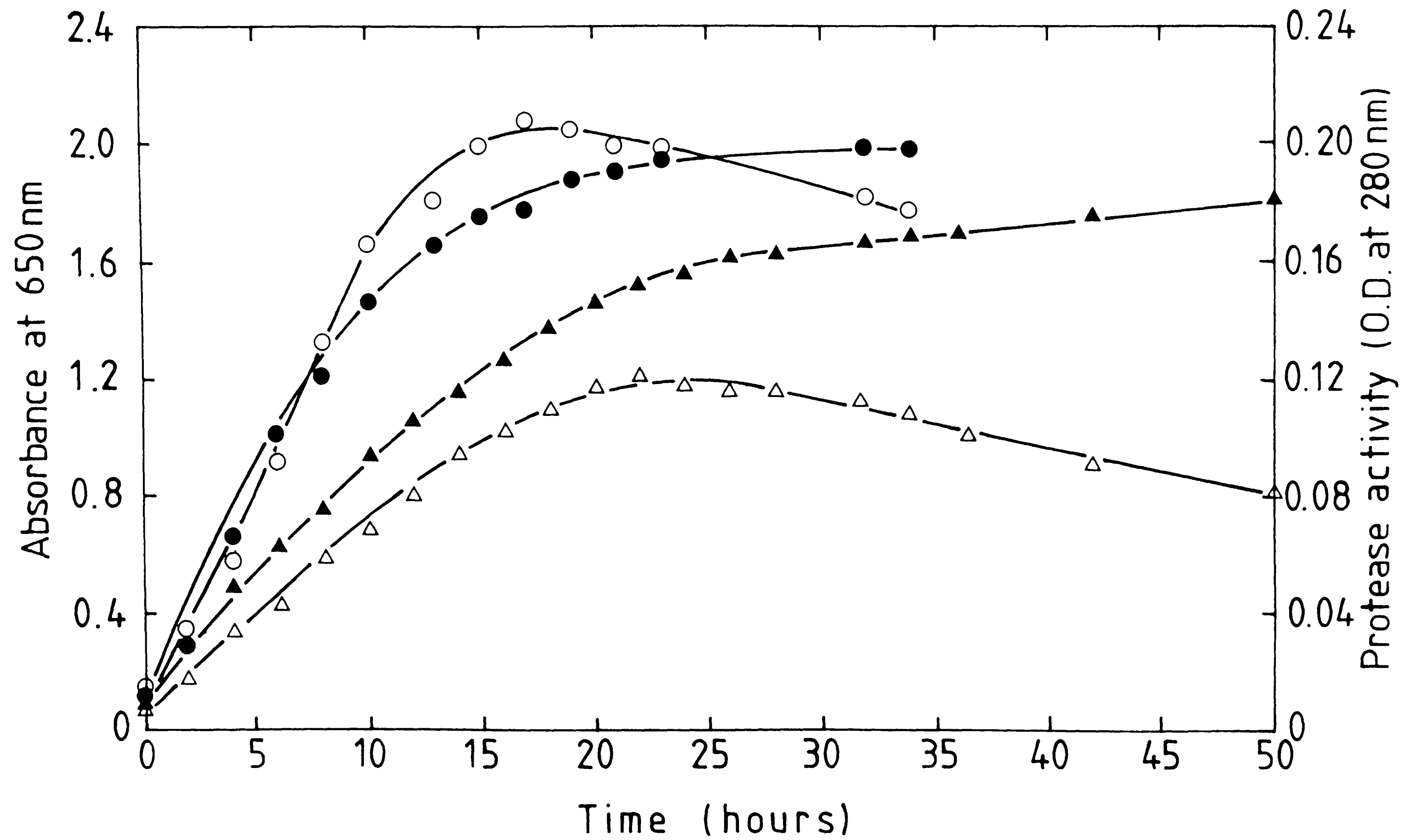


FIGURE 3.2 *THERMUS* STRAIN TOK$_3$ GROWTH RATE AND PROTEASE PRODUCTION.

Cultures of bacteria were grown aerobically in *Thermus* medium at 75°C in 500ml and 1000ml batches in 2 litre flasks.
500ml batch absorbance at 650 (●), protease activity (○)
1000ml batch - absorbance at 650 (▲), protease activity (△).

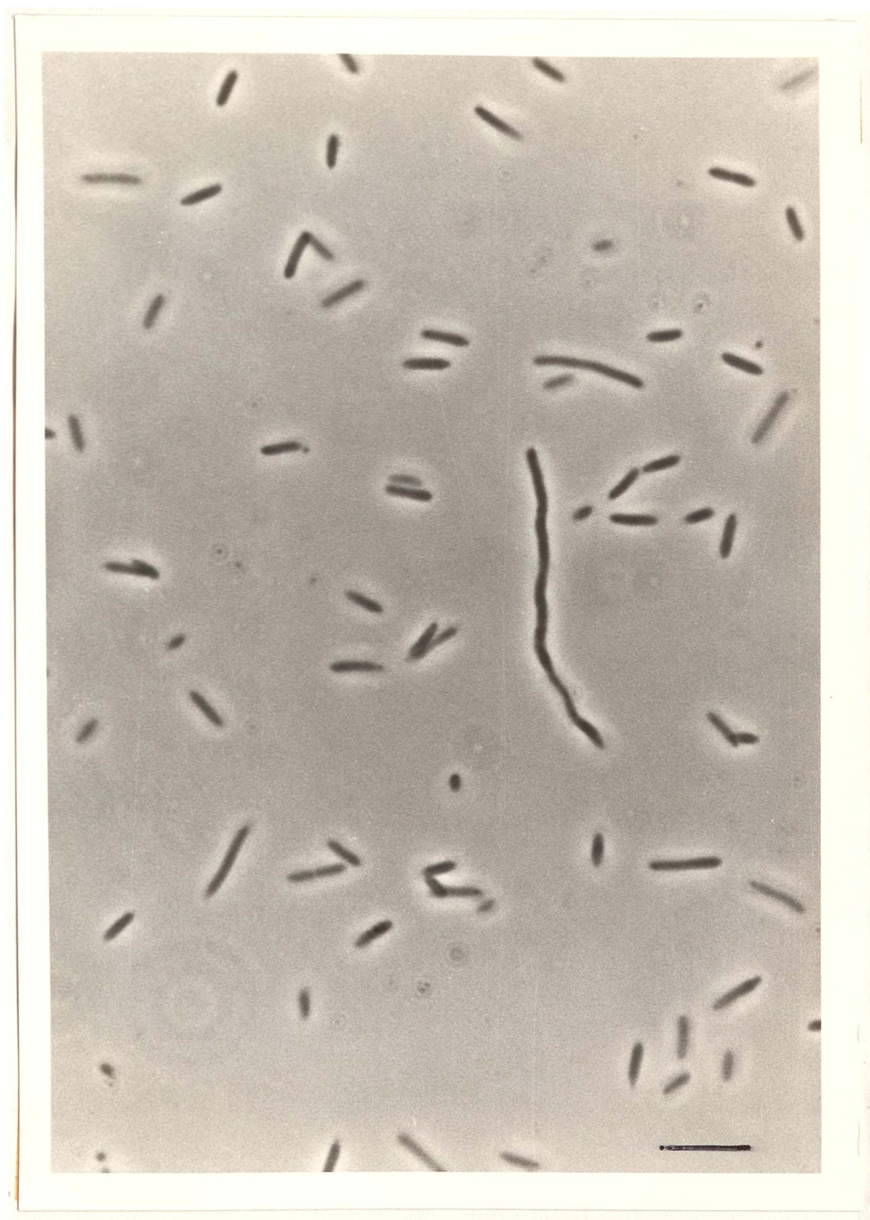


FIGURE 3.3 PHASE CONTRAST PHOTOMICROGRAPH OF *THERMUS* TOK_3 STRAIN.
BAR REPRESENTS 10 μm.

3.8 *THERMUS* STRAIN TOK_3 INTRACELLULAR PROTEASE PRODUCTION

3.8.1 Method:

With a view to evaluating intracellular protease production, a 500 ml volume of the bacteria was grown as described previously. After 24 hours the cell density at 650 nm reached 1.7. The *Thermus* strain Tok_3 cells were harvested by centrifugation (Sorvall RC-2B centrifuge) in the type GSA rotor at 4000g for 30 minutes. The cell pellet was washed twice with 0.1M Tris acetate pH8.0 containing 0.5M NaCl and 10 mM $CaCl_2$, then resuspended in 25 ml of the above buffer, and sonicated for 30 minutes in ice, using a Banson Sonifier, Model B-12. The sonicated sample was then centrifuged at 17,000g in a SS34 rotor for 30 minutes to remove the cell particles. The supernatant was assayed for protease activity by the Kunitz method.

3.8.2 Results:

The results demonstrated that the intracellular protease content was less than 2% of that of extra cellular enzyme.

Extracellular protease = 103000 PU/500ml cell-free supernatant.

Intracellular protease = 1530 Pu/25ml suspension of the sonicated cells obtained from the 500ml bacterial culture.

4. PREPARATION AND PURIFICATION OF THE ENZYME

4.1 DETERMINATION OF PURIFICATION METHOD.

A variety of purification methods were tested in the development of purification protocol for Caldolase.

4.1.1 Ammonium Sulphate Fractionation

4.1.1.1 Method

In order to find the % ammonium sulphate saturation range for enzyme precipitation, two trials were carried out. 60g of ground ammonium sulphate was slowly added with vigorous stirring to 200ml of ice-cold *Thermus* strain Tok_3 cell-free supernatant. The solution was then centrifuged at 16,000g in the Sorvall super-speed RC2-Btype GSA rotor for 30 min. Precipitated proteins was redissolved in 0.1 M Tris acetate pH8.0. Protease activity of both redissolved precipitate and supernatant was determined by the Kunitz method. The ammonium sulphate concentration was sequentially increased to 45, 60 and 70% saturation and in each case the same procedure was followed. In a second trial, ammonium sulphate was dissolved to give saturation levels of 35, 50, 65 and 80%.

4.1.1.2 Results and discussion.

In the first series most protease precipitated in the 45 - 60% saturation range of ammonium sulphate while in the second trial the enzyme recovery was in 50 - 65% (Table 4.1). The results revealed that in the 75% saturation range total enzyme precipitation occurred. It was decided that 70% saturation of ammonium sulphate would be sufficient for Caldolase precipitation and for the routine purification to be used.

Table 4.1 Ammonium Sulphate Fractionation.

$(NH_4)_2SO_4$ Saturation %		Protease Activity %	
		Precipitate	Supernatant
First trial	30	6	93
	45	38	54
	60	44	11
	75	10	1
Second trial	35	15	85
	50	33	50
	65	42	9
	80	9	0

4.1.2 Ultrafiltration

4.1.2.1 Method

One litre of the cell-free supernatant was concentrated to a volume of 67ml using a millipore ultrafiltration unit equipped with a peristaltic pump and a 5sq.ft. membrane with 10,000 molecular weight exclusion.

4.1.2.2 Result

No protease activity was detected in the filtrate. Only 56% of the total initial enzyme activity was detected in the concentrate. The rest was presumably bound to the membrane. In this case enzyme loss due to autolysis must also be taken into consideration. The result indicates that ultrafiltration is an inefficient method for the concentration of Caldolase.

4.1.3 Ion-exchange Chromatography.

4.1.3.1 Methods.

A 5ml volume each of the ion exchange gels DEAE-cellulose, QAE-A25 Sephadex, SP-C25 Sephadex, DEAE-cellulose ~~(N.Z.), DE53 Cellulose, was packed in a separate 10ml syringe~~ or a small column (K16). The columns were then equilibrated with an appropriate buffer (pH ranging from 6 - 9.5). A 5ml sample of enzyme solution (previously dialysed to remove excess salt) passed through each column, which were then washed with the equilibrating buffer to remove unbound proteins. Adsorbed proteins were eluted by 1M NaCl in the buffer. The protease activity and protein concentration of each fraction were determined (see Chapter 3 for methods).

4.1.3.2 Results and Discussion

The results revealed that a large amount of protein and coloured material but little of the enzyme bound to the ion-exchangers used (Table 4.2). The ion-exchange method proved to be an effective purification method without significant loss of enzyme. The technique was a very useful precursor to the affinity chromatography step, where a high concentration of non-enzyme protein and coloured material tended to interfere with protease-binding capacity of the affinity gel.

Table 4.2 Ion-exchange Chromatography.

Gel	Bound protein %	Enzyme Recovered (unbound)	
		Total PU	%
Original enzyme	-	818	100
DEAE-Cellulose (Sigma)	94	711	87
QAE-Sephadex	79	658	82
SP-C25 Sephadex	38	646	79
DEAE-Cellulose (Phoenix Chemicals, N.Z.)	59	650	79
DE53-Cellulose	80	642	78

4.1.4 Adsorption Chromatography

4.1.4.1 Methods

(i) Affinity Chromatography

Affinity chromatography is one of the most powerful techniques for enzyme purification. The following affinity gels were tested: Carbobenzoxy-D-phenylalanyl-triethylene tetraminyl-Sepharose-4B (CBZ-D-phe-TETA-Sepharose-4B (Pierce), 4-Phenylbutylamine-11-Sepharose-4B (Pierce) and Aminocaproyl-p-aminobenzamidine-11-Sepharose-4B (Pierce). One ml from each gel was packed in a small glass column. The column was equilibrated with 0.1 M Tris acetate buffer pH 7.0 containing 10mM $CaCl_2$. 3 ml of enzyme solution (554PU) was applied to each column, followed by washing with the above buffer. The adsorbed material was then eluted with 0.1 M acetic acid pH2.8 (Stevenson and Landman, 1971). The gels were generated with 0.1 M Tris acetate buffer pH 7.0 containing 10mM $CaCl_2$. The

protein concentration and protease activity of each fraction were measured.

(ii) Hydrophobic interaction chromatography

Hydrophobic interaction chromatography is particularly applicable to enzyme solutions which contain a high salt concentration (Ochoa, 1978). Elution of the bound enzyme is frequently achieved by decreasing the ionic strength or decreasing the polarity of the eluent (e.g. with ethylene glycol).

Two hydrophobic gels, Octyl-Sepharose CL-4B and Phenyl Sepharose CL-4B, were tested. The method of Pharmacia Fine Chemical Company (Separation News, 7, 1980) was followed.

One ml gel was packed in a small glass column. 3ml enzyme solution (554PU) in 1M ammonium sulphate was passed through the column, which was then washed with distilled water, 50% and 100% ethyleneglycol. The protease activity of all fractions was determined.

(iii) Immobilised Tannin

Immobilised tannin in the form developed by Tanabe Seigaku Co. Ltd, was tested for Caldolase adsorption.

Using the method of Watanabe *et al*, (1981), one gram of immobilised tannin was packed into a small column. After washing with water, the column was equilibrated with 0.05M sodium phosphate buffer, pH7.5. 3ml enzyme solution (554PU) in the same buffer was applied to the column. After washing the column with the buffer, the adsorbed material was eluted with 0.05M carbonate buffer pH10.0 and subsequently with 0.5M acetate buffer pH4.0. Protease activity of all fractions was assayed.

(iv) Matrex Gel Dye-ligand affinity Chromatography.

A matrex gel dye-ligand kit supplied by Amicon Company was used. Amicon procedure (1981) was followed in this study.

Each matrex gel dye-ligand was first regenerated by washing the column with 12ml of 8M urea (to remove the free dye and tightly bound or precipitated protein remaining from previous runs). The column was then equilibrated by washing with 12ml of 20mM Tris/HCl pH7.5. A 3ml enzyme solution (661PU) was added and allowed to drain. The unbound enzyme was eluted by the addition of 10ml of 20mM Tris/HCl pH7.5 containing 1.5MKCl. The enzyme activity of all fractions was assayed by the standard Kunitz method.

4.1.4.2 Results and Discussion.

The gels tested showed a wide range of adsorption capacities for Caldolase (Table 4.3 and 4.4). When phenyl-sepharose CL-4B was used, enzyme recovery was nil, probably due to tight binding of the enzyme to this gel.

The adsorption and recovery of Caldolase from the majority of affinity gels were unsatisfactory. The sole exception was CBZ-phe-TETA-Sepharose from which the total enzyme applied was adsorbed and then recovered (Table 4.3). In the case of Matrex Gel-dye ligands, the highest binding capacity was displayed by Blue A and Red A (Table 4.4). These gels compared unfavourably with the adsorption capacity of other affinity gels and were discarded.

In the final evaluation, CBZ-phe-TETA Sepharose-4B proved to be the most effective affinity gel, and was routinely used for the purification of the enzyme.

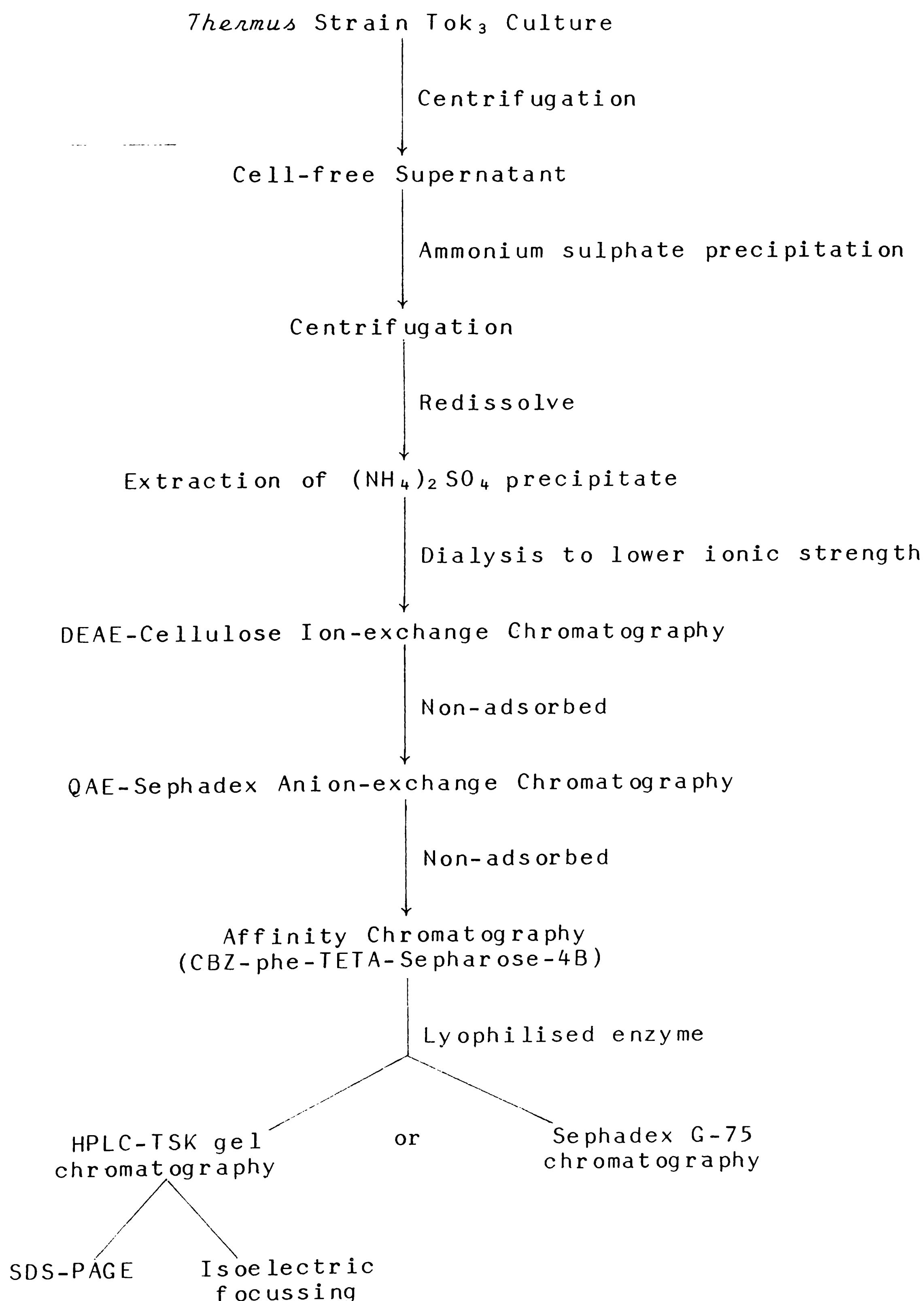
Table 4.3 Affinity Chromatography

Gel	Enzyme Activity Percentage Unbound	Tris wash	Elution buffer wash
BCZ-phe-TETA-Sepharose-4B	0	0	99
4-Phenylbutylamin-11-Sepharose-4B	36	9	47
Aminocarproyl-P-aminobenzamidine-11-Sepharose-4B	41	10	32
Octyl-Sepharose CL-4B	23	12	38
Phenyl-Sepharose CL-4B	0	0	0
Immobilised Tannin	71	8	14

Table 4.4 Matrex Gel Dye-ligand Affinity Chromatography

Gel	Enzyme Activity Percentage Unbound	Tris-wash	Tris-KCl wash
Gel control	88	9.6	0
Blue A	41	19	36
Orange A	75	15	5
Red A	42	13	42
Green A	52	19	22
Blue B	66	15	5.5

OUTLINE OF PROTEASE PURIFICATION



4.2 PURIFICATION SEQUENCE OF CALDOLASE

4.2.1 Preparation of Enzyme Stock

Since the production of extracellular protease by *Thermus* strain Tok_3 was found to be low, large scale bacterial growth was necessary.

A 600 l culture of *Thermus* strain Tok_3 was grown in an 800 l fermenter. Sequential inocula were prepared from a pure bacterial culture using 10% inocula as follows:

Thermus medium was prepared in 100ml amounts in 250ml Erlenmeyer flasks, 1000ml amounts of 2 l flasks, and 20 l amounts in 25 l Quickfit flasks. The latter were autoclaved for 1½hours at 121°C, equilibrated to 75°C in a water bath and filled with previously sterilised adaptors containing sintered glass aerators connected to a sterilised air supply. Condensers were used to minimise evaporation. The fermenter was sterilised with 0.2% formaldehyde, then rinsed 3 times with tap water run through a 0.22µm bacterial filter (Sartobran Capsule). At each inoculating step the culture was gram stained to check purity. The large fermenter was then made up to approximately 600 l of filtered tap water and 20 l concentrated *Thermus* medium, the pH adjusted to 7.5 with 10N NaOH and the temperature brought up to 75°C. After inoculation with 60 l of exponentially growing *Thermus* strain Tok_3, the temperature and pH of the fermenter culture was monitored hourly.

Growth rate was determined by the removal of a sample and measurement of the absorbance at 650nm. At the same time protease activity was measured by the Kunitz method. After 13 hours incubation the culture was harvested by continuous flow centrifugation through a Sharples Model 6 centrifuge

at 17000g and 5 l min^{-1}. The resulting cell-free supernatant was collected in 180 l drums. Protein was precipitated by the addition of ammonium sulphate to 70% saturation and collected after 48 hours by centrifugation as above.

4.2.1.1 Results.

The enzyme secretion reached its maximum level at the late-exponential phase after 9 hours, but had only declined slightly by 13 hours. (Fig.4.1).

4.2.2 Extraction of Ammonium Sulphate Precipitate

The crude extracellular protease obtained from the fermenter was suspended in one litre of 0.1M Tris acetate, pH7.0. The mixture was then homogenised using a Waring blender at high speed for 2 - 3 min. and left overnight at 4 °C. The suspension was then centrifuged at 10,000g in a type GSA rotor using Sorvall RC-2B centrifuge for 30 min. The supernatant containing the major portion of protease (Table 4.5) was removed and freeze-dried or used directly for further enzyme purification. The sediment was then redissolved in 0.1M Tris acetate buffer pH7.0 and protease extraction was carried out twice more as described above. The results are presented in Table 4.1.

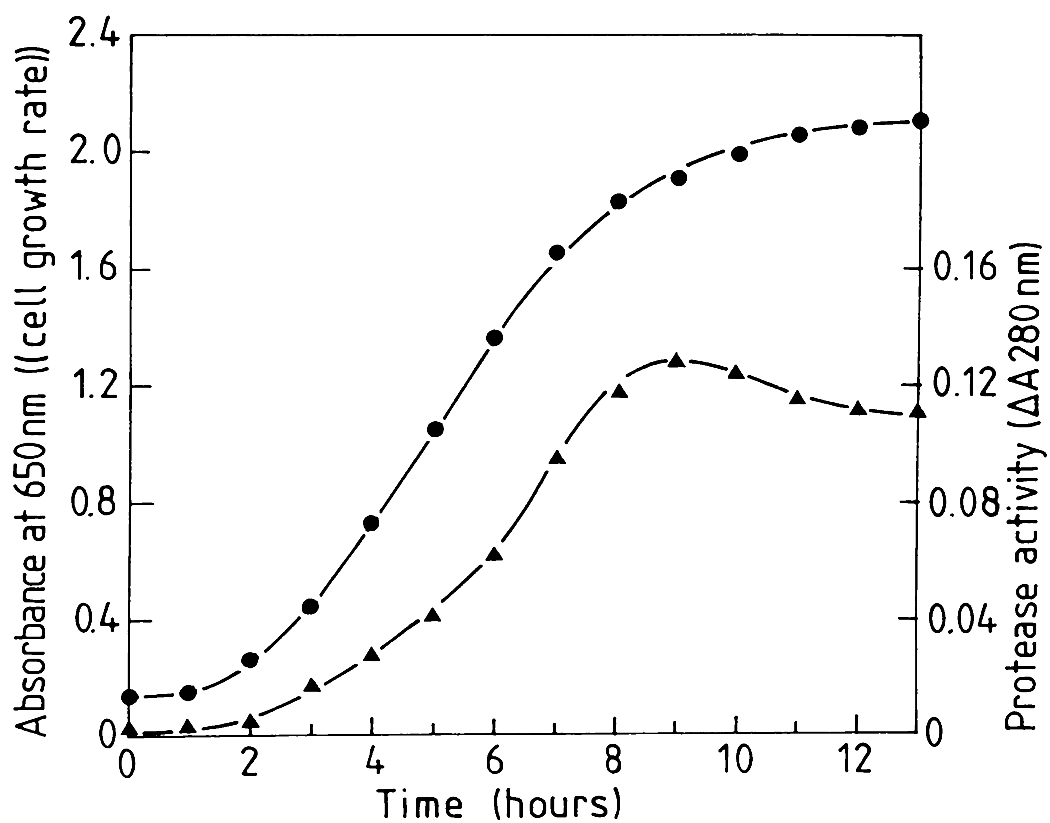


FIGURE 4.1 *THERMUS* STRAIN TOK_3 GROWTH RATE AND PROTEASE PRODUCTION USING 600 LITRE FERMENTER.

Growth rate of bacteria (●) and protease activity of cell-free supernatant (▲) were determined hourly at specified time.

Table 4.5 Extraction of Ammonium Sulphate Precipitate

Extract	Enzyme recovery P.U	Percentage
600 l Fermenter	8,491,500	100
First	2,249,100	26.5
Second	940,950	11
Third	168,300	2

4.2.3 DEAE-Cellulose Chromatography.

5g of lyophilised crude protease (the first extract from ammonium sulphate precipitate) were dissolved in 200ml of 0.1M Tris acetate pH8.0. The enzyme solution was then applied to SEAE-cellulose column (1.6 x 40cm) pre-equilibrated with 0.1M Tris acetate pH8.0 containing 10mM $CaCl_2$. Fractions of 10ml were collected by an ISCO Model UA-5 fraction collector connected to an Absorbance/Fluorescence Monitor. The column was then washed with the equilibrated buffer to remove the unbound materials. Finally the column was eluted with 0 -1.0M NaCl gradient. Protease activity of each fraction was determined using 0.2% casein in 0.2M sodium carbonate-bicarbonate buffer pH9.0. Protein was also measured by absorbance at 280nm in the spectrophotometer or by the method of Peterson (1977). (Fig. 4.2).

The non-adsorbed protein fractions were pooled and used for further purification.

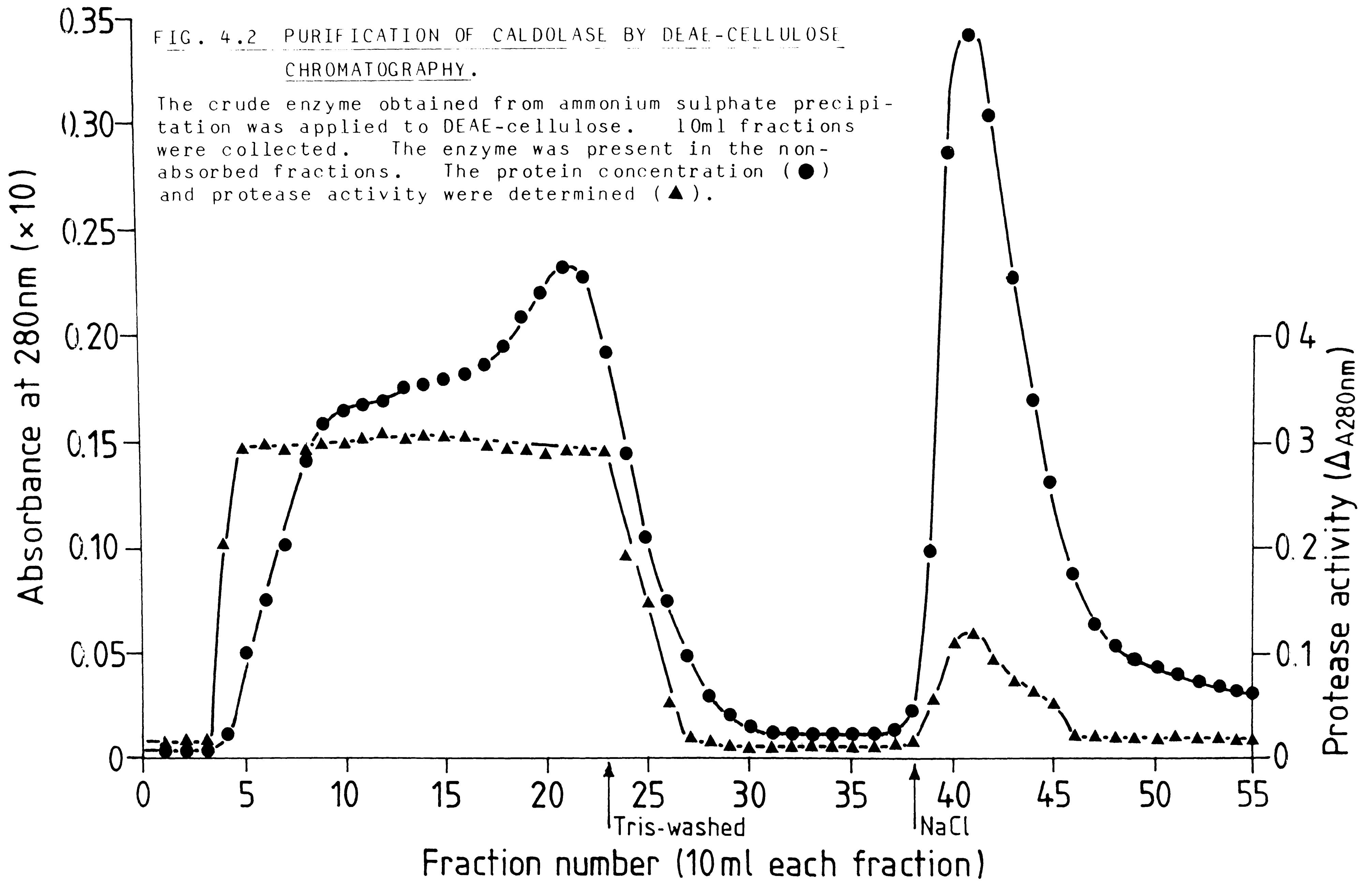


FIG. 4.2 PURIFICATION OF CALDOLASE BY DEAE-CELLULOSE CHROMATOGRAPHY.

The crude enzyme obtained from ammonium sulphate precipitation was applied to DEAE-cellulose. 10ml fractions were collected. The enzyme was present in the non-absorbed fractions. The protein concentration (●) and protease activity were determined (▲).

4.2.4 QAE-Sephadex Anion Exchange Chromatography

The pooled active fractions obtained from the DEAE-Cellulose Purification step, were applied to a QAE-Sephadex anion-exchanger following the procedure described above (Fig. 4.3). The non-adsorbed active fractions were pooled.

4.2.5 Affinity Chromatography

The pooled active fractions from QAE-Sephadex anion-exchange chromatography step were applied to a CBZ-D-phe-TETA Sepharose-4B column (30ml affinity gel in a 1.6 x 40cm column) pre-equilibrated with 0.1M Tris acetate buffer pH7.0. Fractions of 10ml were collected. The column was then washed with the equilibration buffer to remove unbound material. The protease was recovered by the addition of 0.1M acetic acid, pH2.8. The protein concentration and protease activity of all fractions were measured as above (Fig.4.4).

The active fractions were pooled and freeze-dried.

4.2.6 Sephadex G-75 Gel Filtration Chromatography

The lyophilised enzyme, obtained from the affinity chromatography step, was dissolved in 4ml of 0.1M Tris acetate pH8.0 The enzyme solution was then applied to a Sephadex G 75 gel filtration column (2.7 x 100cm) equilibrated with 0.1 Tris acetate pH8.0 containing 0.5M NaCl and 10mM $CaCl_2$. Fractions of 10ml were collected. The protease activity and protein concentration of each fraction were determined (Fig. 4.5).

When it was necessary to prepare samples of salt-free enzyme, the sephadex G-75 column was eluted with 0.1M ammonium-carbonate buffer pH8.0 (a volatile buffer which during freeze-drying process evaporates).

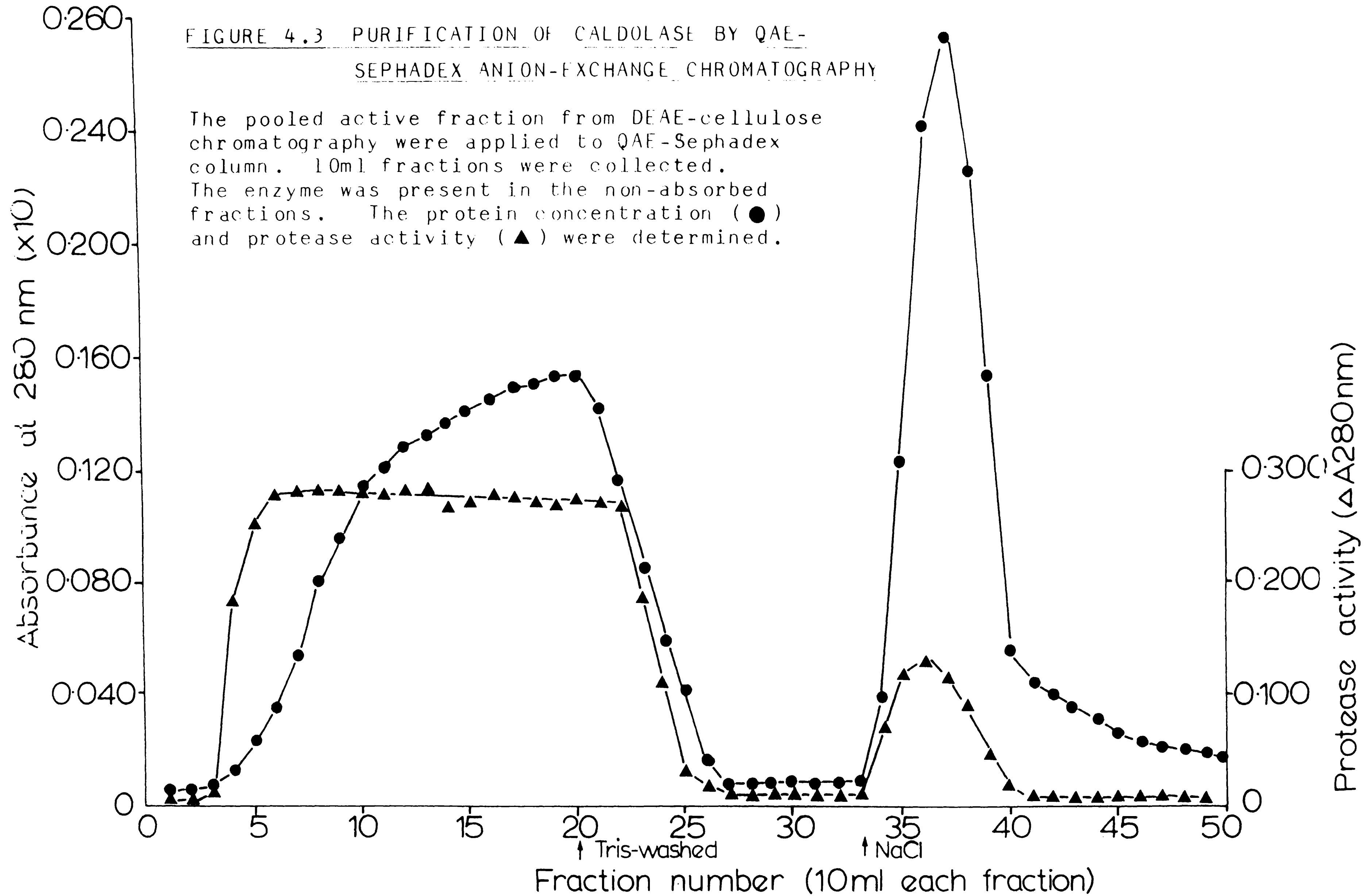


FIGURE 4.3 PURIFICATION OF CALDOLASE BY QAE-SEPHADEX ANION-EXCHANGE CHROMATOGRAPHY

The pooled active fraction from DEAE-cellulose chromatography were applied to QAE-Sephadex column. 10ml fractions were collected. The enzyme was present in the non-absorbed fractions. The protein concentration (●) and protease activity (▲) were determined.

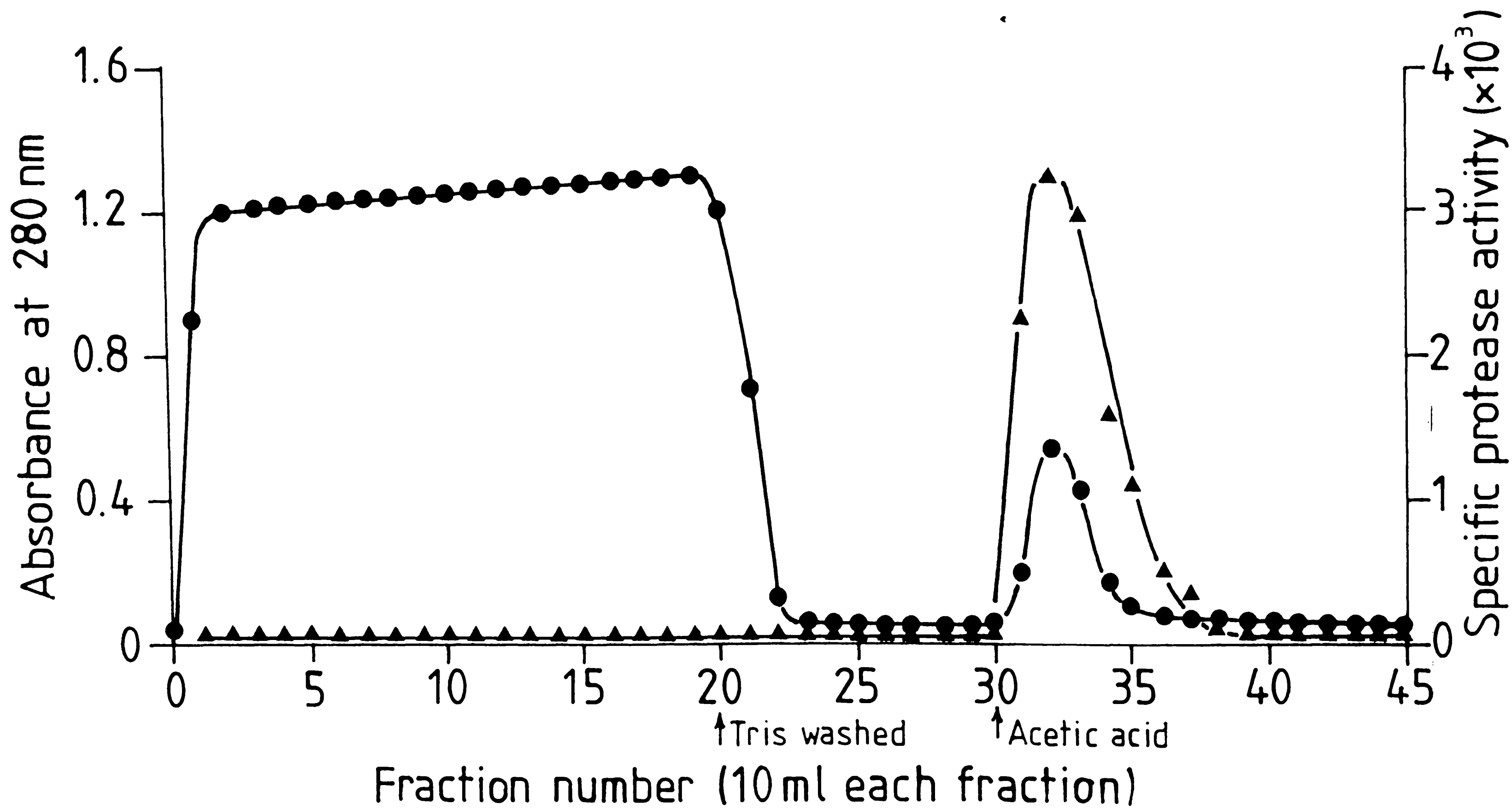


FIGURE 4.4 PURIFICATION OF CALDOLASE BY AFFINITY CHROMATOGRAPHY ON CBS-D-PHE-TETA-SEPHAROSE 4B.

The pooled fractions from QAE-sephadex anion exchange chromatography were applied to the affinity gel. 10ml fractions were collected. The protease was eluted with 0.1M acetic acid, pH 2.8. The protein concentration (●) and protease activity (▲) were determined.

The data resulting from the various steps of Caldolase purification are presented in Table. 4.6.

4.2.7 Results

A large portion of the non-enzyme protein and coloured material was removed by the ion-exchange steps, while little of the protease was lost. (Fig. 4.2 and 4.3). Affinity chromatography with CBZ-D-phe-TETA Sepharose-4B was shown to be the most efficient purification step (Fig. 4.4).

4.2.8 Effect of Low Salt Concentration of Sephadex G-75 Elution Profile

In order to obtain a pure enzyme of low salt concentration the Sephadex G-75 column was eluted with 0.1M Tris pH8.0 containing 0.1M NaCl. The elution profile demonstrated a broad enzyme activity peak (Fig.4.6) This could be attributed to either non-specific binding, causing retardation, (Cowan, 1980) or autolysis of the protease resulting from the very low ionic strength (see Chapter 7 for further details).

TABLE 4.6 PURIFICATION OF CALDOLASE

Step	Volume (ml)	Total Protein (mg)	Total Activity (P.U)	Specific Activity	The Degree of Purification	Recovery %
600 l fermenter	600	-	8491500	-	-	100
Extracted enzyme	3000	-	3358350	-	-	40
5g lyophilised enzyme (a)	200	694	497250	716	1	40
DEAE-cellulose chromatography	208	320.7	461344	1438	2	37
QAE-sephadex anion-exchange chromatography	208	166	441480	2687	3.75	35.5
Affinity chromatography (CBZ-D-phe-TETA sepharose-4B)	48	17.13	414936	24223	33	33.4
(The highest specific activity)				32963		
Sephadex G-75 chromatography	50	7.4	279200	37729	52.7	22
(The highest specific activity)				51205		

(a) 5g of the lyophilised enzyme was dissolved in 200ml of 0.1M Tris acetate pH8.0. This enzyme sample measured about 15% of the total Caldolase extracted from the ammonium sulphate-precipitated sample.

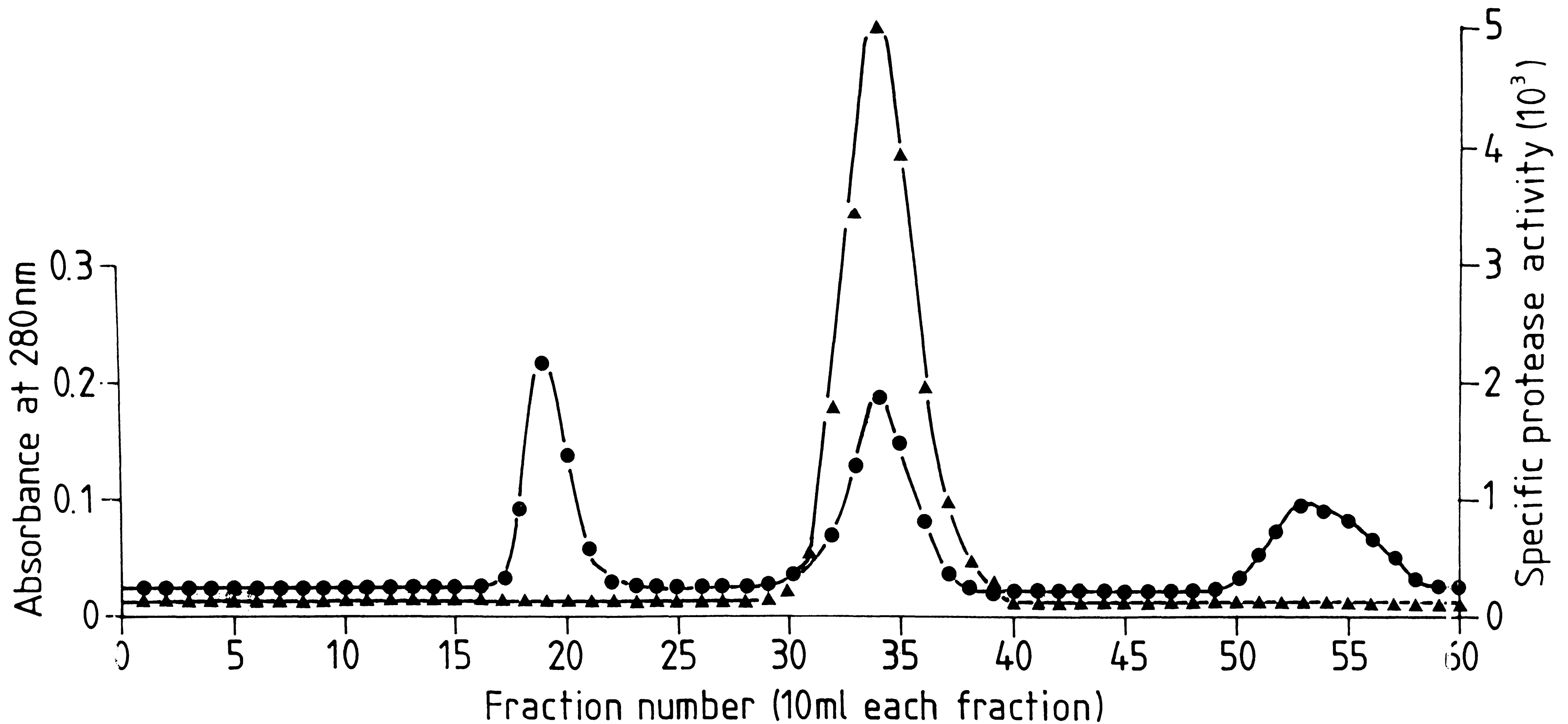


FIGURE 4.5 PURIFICATION OF CALDOLASE BY SEPHADEX G-75 CHROMATOGRAPHY.

The enzyme obtained from affinity chromatography lyophilised and dissolved in 4ml volume Tris acetate pH 8.0 and applied to a sephadex G-75 column. Caldolase was eluted with 0.1M Tris acetate pH 8.0 containing 0.5M NaCl + 10mM $CaCl_2$. 10 ml fractions were collected. The protein concentration (●) and protease activity (▲) were determined.

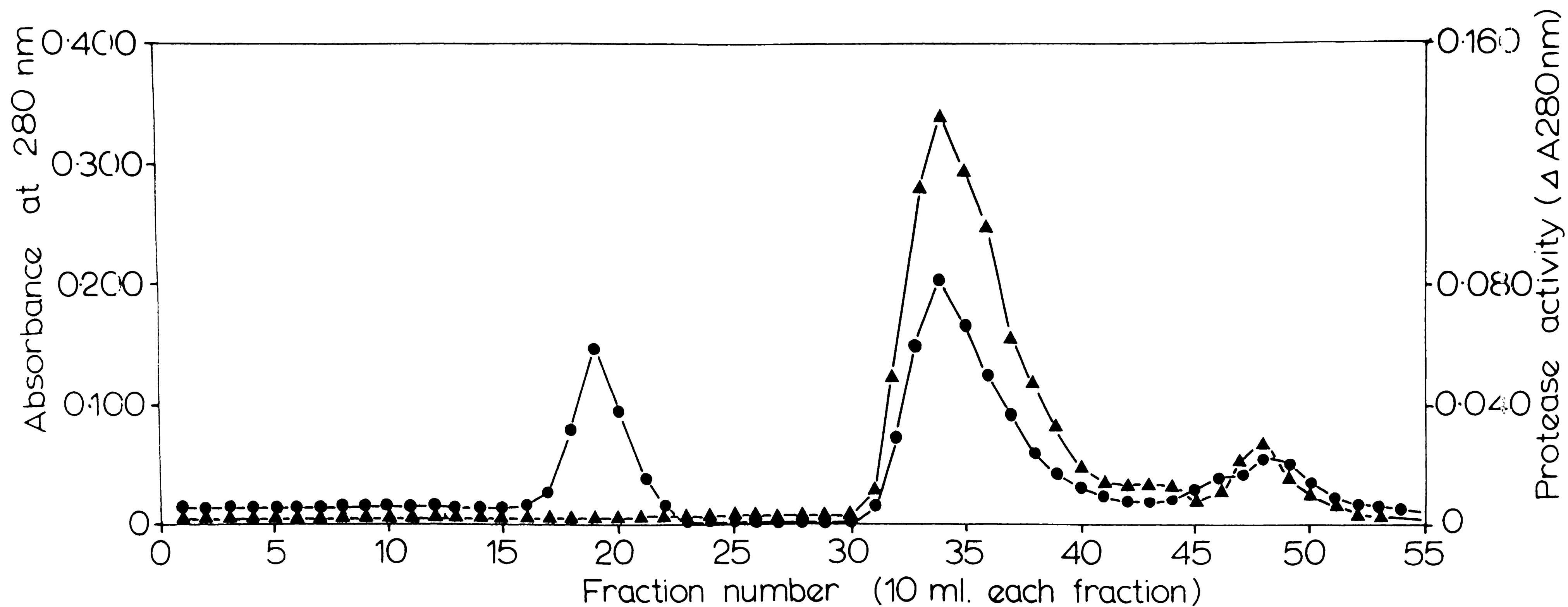


FIG. 4.6 EFFECT OF LOW SALT CONCENTRATION ON SEPHADEX G75 ELUTION PROFILE DURING CALDOLASE PURIFICATION.

A 4ml enzyme solution was applied to a sephadex G-75 column. Caldolase was eluted with 0. M Tris acetate pH 8.0 containing 0.1M NaCl. 10ml fractions were collected. The protein concentration (●) and protease activity (▲) were determined.

5. CALDOLASE CHARACTERISATION.

5.1 MOLECULAR WEIGHT DETERMINATION.

5.1.1 Methods.

The molecular weight of Caldolase was determined by SDS - PAGE, (see Section 2.1), Sephadex G-75 gel filtration chromatography and TSK gel filtration chromatography (see Chapter 4 for details).

5.1.2 Results and Discussion.

The molecular weight of Caldolase was determined by SDS - PAGE and estimated to be 32,000 daltons (Fig. 5.1). On the other hand the molecular weight of the enzyme by Sephadex G-75 and TSK gel filtration column chromatography was estimated to be 25,000 daltons based on comparison with standards of known molecular weights (Mantle, 1978) (bovine serum albumin, ovalbumin, α-chymotrypsinogen A and cytochrome C. Blue dextrane was used for the determination of the void volume) (Fig. 5.2).

TSK G3000SW type is a packed column for high-performance liquid chromatography (HPLC) developed by Toyo SODA Manufacturing Company, Ltd. (Japan). The advantage of HPLC is its high resolution. Substantial differences in molecular weight by different methods have also been described by other authors (Zlotnik *et al*, 1984; Roitsch, 1983; Leach, 1980; Voordouw *et al*, 1974).

Segrest and Jackson (1972) have reported that glycoproteins containing more than 10% carbohydrate behave anomalously during SDS - PAGE when compared to standard proteins. They also noted that:

1. Proteins in general bind constant amounts of SDS per gram when saturated. The protein then has an overall negative charge that masks its intrinsic charge, resulting in a constant charge to mass ratio.
2. Proteins saturated with SDS take on a rod-like configuration, the length of the structure being proportional to its polypeptide chain length and thus its molecular weight.

The binding of SDS to proteins by equilibrium dialysis has been investigated by several authors. (Reynolds & Tanford, 1970; Clarke, 1975). Pitt-Rivers and Impiombato, (1968) have found that:

1. Most of the proteins studied bound 90 - 100% of their weight of SDS.
2. The glycoproteins studied bound 70 - 100% of their weight of SDS, calculated in terms of the polypeptide moiety of the molecule. Therefore, glycoproteins bind less SDS per gram of their weight.

The lower SDS binding results in a decreased charge to mass ratio for glycoproteins versus standard proteins, resulting in a decreased mobility during SDS - PAGE, and thus showing a higher apparent molecular weight.

Anomalous behaviour of proteins is also apparent during gel filtration chromatography. The partial specific volume, frictional ratio, and association/dissociation reactions will all affect the way the molecular weight relates to the Stokes radius of the molecule (Smith, 1963; Andrews, 1965; Siegel & Monty, 1966; Mantle, 1978). Siegel and Monty, having studied the gel filtration behaviour of a number of proteins,

found that ferritin revealed the most significant deviation from the calibration curves when the elution volume was plotted against log molecular weight. This was due to the low partial specific volume ($0.59cm^3/g$) of ferritin compared to most proteins which exhibit a value of approximately 0.73 (Mantle, 1978).

The frictional ratio, which represents the ratio of resistance actually met by the overall shape of the molecule, will also have an effect (Siegel & Monty, 1966). For example, fibrinogen, which has a frictional ratio of 2.35 compared to most proteins with a mean value of about 1.25, elutes anomalously with regard to molecular weight.

Association/dissociation reaction in proteins undergoing concentration or pH-dependence may be responsible for anomalous elution volumes. This phenomenon can be overcome by selecting conditions where one form predominates (Mantle, 1978).

Andrews (1965) has found a strong correlation between molecular weight and gel filtration behaviour of carbohydrate-free proteins. In contrast, the presence of carbohydrate in glycoprotein molecules causes anomaly in gel filtration behaviour. This is probably due to the expanded structures of glycoproteins compared with typical globular proteins, resulting perhaps from a greater hydration in solution of carbohydrate chain as compared with polypeptide chains.

In view of the high carbohydrate content of Caldolase, the anomalous behaviour of this enzyme during gel filtration and SDS - PAGE is perhaps not surprising. Therefore, the molecular weight determination from the above techniques must be regarded as approximate.

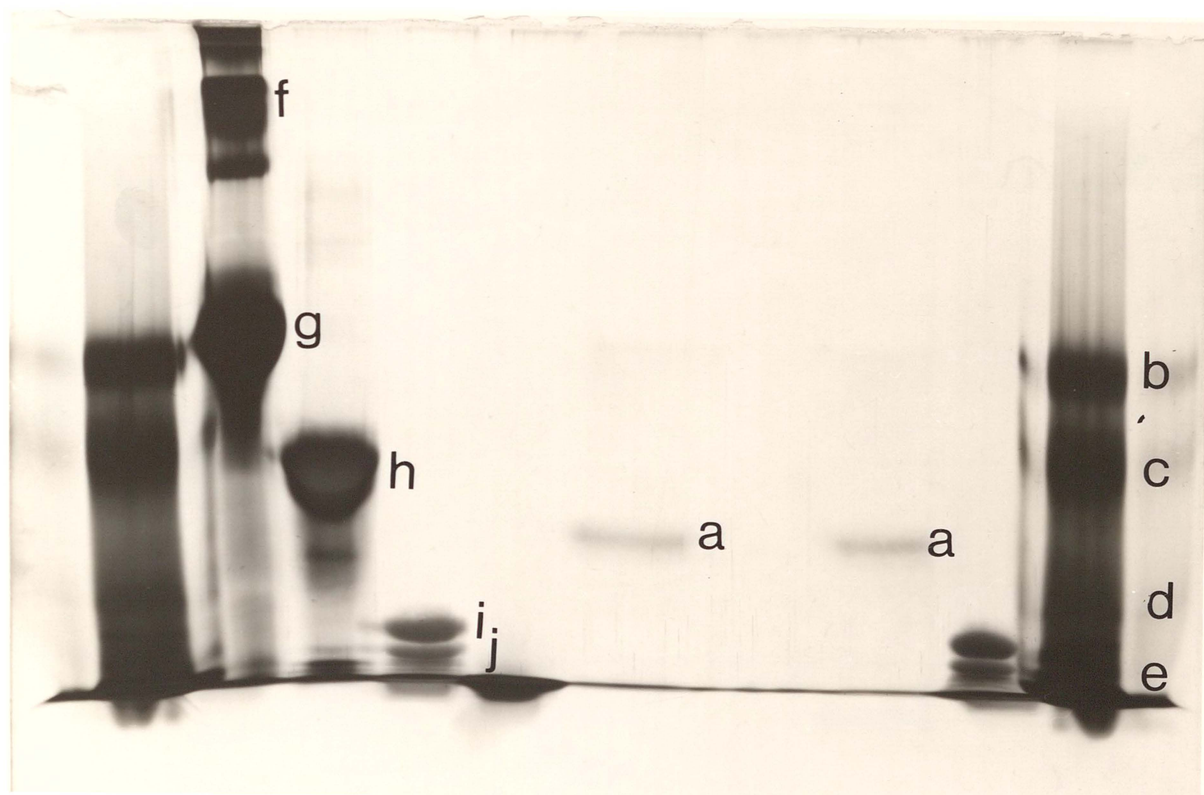


FIGURE 5.1 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CALDOLASE

(a) Caldolase (b) Bovine serum albumin (66,000 daltons), (c) Ovalbumin (45,000 daltons), (d) α-Chymotrypsinogen (25,000 daltons), (e) Cytochrome C (12,000 daltons), (f) Bovine serum albumin (dimer, 132,000 daltons), (g) Bovine serum albumin (monomer, 66,000 daltons), (h) Albumin (from chicken egg, 45,000 daltons), (i) Carbonic anydrase (from bovine erythrocytes, 29,000 daltons), (j) α-Lactalbumin (from bovine milk, 14,200 daltons).

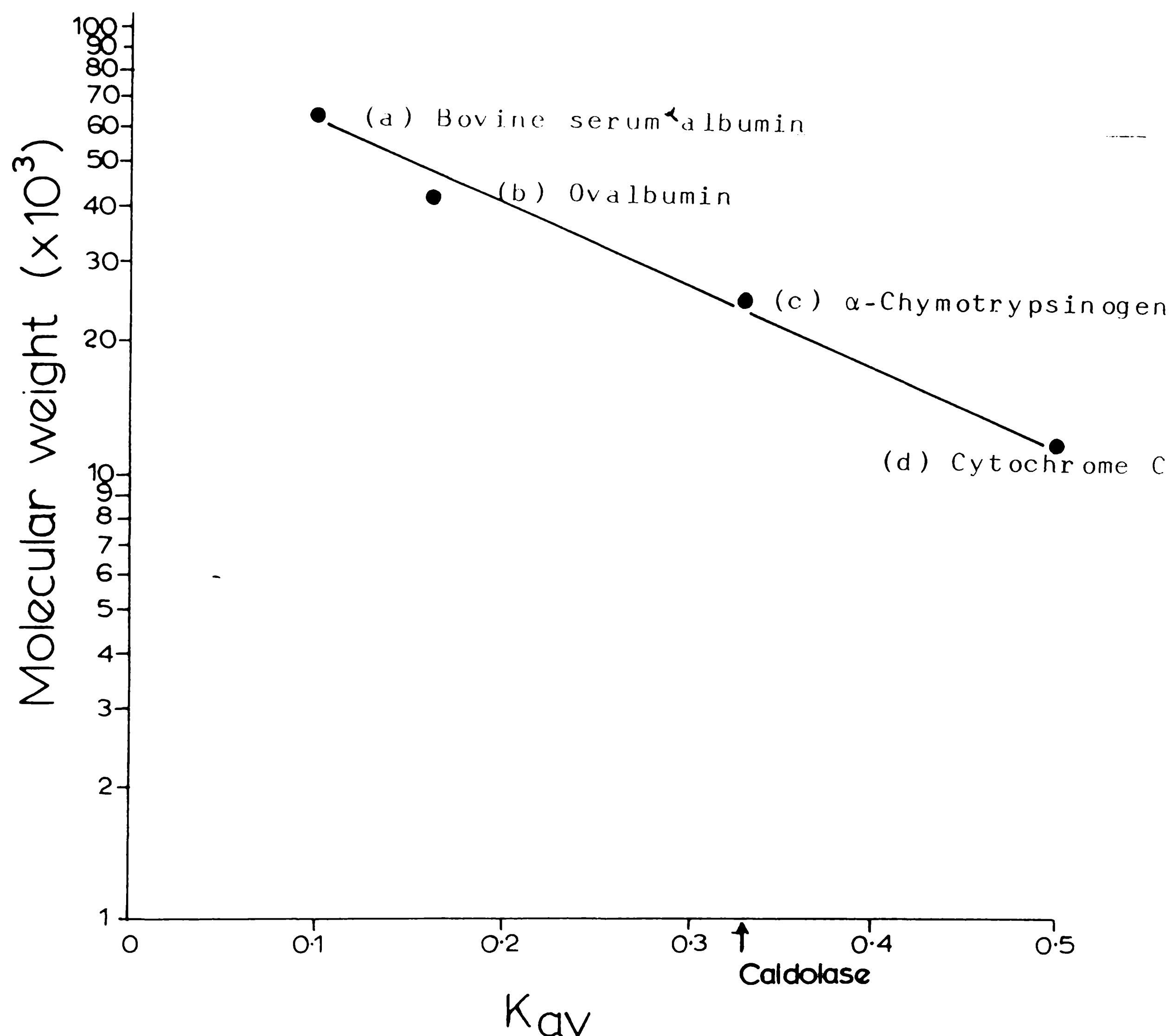


FIGURE 5.2 SEPHADEX G-75 CHROMATOGRAPHY OF STANDARD MARKERS

The mixture of standard markers (4ml) was applied to a sephadex G-75 column and eluted with 0.1M Tris acetate pH 8.0 containing 0.5M NaCl + 10mM $CaCl_2$. 10ml fractions were collected.

The standard marker profile was obtained by using $K_{av} = \frac{Ve - Vo}{Vt - Vo}$ formula, where K_{av} distribution coefficient, Ve = elution volume of protein, Vo void volume and Vt = total volume of column (Mantle, 1978).

5.2 ISOELECTRIC FOCUSSING

5.2.1 Method.

The method used has been described in Section 2.2

5.2.2 Result and Discussion.

Isoelectric focussing of Caldolase revealed a single band with an isoelectric point (pI) of 8.9 when compared with the standards of known isoelectric points. (Fig. 5.3; 5.4).

Although most serine proteases have basic isoelectric points (Keil, 1971; Markland & Smith, 1971), some possess an acidic pI (Renko *et al*, 1981; Ogrydziak & Scharf, 1982).

5.3 CARBOHYDRATE DETERMINATION

5.3.1 Method

The carbohydrate content of the purified Caldolase was analysed by the phenol/sulphuric acid method (Dubois *et al*, 1956). Two ml of the purified enzyme (70 μg ml^{-1}) were pipetted into each test tube (15 ml centrifuge glass tube), and 0.05 ml of 80% phenol was added prior to the addition of 5 ml of concentrated sulphuric acid. The mixtures were left for 10 minutes at room temperature, they were then shaken and placed for 20 minutes in a water bath at 30°C. The absorbance of each sample was measured at 490 nm.

5.3.2 Result and Discussion

The carbohydrate concentration of Caldolase, using glucose as a standard, was determined to be approximately 10%, equivalent to 16 mol of hexose per mol protein based on a molecular weight of 25,000. (Fig. 5.5).

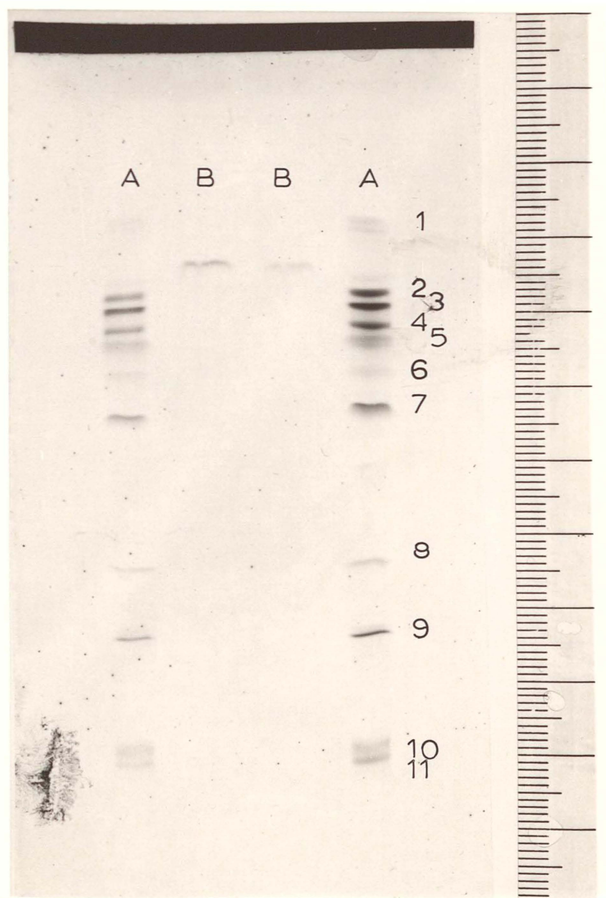


FIGURE 5.3 THE ISOELECTRIC POINT OF CALDOLASE

The pI of enzyme was determined using 5% polyacrylamide gel (Servalyte pH 3 - 10) by comparison with a broad pI calibration kit (pH 3 - 10) (Pharmacia Fine Chemicals). (B) Caldolase, (A) standard markers [1. trypsinogen (pI - 9.30), 2. lentil lectin-basic band (pI - 8.65), 3. lentil lectin-middle band (pI - 8.45), 4. lentil lectin-acidic band (pI - 8.15), 5. myoglobin-basic band (pI - 7.35), 6. myoglobin-acidic band (pI - 6.85), 7. human carbonic anhydrase B (pI - 6.55), 8. bovine carbonic anhydrase B (pI - 5.85), 9. β-lactoglobulin A (pI - 5.20), 10. soybean trypsin inhibitor (pI - 4.55), 11. amyloglucosidase (pI - 3.50)].

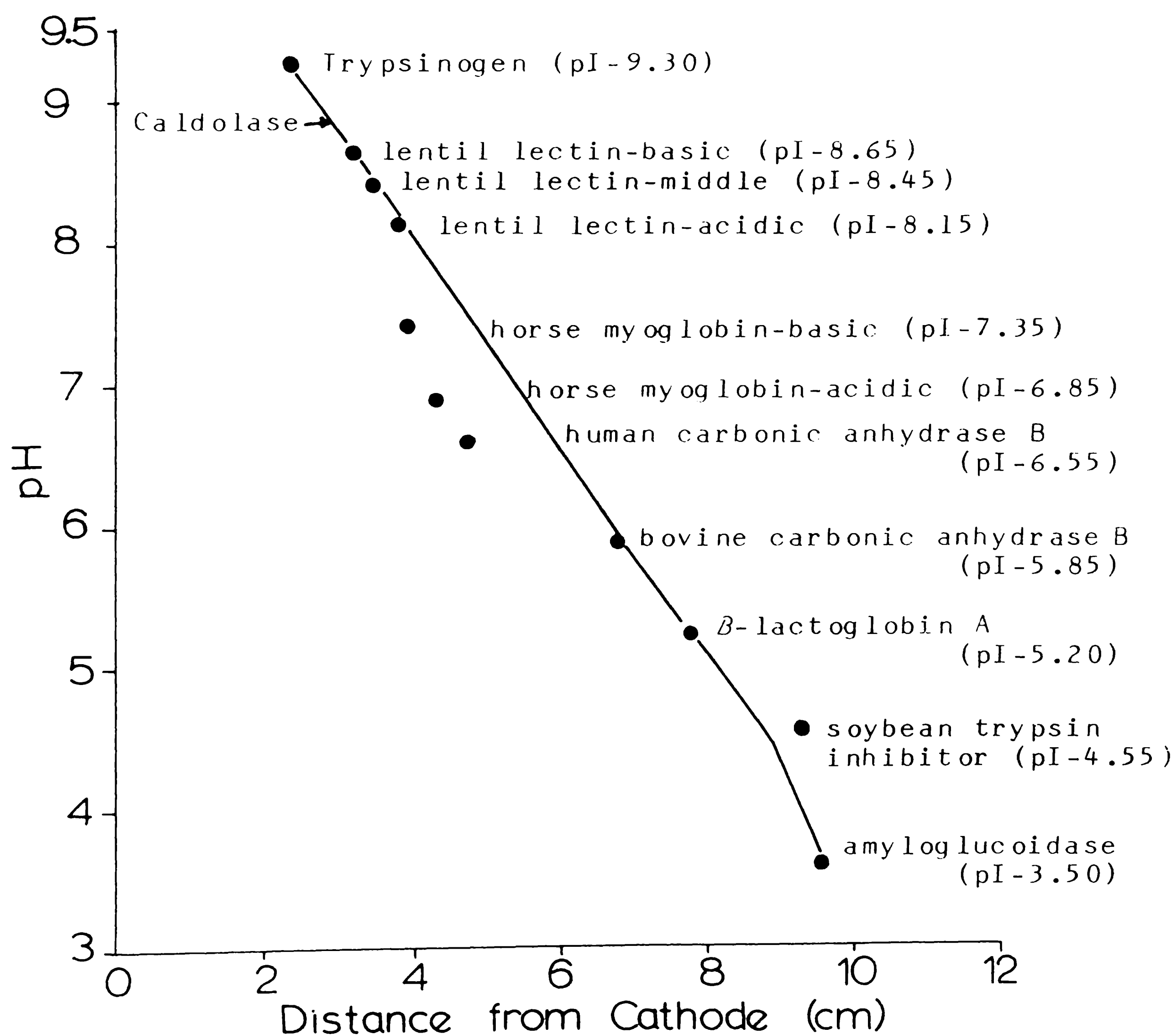


FIG. 5.4 THE STANDARD MARKERS PROFILE
using the broad pI calibration kit (Pharmacia Fine Chemicals) on a 5% polyacrylamide gen. (Servalyte pH 3-10).

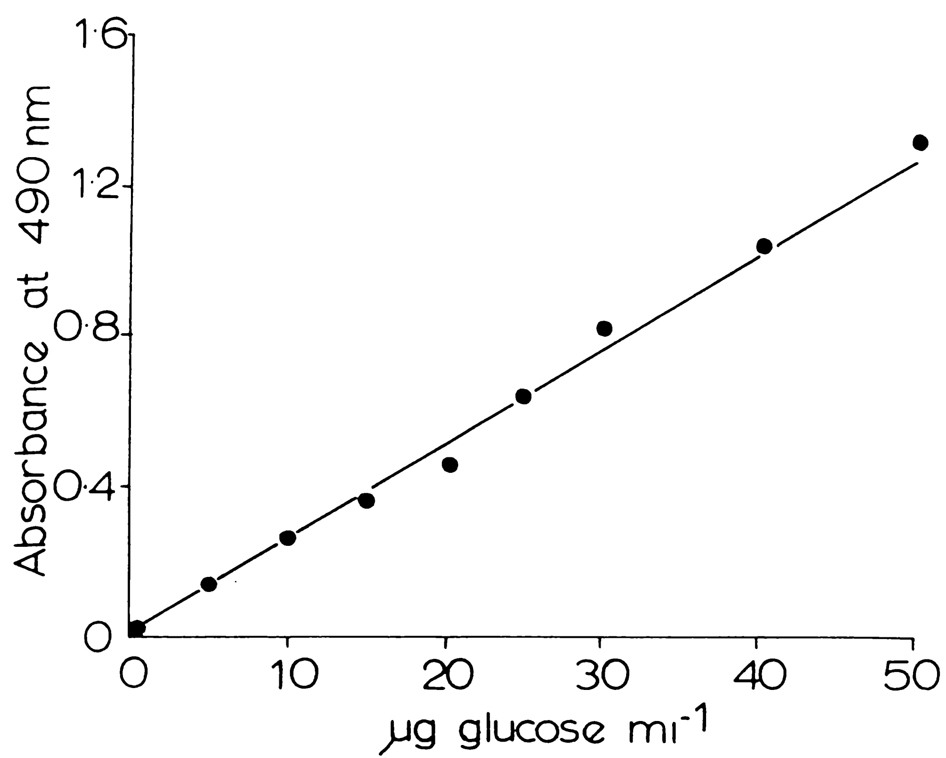


FIGURE 5.5 THE GLUCOSE STANDARD PROFILE

The standard curve for glucose at specified concentrations was prepared as outlined in section 5.3.

Microbial extracellular proteases vary in both the presence and percentage of carbohydrate content. Carbohydrate is absent from some extracellular proteases, e.g., subtilisins (Ottesen & Svendsen, 1970) and present in others: Bacillopeptidase F from *Bacillus subtilis* strain 168 (Roitsch & Hageman, 1983), Caldolysin contains 13% carbohydrate (Cowan & Daniel, 1982a). Ogrydziak & Scharf (1982) have noted that the carbohydrate content of an alkaline protease from *Saccharomycopsis lipolytica* was 15% when the organism was grown in a medium containing proteose-peptone. By contrast, when a carbohydrate-free protein (lysozyme) was substituted in the growth medium, the protease contained only about 1.8% carbohydrate. This indicates that the purified protease extracted from the first culture medium was contaminated with a large amount of non-covalently-bound carbohydrate.

It is possible that the high content of carbohydrate in extremely thermophilic extracellular proteases is involved in the molecular stabilisation of these enzymes. The suggestion has been proffered that carbohydrate in glycoproteins is instrumental in stabilising the three-dimensional structure of the molecule and protecting the protein against proteolysis (Pazur *et al*, 1970; Nakamura & Hayashi, 1974).

5.4 SULPHYDRYL GROUPS AND DISULPHIDE BONDS IN CALDOLASE

5.4.1 Methods.

i Free sulphydryl (-SH) group content:

Free sulphydryl was determined using the method of Robyt *et al*, (1971). Two ml of enzyme solution (35 μg ml^{-1}) were added to 0.6 ml of 2mM 5 - 5′ dithiobis - (2-nitrobenzoic acid)

(DTNB). The mixtures were then incubated at 22°C for 30 minutes. The absorbance was measured by spectrophotometer at 410 nm against an enzyme-free blank.

ii The content of cystine

For the determination of the cystine content of Caldolase the method of Anderson and Wetlaufer (1975) was employed. 0.25 ml enzyme (35 μg ml^{-1}) was mixed with 0.25 ml of 6N NaOH and incubated for 30 minutes at 22°C. Following the disulphide bonds cleavage, the reaction was terminated by the addition of 0.5 ml of 6N H_2PO_4 containing 2mM EDTA and 100 ml of 2mM DTMB. The absorbance was monitored at 410 nm against an enzyme-free blank.

5.4.2 Results and Discussion

Caldolase apparently possessed no free sulphydryl groups. From the absorbance value ($\Delta 410 = 0.001$) and the molar extinction coefficient of CNT ($\varepsilon = 11400$) Robyt *et al*, 1971), it was calculated that the ratio of CNT produced per mole of enzyme corresponded to less than one (0.062) sulphydryl group.

The above result is in agreement with the conclusion derived from the response of the enzyme to cysteine inhibitors.

The cystine determination experiment revealed an absorbance value of $\Delta A = 0.075$. Based on the molar extinction coefficient of CNT ($\varepsilon = 11400$) (Robyt *et al* , 1971) and Anderson & Wetlaufer's (1975) stoichiometric relation of 1.2 ± 0.3 mole CNT per mole S - S, the presence of 4 disulphide bonds per molecule of Caldolase is implied. However, neither mercaptoethanol nor dithiothreitol had a significant effect on enzyme stability (see Chapter 6 for further detail). This indicates that either disulphide bonds are not involved in the

molecular stabilisation of Caldolase or S - S bonds are well protected from the reagents.

Disulphide bonds in Caldolysin accounted for a significant proportion of the intrinsic stability of the tertiary structure. The conclusion reached was that the cystine contribution to Caldolysin stability was about 31% of the total free energy of stabilisation (Cowan, 1980).

Some extracellular proteases (Thermolysin, subtilisins) possess neither sulphydryl groups nor disulphide bonds. The presence of cysteine has been reported in some extracellular proteases (Ogrydziak & Scharf, 1982; Stepanov *et al*, 1981). Harris (1976) suggested that the presence of cysteine could be a potential source of instability (due to oxidation or disulphide exchange reaction), and he concluded that thermophilic enzymes contain fewer SH groups than their mesophilic counterparts. It is entirely possible, however, that SH groups may be buried within the interior of the protein molecule and thus less likely to be a potential source of instability. Therefore, it cannot be ruled out that a thermophile protein may be found to have as many SH groups as its mesophilic counterpart. In the case of alcohol dehydrogenase from *Bacillus stearothermophilus* which possesses the same number of cysteine residues as its mesophilic analogue, all of its SH groups are localised inside the protein molecule and are unreactive, particularly to oxidation (Bridgen *et al*, 1973).

5.5 METALS IN CALDOLASE

5.5.1 Methods

Purified lyophilised enzyme was dissolved in 10 mM EDTA and incubated at room temperature for 30 minutes. The mixture was then applied to a Sephadex G-75 gel filtration column (1.6 x 65cm) equilibrated with 0.1M Tris acetate pH8.0 containing 0.5M NaCl. Two peaks were obtained, the first containing protease and the second EDTA. Fractions of each peak were pooled and assayed for enzyme activity. Protein concentration of the pooled enzyme peak was 64 μg ml^{-1}. In addition the presence of Ca^{2+} and Zn^{2+} in both peaks was tested by atomic absorption using Ca^{2+} and Zn^{2+} as the standards at concentrations of 0.1 - 10 μg ml^{-1} and 0.1 - 0.5 μg ml^{-1} respectively.

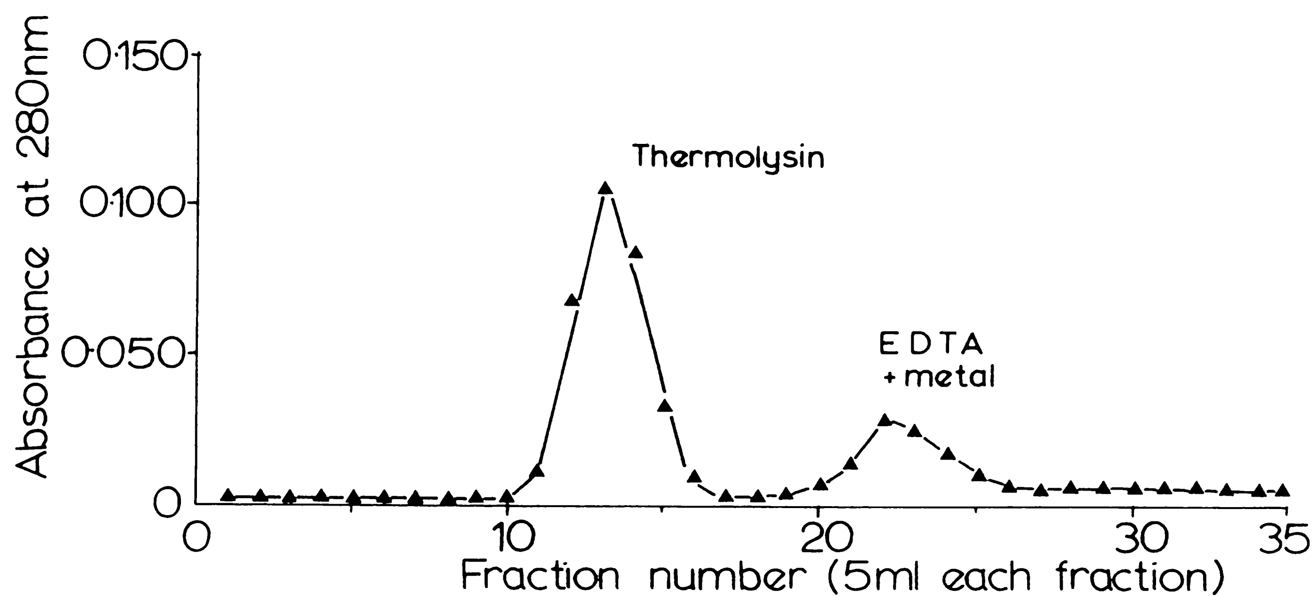
A similar experiment was carried out using thermolysin as a positive control (the protein concentration of the pooled enzyme peak was 83 μg ml^{-1}).

5.5.2 Results and Discussion

The enzyme activity was detected only in the first peak fractions. The mole ratio of Ca^{2+} or Zn^{2+} to Caldolase was less than a one to one correspondence (Table 5.1 and Fig. 5.6). Throughout the above treatment the activity and thermostability of Caldolase remained unaffected when the enzyme was incubated at 75°C for 30 minutes and the protease activity measured (The data have not been shown).

The following conclusions were drawn:

(i) Caldolase retained full activity and thermostability following EDTA treatment, indicating that no EDTA-susceptible essential metal was present in Caldolase.



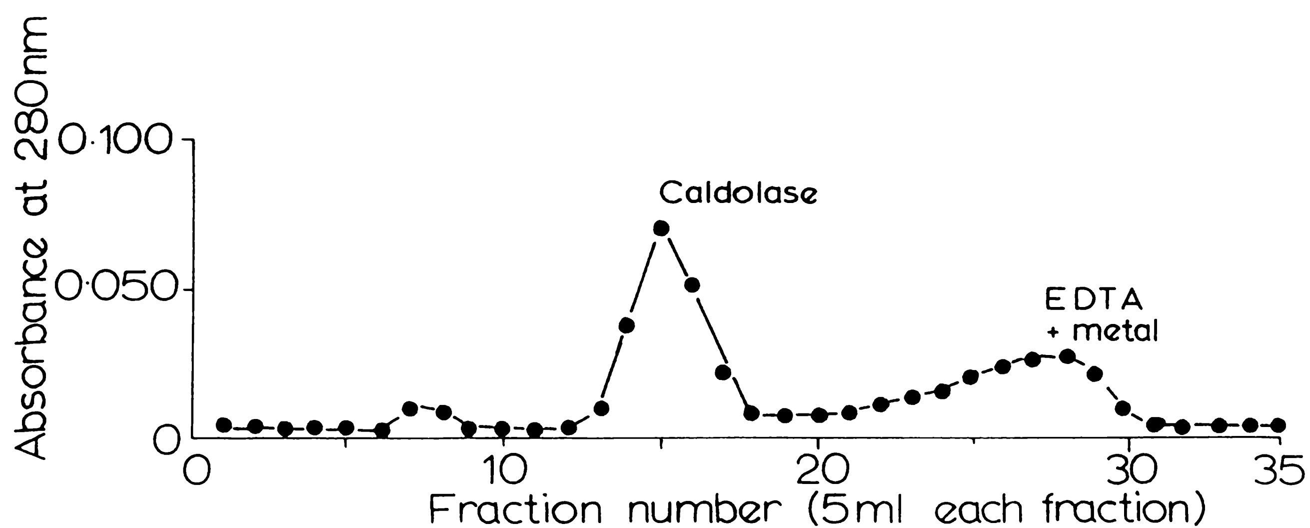


FIGURE 5.6 DETECTION OF Ca^{2+} AND Zn^{2+} IONS IN CALDOLASE AND THERMOLYSIN

Sephadex G75 chromatography of EDTA-treated Caldolase (●) and Thermolysin (▲) was carried out as described in section 5.5.1

(ii) Ca^{2+} and Zn^{2+} were present in the Caldolase peak in insignificant amounts ($<$ 0.2mol Ca^{2+}/mole Caldolase and 0.03 mole Zn^{2+}/mole of enzyme).

When a similar experiment was carried out using thermolysin, the presence ~~of both Ca^{2+} and Zn^{2+} in the EDTA peak~~ (Table 5.1) indicated that both metals were present in thermolysin and that they could be easily removed by EDTA. Treatment by EDTA entirely inactivated thermolysin. It is well known that Ca^{2+} is involved in the stability and Zn^{2+} in the catalytic activity of thermolysin (Feder *et al*, 1971).

Table 5.1 Metals in Caldolase and Thermolysin

Enzyme	Metal	Enzyme Peak $\mu g\ ml^{-1}$	EDTA Peak $\mu g\ ml^{-1}$
Caldolase	Ca^{2+}	0.02	0.09
	Zn^{2+}	0.005	0.046
Thermolysin	Ca^{2+}	0.016	1.72
	Zn^{2+}	0.0028	0.32

5.6 AMINO ACID COMPOSITION OF CALDOLASE

5.6.1 Methods

Caldolase hydrolysis was carried out with 6M HCl containing 7% thioglycolic acid (Böhlen, 1983) in the sealed, evacuated ampoules for 24 and 72 hours at 110°C. Thioglycolic acid is known to protect methionine, tyrosine, and tryptophan from partial oxidation and to reduce cystine to cysteine (Böhlen, 1983). The acids were then removed by Rotory evaporation at 98°C followed by three washes with double-distilled water to eliminate any traces of acids and to neutralise the hydrolysed sample.

Cysteine cannot be directly analysed quantitatively using o-phthalaldehyde (OPA) due to the low fluorescence yield of the cysteine fluorophor. Since performic acid is able to convert cysteine to cysteic acid which forms a highly fluorescent derivative with OPA and can be easily detected, therefore a second enzyme sample was used for the analysis of proline and cysteine (as cysteic acid). In this assay the cysteine was converted to cysteic acid with performic acid by adding 90% of formic acid and 10% hydrogen peroxide to the dried enzyme sample. The reaction was allowed to proceed for 15 minutes at room temperature. After drying the ampoule under vacuum, 1 ml of 6M HCl was added. The oxidised enzyme was hydrolysed as above.

The amino acid composition of each sample was determined using a Waters HPLC amino-acid analyser with post column reaction, with OPA for detection.

5.6.2 Results and Discussion

The amino acid composition of Caldolase is presented in Table 5.2. No pronounced differences were observed when the amino acid composition of Caldolase was compared with a number of thermophilic and mesophilic serine proteases. (Table 5.2).

Others have already reported the higher ratio of arginine and lower lysine residues possessed by thermophilic enzymes compared to their mesophilic counterparts (Frank *et al*, 1976; Kagawa *et al*, 1976). Caldolase also possesses these features (Table 5.2) when compared to other mesophilic proteases; the exception is the serine protease from *Thermoactinomyces vulgaris*, (Stepanov *et al*, 1980), which does not follow this rule (Table 5.2). Attempts to determine cystine, proline and hydroxy-proline residues in Caldolase were largely unsuccessful.

5.7 PH OPTIMUM OF CALDOLASE

5.7.1 Methods

The influence of pH on Caldolase activity was examined over a range of pH 6 - 11 in the Universal buffer (Dawson *et al*, 1969). The pH of each sample buffer was adjusted at 75°C and 0.2% (w/v) casein was then dissolved in the buffers. The protease activity was determined at 75°C using the standard Kunitz method. In addition the following buffers, 0.1M HEPES (pH 6.5 - 10), 0.1M Bicine (pH 6.5 - 9.5) and 0.1M sodium carbonate-bicarbonate (pH 8.5 - 10.3) were employed. Each buffer sample was prepared as above and the protease activity measured.

Table 5.2 Partial Amino Acid Composition of Caldolase and some other Serine Proteases.

Amino Acid	Caldolase No.	Caldolase %	Serine Protease *The. Vulgaris* (b) %	Subtilisins (c) Carlsberg %	Subtilisins (c) BPN′ %	*Steptomyces rinosus* Serine Protease % (d)	Chymotrypsin C (e) %
Lys	5	2.4	4	3.2	4	7.7	3.1
His	6	2.9	1.5	1.8	2	2.8	2.2
Arg	7	3.3	2	1.5	0.7	1.4	3.1
Asp	21	10	12	10	10	7.7	9.9
Thr	19	9	8	7	4.7	7.7	6.3
Ser	17	8	9	11.5	13.6	8.2	9
Glu	10	4.8	6	4.4	5.5	10	9.5
Pro	ND[a]	ND[a]	6	3.2	5	3.3	5.4
Gly	29	14	11	12.7	12	14.4	11.3
Ala	31	15	14	15	13.5	10	5.4
Half-Cys	ND[a]	ND[a]	0.7	0	0	0.96	3.1
Val	19	9	7.4	11	11	6.7	8.5
Met	2	1	0.4	2	2	0.48	0.45
Ile	8	3.8	5	3.6	4.7	4.3	5.4
Leu	16	7.7	3	5.8	5.5	5.3	8.6
Tyr	9	4.3	6	4.7	3.6	4.8	2.7
Phe	4	1.9	1.5	1.5	1	2.4	1.8
Trp	3	1.4	2.4	1	1	0.96	3.6
	206	100	100	100	100	100	100

(a) ND = not determined

(b) Stepanov *et al*, (1980)

(c) Markland & Smith (1971)

(d) Renko *et al*, (1981)

(e) Hess, (1971)

5.7.2 Results and Discussion

Caldolase exhibited a broad pH profile for activity with the optimum being about 9.5 (Fig. 5.7). Dilution of the Universal buffer at high pHs did not produce a sufficient decrease in the ionic strength to have any significant effect on enzyme activity (conductivity of the buffers was measured by conductivity meter).

The enzyme activity at lower and higher pH is greatly dependent upon the type of buffers (Fig. 5.7).

5.8 EFFECT OF INHIBITORS ON CALDOLASE

A number of different mechanisms are responsible for enzyme inhibition.

(i) Trapping mechanisms (e.g. by macroglobulins)

(ii) Binding of inhibitors to the active site of the enzyme e.g., the inhibition of serine proteases by DFP and PMSF.

(iii) Removal of the active site metal (Ca^{2+}, Zn^{2+}, etc.) by metal chelators.

5.8.1 Methods

The effect of a variety of inhibitors on Caldolase was evaluated using two techniques: i The Kunitz method (Table 5.3), ii Plate assay (Fig. 3.2).

(i) The standard Kunitz method.

Concentrated solutions of inhibitors were prepared in 0.1M Tris acetate pH 8.0. Some inhibitors (PMSF, Di-phenyl carbamyl-chloride) which could not be solubilised in the buffer

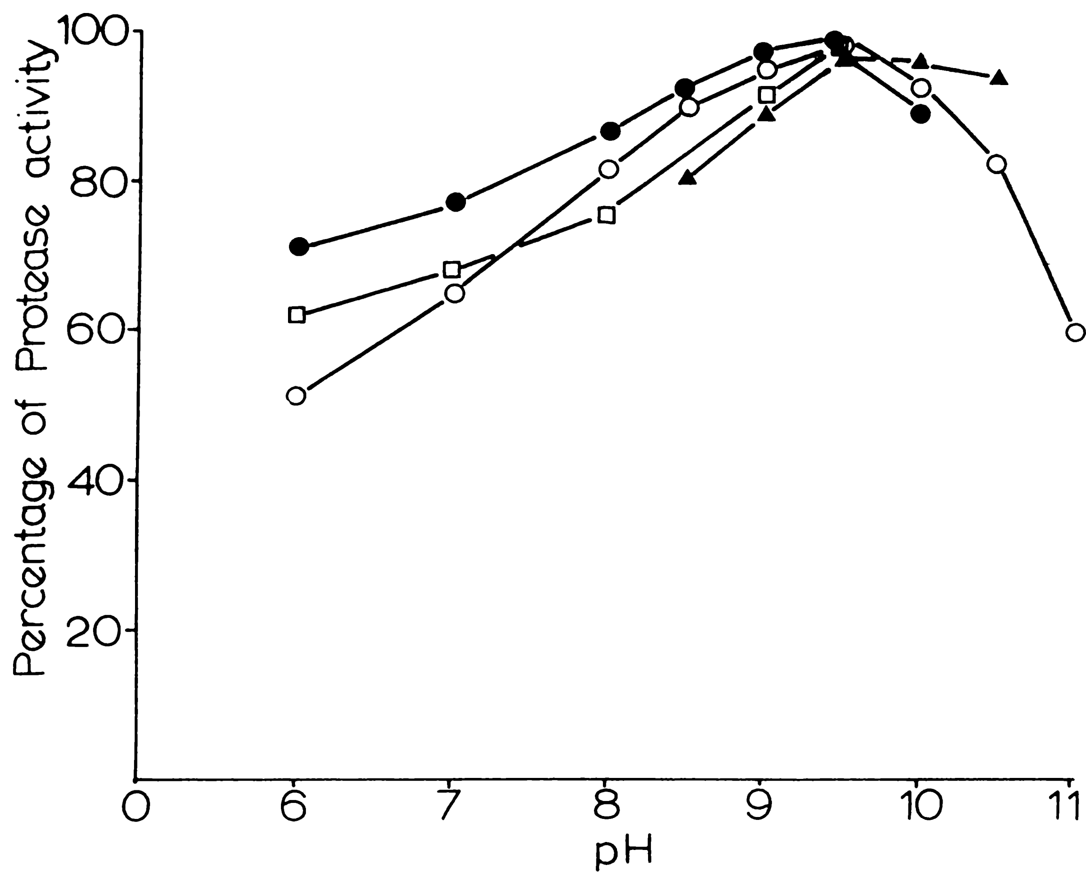


FIGURE 5.7 THE EFFECT OF pH ON PROTEOLYTIC ACTIVITY OF CALDOLASE

The enzyme reaction was carried out in the following buffers at specified pHs using 0.2% (w/v) casein as outlined in section 5.7: Hepes (●), Bicine (□), Sodium carbonate (▲) and Universal buffer (○).

were dissolved in one of the following solvents: n-propanol, dimethyl sulphoxide, 2-methoxy ethanol, 1-4-dioxan or methanol. When such reagents were used the control sample contained equal amounts of solvent.

P-chloromercuribenzoate and 4-hydroxymercuribenzoate were dissolved in dilute alkali (pH 10 - 11), then pH adjusted to 8.0. The enzyme (15 $\mu g\ ml^{-1}$) was preincubated with inhibitors at the specified concentrations (Table 5.3) for a period of 1 - 2 hours at room temperature. The protease activity of each sample was determined at 75°C using 0.2% casein in 0.1M Tris acetate pH 8.0. In the case of inhibitors absorbing at 280nm (O-phenanthrolin, iodoacetic acid, N-ethyl-5-phenyl isoxazolium, etc.), 0.1% azo-casein was substituted for casein and the absorbance measured at 440nm.

Since gramicidin precipitates in the presence of NaCl (Shannon *et al*, 1982), it was dissolved in 0.1M Bicine buffer pH 8.0. The enzyme was also prepared in this buffer.

(ii) Plate Assay

The initial screening of proteases was based on the incorporation of various protease inhibitors in casein-agar plates. (See Chapter 2 for detail)

5.8.2 Results and Discussion

Caldolase was strongly inhibited by low concentrations of DFP, PMSF and $HgCl_2$ (Table 5.3) and inhibited by relatively high concentrations of DPCC, iodoacetic acid, HMBA, $CuCl_2$ and Chymostatin (Table 5.3). It was not inhibited by metal chelators, trypsin inhibitors, TPCK and Pepstatin A (Table 5.3).

The above results indicate that Caldolase is a serine protease

The most interesting feature of this enzyme is that, unlike metal proteases, chelating agents have no effect, suggesting that neither Ca^{2+} nor Zn^{2+} are required for enzyme activity or stability. TPCK and TLCK, which inhibit chymotrypsin and trypsin respectively, have no effect on Caldolase. Similar results were found in subtilisins (Ottesen & Svendsen, 1970), but the related compound benzyloxy carbonyl phenylalanine bromomethyl ketone (ZPBK) was able slowly to inactivate the subtilisins by reaction with an essential histidine residue in the catalytic centre (Shaw & Ruscica, 1968). Since Caldolase was strongly inactivated by Hg^{2+} (known to form a covalent bond to protein-SH, Co^{2-}, histidine) (Mahler & Cordes, 1971a), therefore it is possible that an essential histidine residue is present at the active site of Caldolase. In this case Caldolase shows a similarity to subtilisins. Iodoacetic acid was only able to inhibit Caldolase at high concentrations and at high temperatures (Table 5.3). It is therefore likely that since iodoacetic acid reacts with side-groups of amino acids other than cysteine, such as the imidazole group of histidine, the ε-amino group of lysine, the phenolic hydroxyl of tyrosine (Means & Feeney, 1971), a reaction between iodoacetic acid and a residue other than cysteine is responsible for the loss of Caldolase activity.

TABLE 5.3 EFFECT OF INHIBITORS ON CALDOLASE.

Inhibitor (class)	Inhibitor	Pre-Incubation Time	Concentration of Inhibitor	Enzyme Inhibition %
Serine inhibitors	PMSF (Phenyl methyl sulphonyl fluoride)	60 mins.	0.1mM	84
			1 mM	92
			5 mM	100
	DFP (Di-isopropyl fluorophosphate)	105 "	0.1mM	30
			1 mM	90
			5 mM	100
	DPCC (Di-phenyl carbamyl-chloride)	60 "	1 mM	32
			5 mM	98
			10 mM	100
Trypsin inhibitors	Soybean trypsin inhibitor	60 "	0.1mg ml^{-1}	0
	Lima bean	60 "	0.1mg ml^{-1}	0
	Chicken egg white	60 "	0.1mg ml^{-1}	0
	Anti-trypsin	60 "	0.1mg ml^{-1}	5
	Leupeptin	60 "	1 mM	0
	Gramicidin S HCl	60 "	0.1mg ml^{-1}	0
	Anti-pain	60 "	0.1mg ml^{-1}	0
	TLCK (N-α-p-Tosyl-L-lysinechloromethyl ketone)	60 "	1 mM	0
		60 "	5 mM	2
Chymotrypsin inhibitors	Chymostatin	60 "	0.1mg ml^{-1}	66
		60 "	0.5mg ml^{-1}	78
	TPCK (N-α-p-Tosyl-L-phenylalanine Chloromethyl ketone)	60 "	1 mM	0
		60 "	5 mM	0
Metal chelators	EDTA (Ethylene diaminetetra acetic acid di-sodium salt)	120 "	0.1 mM	0
		120 "	1 mM	0
		120 "	5 mM	2
		120 "	10 mM	3
	EGTA Ethylene glycol-bis (β-amino-ethyl ether) N-N -tetra acetic acid	120 "	0.1 mM	0
		120 "	1 mM	0
		120 "	5 mM	0
		120 "	10 mM	0
	O-phenanthroline	120 "	0.1 mM	0
		120 "	1 mM	1

Continued...

TABLE 5.3 Continued.

Inhibitor (class)	Inhibitor	Pre-Incubation	Concentration of Inhibitor	Enzyme Inhibition %
	NEPIS (N-Ethyl-5-phenyl-Isoxazolium-3'-Sulphonate)	120 mins.	1 mM	0
		120 "	5 mM	0
Cysteine inhibitors	Iodo acetic acid	120 " (22°C)	1 mM	3
			5 mM	4
			10 mM	58
		6 " (75°C)	1 mM	3
			5 mM	19
			10 mM	97
	PCMB (p-chloro-mercuribenzoate)	90 "	0.1 mM	0
		90 "	1 mM	0
		90 "	5 mM	0
		90 "	10 mM	0
	HMB (4-hydroxy-mercuribenzoate)	60 "	1 mM	0
		60 "	5 mM	0
		60 "	10 mM	0
	N-Ethylmaleimide	60 "	1 mM	0
			5 mM	0
			10 mM	0
$CuCl_2$			1 mM	0
			5 mM	4
			10 mM	39
$HgCl_2$			1 mM	70
			5 mM	93
			10 mM	100
Aspartic protease inhibitor	Pepstatin A		1 mM	0
			2 mM	0

5.9 TEMPERATURE-ACTIVITY RELATIONSHIP IN CALDOLASE

Some thermophilic proteins undergo conformational changes between 40° - 70°C while still retaining their catalytic activity. Many mesophilic enzymes are subject to denaturation at these temperatures (Zuber, 1979; Cowan & Daniel, 1982a; Mozhaev & Martinek, 1984). The latter authors suggested that at high temperatures (55° - 70°C) the thermophilic enzymes rearrange their structures in order to strengthen the hydrophobic interactions.

An investigation was carried out to determine the effect of temperature on the catalytic activity of Caldolase using casein and a synthetic peptide (N-succinyl-ala$_3$-p-nitroanilide) as substrates. Data for an Arrhenius plot were obtained from the reaction of the enzyme (5 μg ml^{-1}) with 0.2% casein in 0.1M Tris acetate pH 8.0. Short incubation times were used at high temperatures (80° - 100°C) to minimise the effect of enzyme autolysis. High enzyme activity at high temperatures also enabled the use of a very diluted enzyme sample to further reduce the significance of autolysis. By contrast, at lower temperatures longer periods were found to be essential since the enzyme activity was low.

To eliminate the problem of substrate denaturation during temperature elevation, the synthetic peptide N-succinyl-ala$_3$-p-nitroanilide was also used as the substrate, since, unlike casein, the peptide will show minimal conformational changes in response to temperature changes. The Arrhenius plots for casein and the peptide substrate exhibit a sharp discontinuity at about 92°C due to denaturation (Fig. 5.8; 5.9). The remainder of the plot is clearly not a straight line, especially for the peptide substrate. However the plots seem to fit a

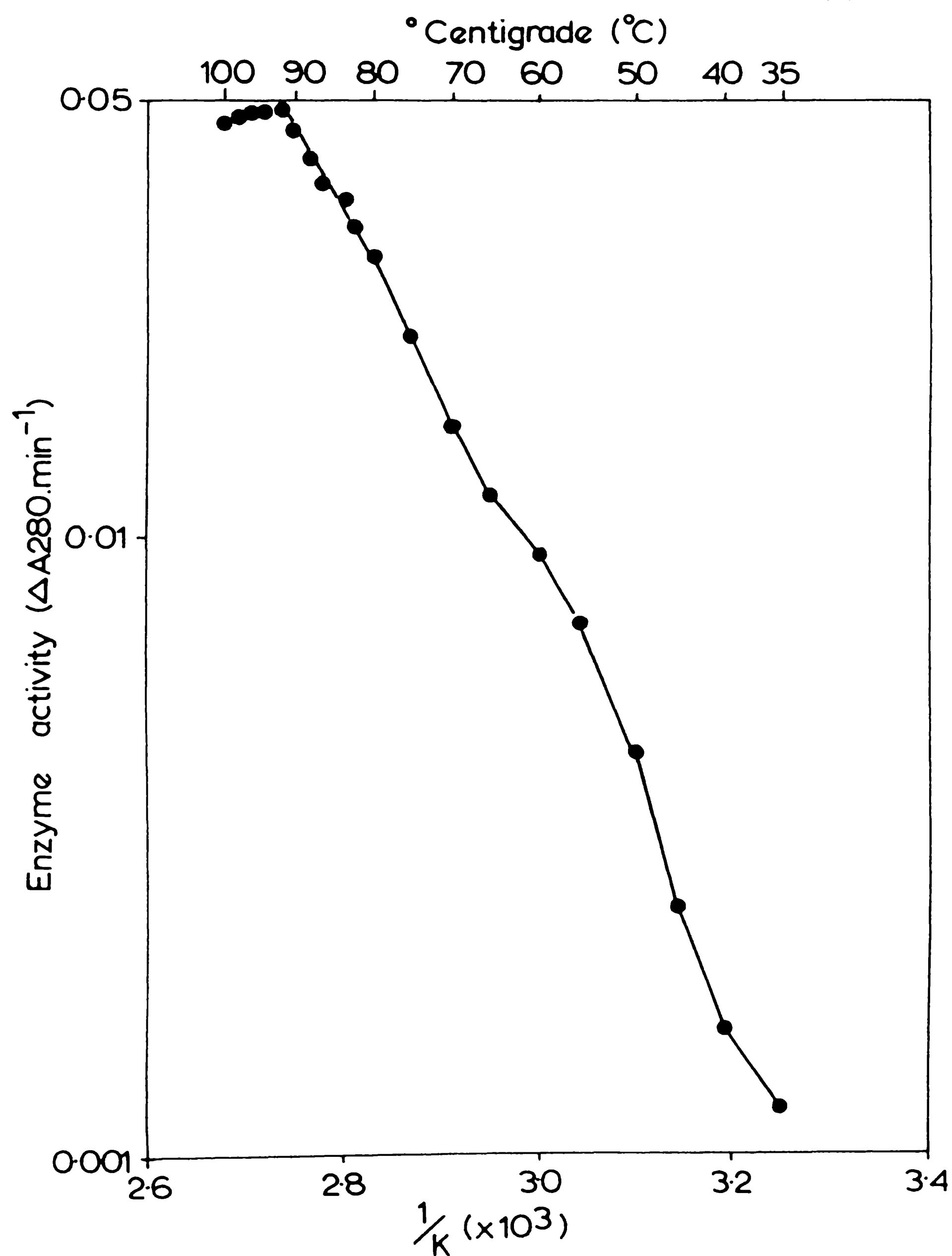


FIGURE 5.8 ARRHENIUS PLOT FOR CALDOLASE

Temperature-activity relationship in the enzyme was determined at indicated temperatures with 0.2% (w/v) casein in 0.1 Tris acetate pH 8.0.

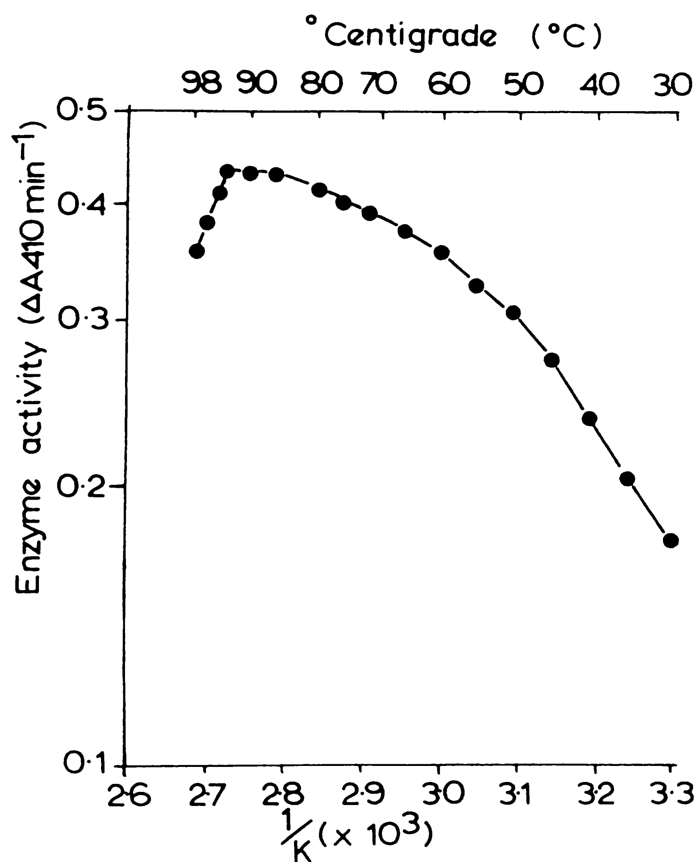


FIGURE 5.9 ARRHENIUS PLOT FOR CALDOLASE

Temperature-activity relationship in the enzyme was determined at designated temperatures with N-succinyl-ala-ala-ala-p-nitroanalide.

curve as well as, or better than, a number of straight lines. Attempts are often made to fit straight line to such curves, but the evidence for a discontinuity at any particular temperature or temperatures seems poor in this case (e.g. Wolfe & Bagnall, 1979). Nevertheless, the change in slope of the line clearly represents a change in property of the enzyme with temperature. As discussed in Section 7.4.3, hydrophobic bonds are involved in the stability of Caldolase. It is also well known that the strength of hydrophobic interactions changes with temperature (Brandts, J.F., 1967). The dielectric constant of the solvent also changes with temperature, and this will influence protein conformation.

It seems from the Arrhenius plots that Caldolase does not undergo a sudden conformational change, but rather experiences a gradual change in proportion to an increase in temperature. These are subtle, as evidenced by the fact that no difference was observed in Km value at various temperatures (45^{0} - 85^{0}C).

Calculated activation energies are Ea(<75^{0}C) = 132kJ mol^{-1}; Ea(>75^{0}C) = 57kJ mol^{-1} using casein as the substrate.

5.10 MICHAELIS-MENTEN KINETICS

5.10.1 Methods

The effect of substrate concentration on Caldolase was determined by Kunitz method using casein and azo-albumin substrates at concentrations ranging from 0.1 - 2% (w/v). A chromogenic synthetic peptide substrate (N-succinyl-ala-ala-ala pNA) and an ester substrate (CBZ-phe-pNE) at concentrations of 0.05 - 2mM were also employed.

5.10.2 Results and Discussion

The Michaelis-Menten relationship (V vs S) showed strong substrate inhibition for the protein substrates (Fig. 5.10). In the Lineweaver-Burk plots (1/v vs 1/S), deviations from linearity at high substrate concentrations occurred as a result of this (Fig. 5.11; 5.12). The inhibition constant (K_S') of the binding between Caldolase and casein was estimated to be 2.8mg ml^{-1} when 1/v against S was plotted (Fig. 5.13).

No substrate inhibition was observed when peptide and ester substrates were used (Fig. 5.14; 5.15), indicating that substrate inhibition using casein and azo-albumin substrates may be due to steric hindrance rather than binding to the active site of the enzyme.

A Km value of 1.42×10^{-3}M was calculated for N-succinyl-ala-ala-ala-pNA (Fig. 5.14). The Km value for CBZ-phe-pNE was 1.66×10^{-3}M (Fig. 5.15).

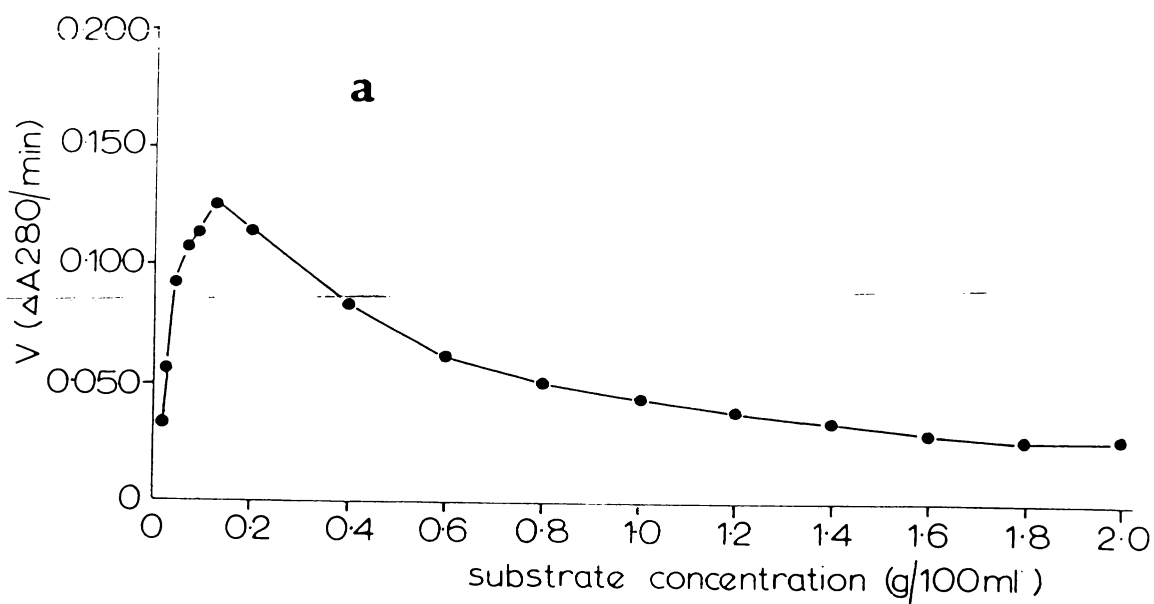
5.11 THE INFLUENCE OF TEMPERATURE ON KM

5.11.1 Method

The rate of hydrolysis of various concentrations of casein by caldolase at a range of temperatures (85°, 75°, 65° and 45°C) was determined.

5.11.2 Results and Discussion

The Michaelis-Menten relationship revealed strong substrate inhibition at all temperatures tested (Fig. 5.16; 5.17). No significant changes in Km values at various temperatures were observed (Fig. 5.17).



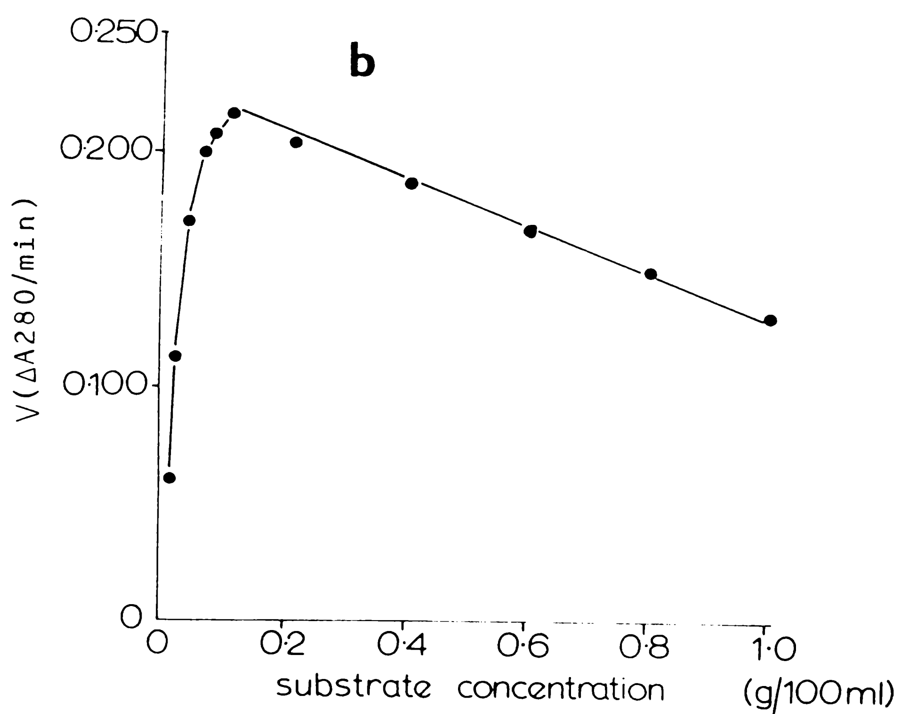


FIGURE 5.10 MICHAELIS-MENTEN PLOTS FROM THE HYDROLYSIS OF CASEIN (a) AND AZO-ALBUMIN (b) BY CALDOLASE.

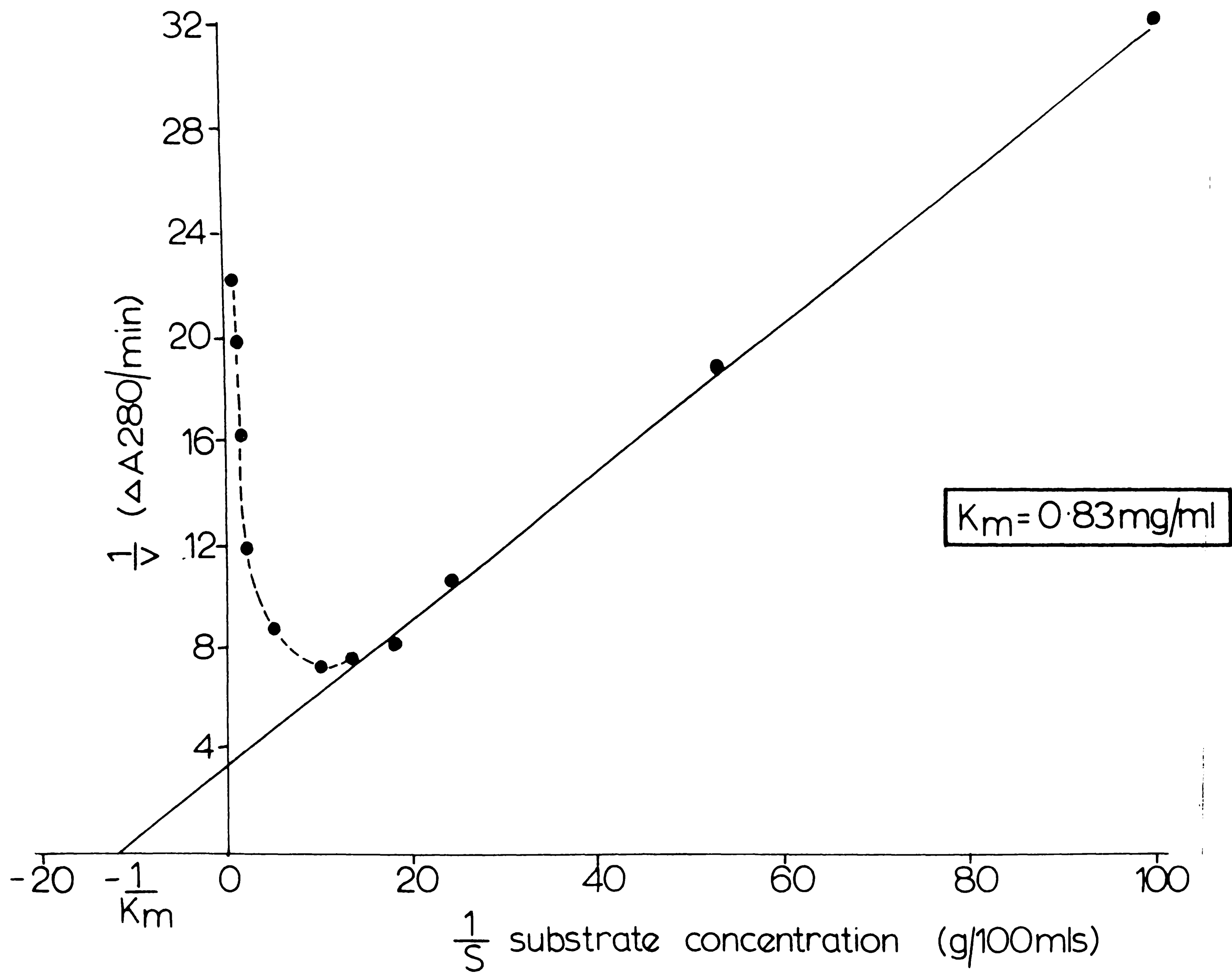


FIGURE 5.11 LINEWEAVER-BURK PLOT FOR THE HYDROLYSIS OF CASEIN BY CALDOLASE

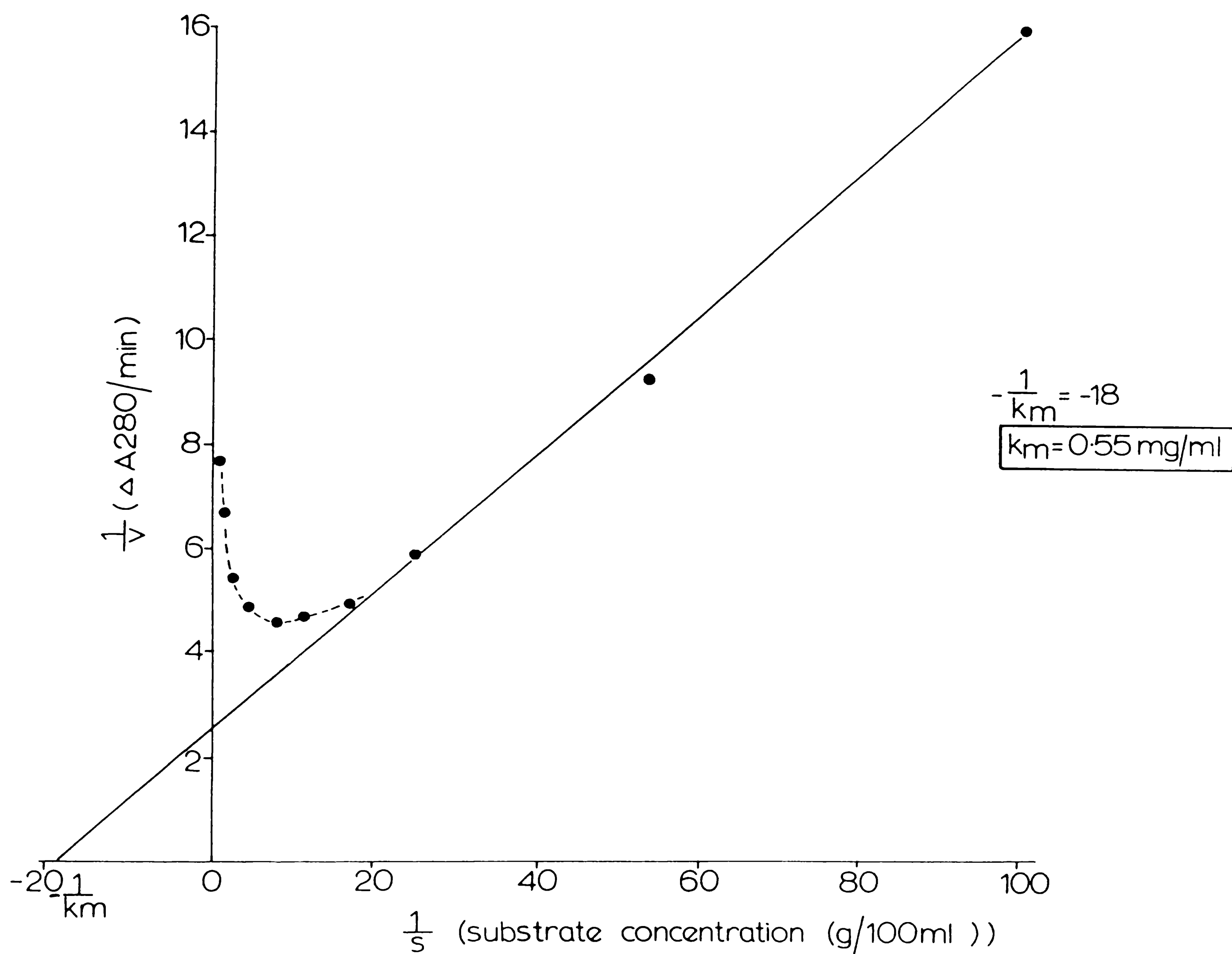


FIGURE 5.12 LINEWEAVER-BURK PLOT FOR THE HYDROLYSIS OF AZO-ALBUMIN BY CALDOLASE

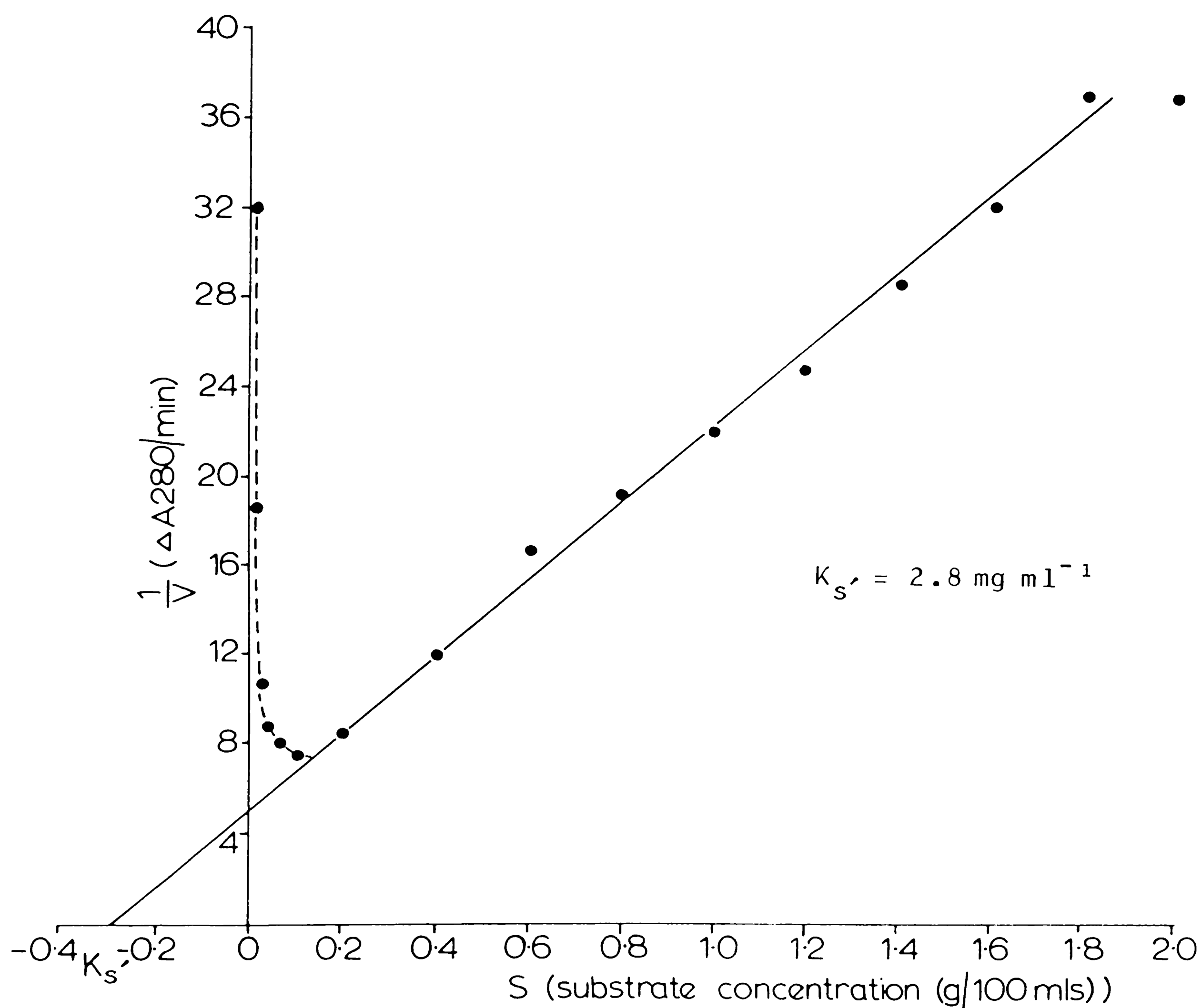


FIGURE 5.13 DETERMINATION OF THE INHIBITION CONSTANT ($K_{s'}$) FROM DATA IN FIGURE 5.11 (HYDROLYSIS OF CASEIN.

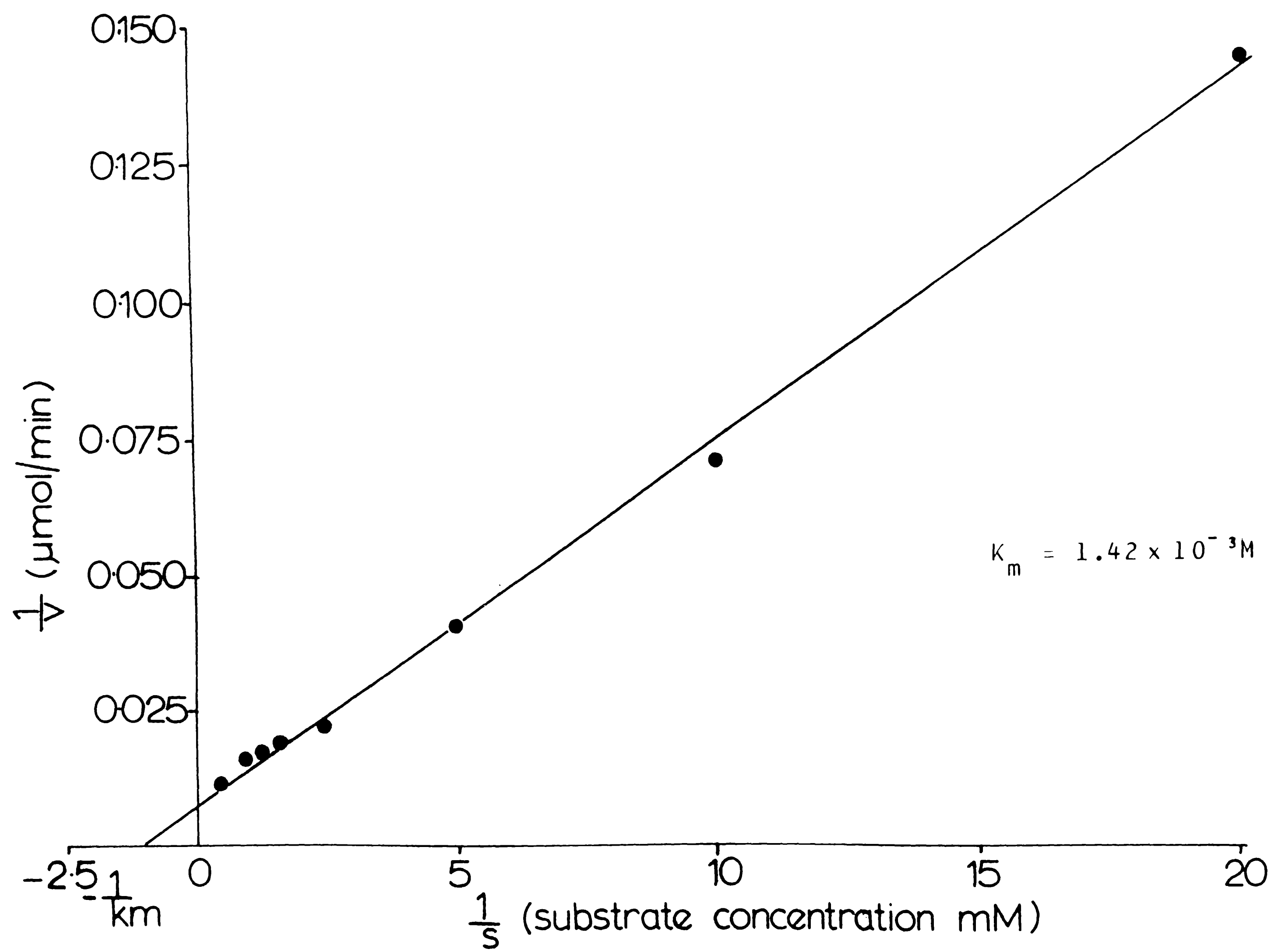


FIGURE 5.14 LINEWEAVER-BURK PLOT FROM THE HYDROLYSIS OF N-succinyl-ala-ala-ala-p-nitroanalide.

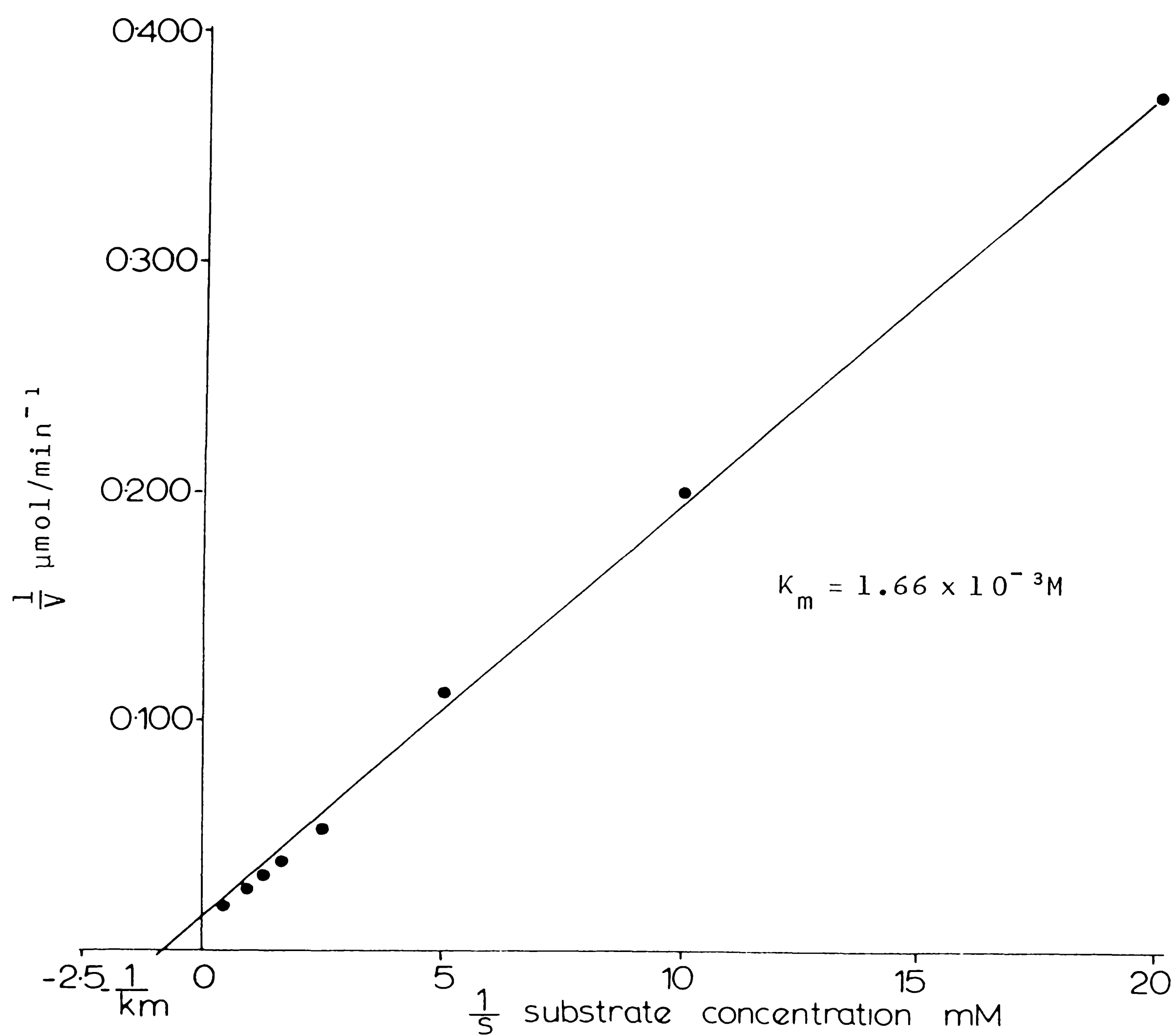


FIGURE 5.15 LINEWEAVER-BURK PLOT FROM THE HYDROLYSIS OF N-CBZ-L-TRYPTOPHANE P-NITROPHENYL ESTER

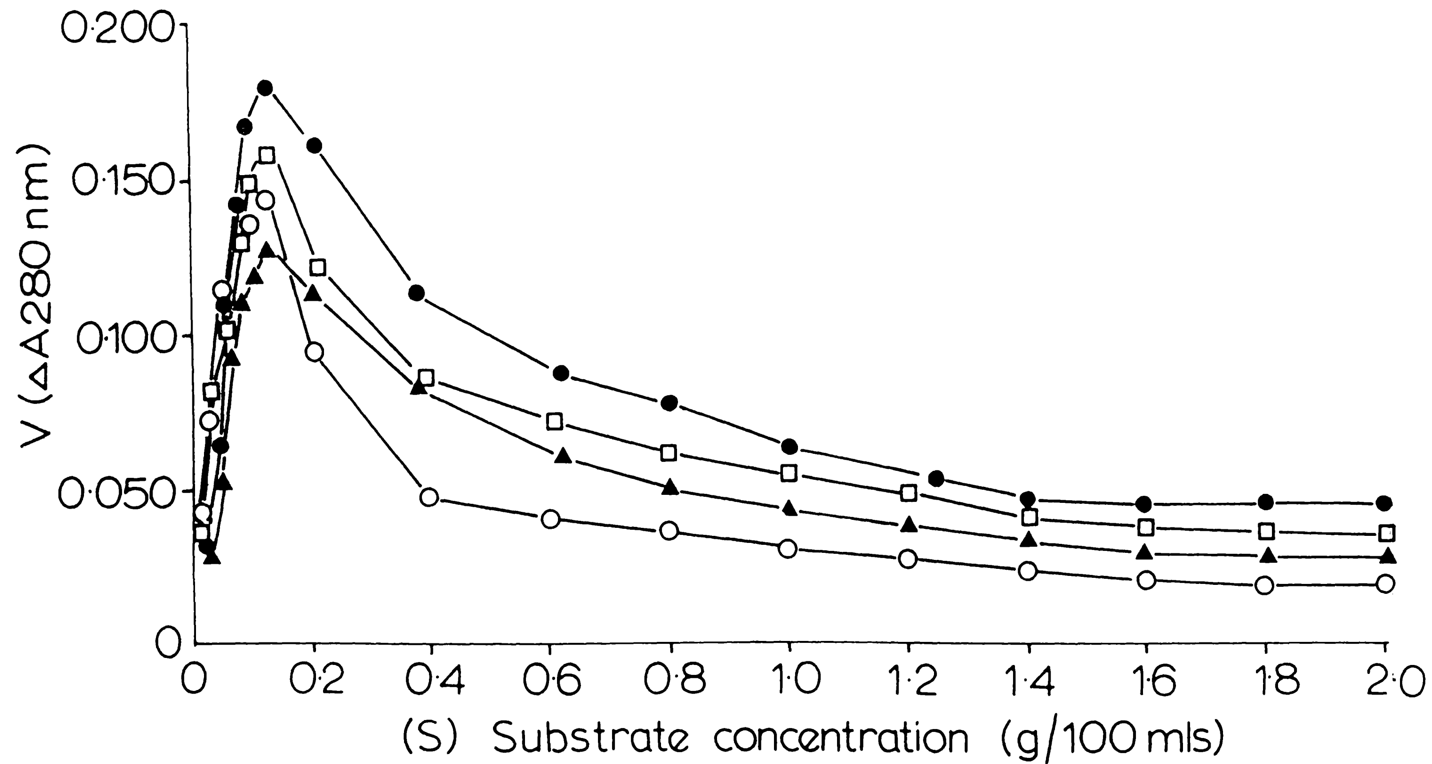


FIGURE 5.16 THE INFLUENCE OF TEMPERATURE ON THE Km FOR CALDOLASE

Michaelis-Menten plots from hydrolysis of casein by the enzyme at 45 °C (○), 65 °C (□), 75 °C (▲), and 85 °C (●).

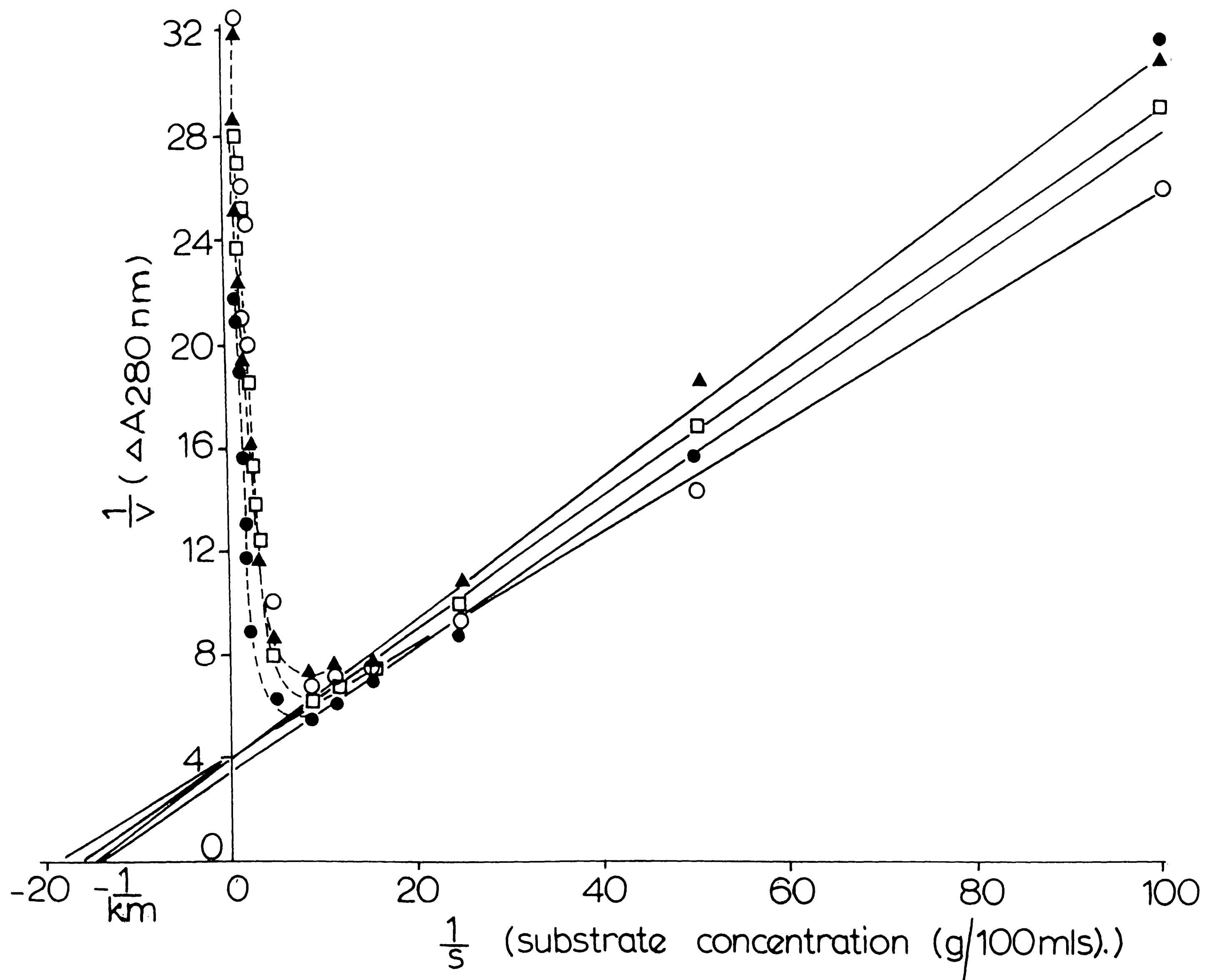


FIGURE 5.17 THE INFLUENCE OF TEMPERATURE ON Km FOR CALDOLASE.

Lineweaver-Burk plot from the hydrolysis of casein by the enzyme at 45°C (○), 65°C (□), 75°C (▲) and 85°C (●).

6. SUBSTRATE SPECIFICITY

A considerable range of proteins and synthetic peptides are available as substrates for estimation of protease activity. Included below are a large number of proteins, dye-bound proteins, synthetic peptides, chromophore-linked synthetic peptides and esters which were utilised as substrates for Caldolase.

6.1 Hydrolysis of Proteins

The hydrolysis of proteins by Caldolase was evaluated by two methods:

i Agar plate method (a qualitative assay), and

ii The standard Kunitz assay (quantitative)

6.1.1 Agar Plate Method

6.1.1.1 Method

A number of proteins and dye-bound proteins (casein, fibrin, haemoglobin, azocoll, elastin and elastin-congo red) were dissolved in agar and the protease assays carried out as described in Chapter 2.

6.1.1.2 Results and Discussion

Caldolase was able to hydrolyse casein, fibrin, haemoglobin, elastin, azocoll and elastin-congo red (Fig. 6.1, 6.2 and Table 6.1).

To provide a comparison to this enzyme several other proteases were used as the controls. These included Thermolysin at 40 μg ml^{-1}, Papain, Trypsin, a metalloprotease from *Bacillus polymyxa* and Subtilisin BPN′ each at a concentration

of 80 μg ml^{-1}. A comparison of zone diameters formed on plates containing various substrates is given in Table 6.1. Caldolysin showed a very weak activity toward elastin and elastin-congo red. Trypsin apparently was not able to hydrolyse these two substrates (Fig. 6.2). The remaining enzymes were capable of hydrolysing all the substrates (Fig. 6.1, 6.2).

6.1.2 The Kunitz Assay

6.1.2.1 Method

The standard Kunitz method (see Chapter 4 for details) was used for hydrolysis of casein. In addition to casein, several other substrates such as fibrin (Abdel-Fattah and Ismail, 1984), collagen, albumin, ovalbumin and haemoglobin (Cowan, 1980) have been utilised as substrates for Caldolase. Protease activity was determined at 75°C and 35°C for 15 and 30 minutes respectively.

6.1.2.2 Results and Discussion

The purified Caldolase hydrolysed several protein substrates (Table 6.2). The enzyme had a very weak activity toward elastin, while collagen showed the highest degree of susceptibility to proteolysis. In contrast Thermolysin has a strong elastase activity (Morihara & Tsuzuki, 1966). As Table 6.2 shows, at 75°C the rate of hydrolysis of all substrates tested, when compared with casein, was higher than at 35°C. This will be due to the structural changes of these substrates at high temperatures, resulting in increased accessibility of susceptible peptide bonds to the enzyme as well as enhancement of reaction rate by temperature. Casein can also not be excluded from this phenomenon.

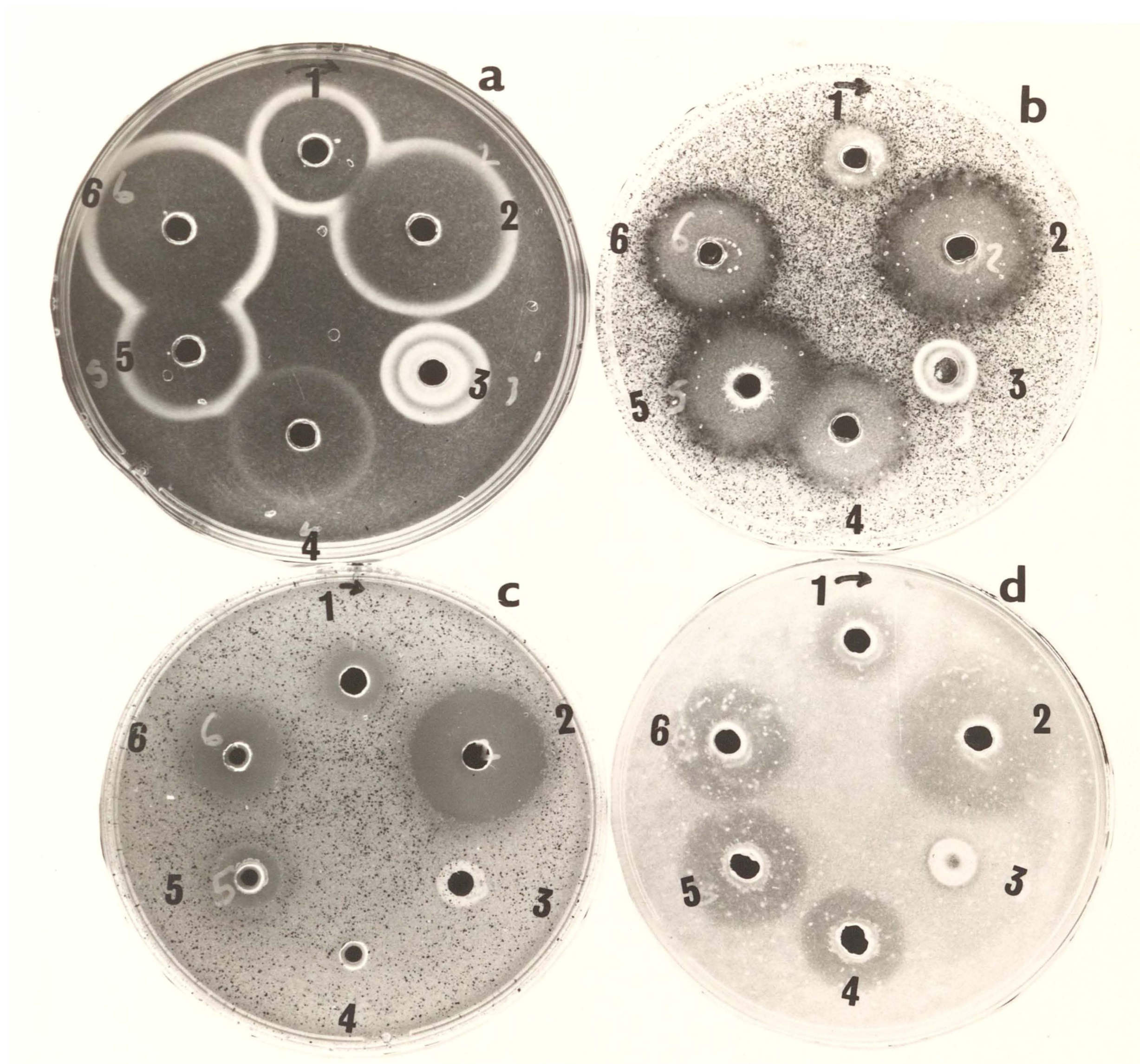


Fig. 6.1 Zones formed on plates containing casein (A), Azocoll (B), Elastin-congo red (C) and Fibrin (D) after 18 hours of incubation at 50°C with Caldolase at 12 $\mu g ml^{-1}$ (1) Thermolysin at 40 $\mu g ml^{-1}$ (2) Papain (3), Trypsin (4), a metal protease from *B. polymyxa* (5), and (6) subtilisin BPN′ each at 80 $\mu g ml^{-1}$.

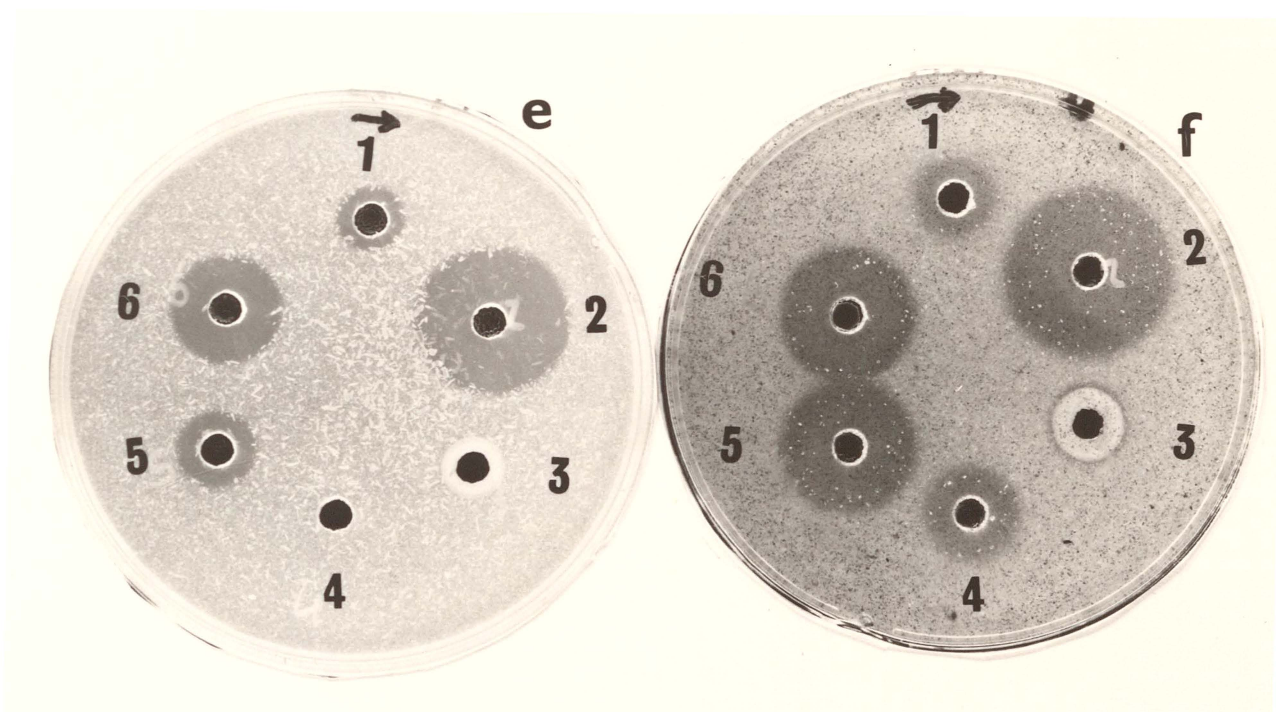


Fig.6.2 Zones formed on plates containing Elastin (E) and Haemoglobin (F) after 18 hours of incubation at 50°C with Caldolase at 24 μgml^{-1} for elastin and 12 μgml^{-1} for haemoglobin (1), Thermolysin at 40 μgml^{-1} (2), Papain (3), Trypsin (4), a metal protease from *B.polymyxa* (5), and (6) subtilisin BPN′ each at 80 μgml^{-1}.

Table 6.1 Zone Diameters Formed (mm) On Plates Containing Various Substrates.

Substrate	Caldolase (a)	Thermolysin (b)	Trypsin (c)	Metal protease from *B. polymyxa* (c)	Subtilisin BPN' (c)
	ENZYMES				
Casein	15	22	16	16	24
Azocoll	14	21	15	19	15
Elastin-congo red	5	11	0	4	10
Fibrin	9	19	11	14	14
Elastin	5	15	0	7	10
Haemoglobin	7	18	9	14	14

(a) Caldolase at 12 $\mu g ml^{-1}$ for casein, azocoll, haemoglobin and fibrin; and 24 $\mu g ml^{-1}$ for elastin and elastin-congo red

(b) Thermolysin at 40 $\mu g ml^{-1}$

(c) Enzymes at 80 $\mu g ml^{-1}$

6.2 HYDROLYSIS OF DYE-LINKED PROTEINS

6.2.1 Methods

Each protein was dissolved at a concentration of 0.1% (w/v) in 0.1M Tris acetate pH 8.0. The amount of chromophore remaining in the supernatant after TCA precipitation of unhydrolysed protein was measured at 440nm (azo-casein, azo-albumin and azocoll) or at 495 (elastin-congo-red) (Cowan, 1980; Shotton, 1970). Enzyme activity was expressed as ΔA 440nm min^{-1} or ΔA 495nm min^{-1} based on the type of the chromogenic substrate being used. Protease activity was carried out at 75^{0}C and 35^{0}C for 15 and 30 minutes respectively.

6.2.2 Results and Discussion.

The susceptibility of azo-albumin and azocoll were greater than that of azo-casein in contrast to the results for the unmodified substrates. The hydrolysis of elastin-congo red was very poor (Table 6.2).

Table 6.2 Relative Rates of Proteins Hydrolysis by Caldolase

Substrate	Temperature (°C) 75	35
Casein	100 [c]	100 [c]
Bovine Serum Albumin	62	34
Ovalbumin	31	8
Haemoglobin	99	68
Collagen Type I (Lot 108C-8010) [d]	61	31
Collagen Type I (Lot 21F-8000) [d]	154	67
Fibrin	52	39
Elastin	> 1	> 1
Azo-casein [a]	100	100
Azo-albumin [a]	133	127
Azo coll [a]	124	115
Elastin-Congo Red [b]	1.8	1.2

(a) O.D = 440nm

(b) O.D = 495nm

(c) Actual rates of Casein hydrolysis at 75°C and 35°C were $\Delta A280\ min^{-1} = 0.069$ and $\Delta A280\ min^{-1} = 0.0027$ respectively.

(d) Both collagens, purchased from Sigma Company, were of the same type but with different lot numbers. They gave two different inexplicable results.

6.3 CONTINUOUS PROTEASE ASSAY USING CHROMOGENIC PEPTIDE SUBSTRATES.

6.3.1 Method

A number of synthetic chromogenic peptide substrates were evaluated for Caldolase activity. Each substrate was dissolved at a concentration of 0.25mM in 0.1M Tris acetate pH 8.0 containing 10% methoxy ethanol (Bergstrom, 1977). 0.9ml of substrate was preincubated at 70°C in 1 cm quartz cuvettes in a thermostated compartment of a Unicam SP1800 Ultraviolet Spectrophotometer equipped with a chart-recorder. The reaction was then initiated by the addition of 100 µl enzyme (25 µl ml^{-1}). The hydrolysis of the substrate was continuously monitored at 410nm. The rate of substrate hydrolysis was calculated from the molar absorption coefficient of the chromophore for p-nitroaniline (ε_M^{440} = 9400M^{-1} cm^{-1}) (Bergström, 1977). The protease activity was expressed as mM min^{-1} of the substrate utilised.

6.3.2 Results and Discussion

Caldolase was capable of cleaving several synthetic peptides (Table 6.3). Of these, benzoyl-phe-val-arg-pNA showed the greatest susceptibility toward enzyme hydrolysis, while hydrolysis of benzoyl-pro-phe-arg-pNA proceeded very slowly. The enzyme failed to hydrolyse several of the smallest synthetic peptides in Table 6.3. This suggests that the minimum requirement of Caldolase for hydrolysis of a synthetic peptide substrate is at least four groups (amino acid residues and terminal blocking group). A similar conclusion has been reached by Cowan and Daniel (1982a) for Caldolysin. *Myxobacter* has a size requirement of at least a tetrapeptide (Jackson & Wolfe, 1968).

Table 6.3 Hydrolysis of Synthetic Peptides By Caldolase

Substrate	Rate of hydrolysis ($mM\ min^{-1}$)
Benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide HCl	9.23×10^{-2}
Benzoyl-L-valyl-glycyl-L-arginine-p-nitroanilide HCl	1.4×10^{-2}
Tosyl-glycyl-L-prolyl-arginine-p-nitroanilide acetate	1.69×10^{-3}
N-CBZ-glycyl-L-prolyl-L-arginine-p-nitroanilide	1.19×10^{-3}
N-succinyl-alanyl-alanyl-L-alanine-p-nitroanilide	0.989×10^{-3}
N-CBZ-glycyl-L-prolyl-L-arginine-p-nitroanilide	0.923×10^{-3}
N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide	very low
Benzoyl-DL-arginine-p-nitroanilide	no hydrolysis
N-succinyl-L-phenylalanine-p-nitroanilide	" "
N.acetyl-DL-phenylalanine-p-nitroanilide	" "
N-CBZ-L-phenylalanine-p-nitroanilide	" "

6.4 CONTINUOUS ESTERASE ASSAY USING CHROMOGENIC ESTER SUBSTRATES.

The assay is based on the cleavage of an ester bond in a small molecular weight synthetic substrate and measurement of the rate of liberation of the coloured p-nitrophenol in a spectrophotometer.

6.4.1 Method

Esterase activity of Caldolase (25 µg ml^{-1}) was determined using the method of Abdelal *et al* (1977). The reaction mixture contained 0.5 ml of 0.6 M Tris-HCl in methanol pH 8.0, 50 µl of 5 mM substrate in dioxane, and 350 µl deionised water. The rate of hydrolysis was measured in a similar manner to that mentioned for the chromogenic peptide substrate assay. The incubation temperature used was 50°C and p-nitrophenol released was measured at 400 nm. The molar extinction coefficient for p-nitrophenol is (ε_M^{440} = 18750) at pH 8.0 (Martin, *et al*, 1959). The esterase activity was expressed as mM min^{-1} of the substrate utilised.

6.4.2 Results and Discussion

Caldolase was able to hydrolyse all synthetic ester substrates tested (Table 6.4). Most of these substrates showed a great susceptibility toward the enzyme. Owing to the instability of some of the ester substrates, such as CBZ-lys-pNE, the esterase activity was carried out at 50°C. In contrast to the synthetic peptide substrates, Caldolase hydrolysed a large number of synthetic ester substrates containing three groups (amino acid and blocking group).

Table 6.4 Hydrolysis of Synthetic Ester Substrates by Caldolase

Substrate	Rate of hydrolysis (mM min^{-1})
N-CBZ-L-alanine-p-nitrophenyl ester ((N-Carbobenzoxy-L-alanine-p-nitrophenyl ester))	7.1×10^{-2}
N-CBZ-L-tyrosine-p-nitrophenyl ester	6.2×10^{-2}
N-CBZ-L-phenylalanine-p-nitrophenyl ester	6.0×10^{-2}
N-CBZ-L-leucine-p-nitrophenyl ester	5.94×10^{-2}
N-CBZ-L-tryptophan-p-nitrophenyl ester	5.59×10^{-2}
N-CBZ - lycine-p-nitrophenyl ester	3.91×10^{-2}
CBZ-asparagine-p-nitrophenyl ester	3.2×10^{-2}
CBZ-β.cysteine-p-nitrophenyl ester	2.93×10^{-2}
CBZ-β.asparagine-p-nitrophenyl ester	2.84×10^{-2}
N-CBZ-L-valine-p-nitrophenyl ester	1.47×10^{-2}
N-CBZ-β.alanine-p-nitrophenyl ester	1.0×10^{-2}
N-CBZ-D-norleucine-p-nitrophenyl ester	9.9×10^{-3}
N-CBZ-L-isoleucine-p-nitrophenyl ester	1.32×10^{-3}
N-CBZ-L-proline-p-nitrophenyl ester	1.05×10^{-3}
Nα-CBZ-L-Lysine-p-nitrophenyl ester HCl	*

* Too unstable at 50°C to determine enzymic rate of hydrolysis.

6.5 COMPARISON STUDIES OF PROTEASE AND ESTERASE ACTIVITY OF CALDOLASE.

A series of experiments were designed to determine whether Caldolase possessed esterase activity or whether the esterase was present as a contaminant in the highly purified protease.

i The eluted fractions from the Sephadex G.75 gel filtration column were assayed for protease and esterase activity. The results revealed that the ratio of protease to esterase activity of all fractions were similar (Fig.6.3).

ii The highly purified lyophilized Caldolase was re-run through an HPLC gel permeation column, The eluted fractions showed the same ratio of protease to esterase activity. (results have not been shown).

iii Inhibitor study: The purified Caldolase was incubated in 5mM of EDTA, EGTA, p-chloromercuribenzoic acid (PCMBA), PMSF and di-phenyl carbamoyl chloride (DPCC) at room temperature for 90 minutes. No significant differences were found between the susceptibility of the two activities (Table 6.5)

iv Thermostability: Caldolase samples (25 $\mu l\ ml^{-1}$) were incubated at room temperature, $90^{0}C$ and $95^{0}C$ for a period of 50 min. The protease and esterase activity of each sample were determined. The ratio of protease to esterase activity was essentially the same (Table 6.6).

The most likely conclusion which can be drawn from these results is that Caldolase possesses esterase activity. This conclusion is supported by evidence that only a single protein is present (see Chapter 5).

Table 6.5 Inhibitors Assay

Inhibitor	Protease (% inhibition)	Esterase (% inhibition)
EDTA	2.8	0
EGTA	0	0
PCMBA	0	0
PMSF	72	83
DPCC	97	100

Table 6.6 Temperature Effect On Protease and Esterase Activity

Temperature	Protease (% inactivation)	Esterase (% inactivation)
Room temperature	0	0
90°C	54	57
95°C	78	72

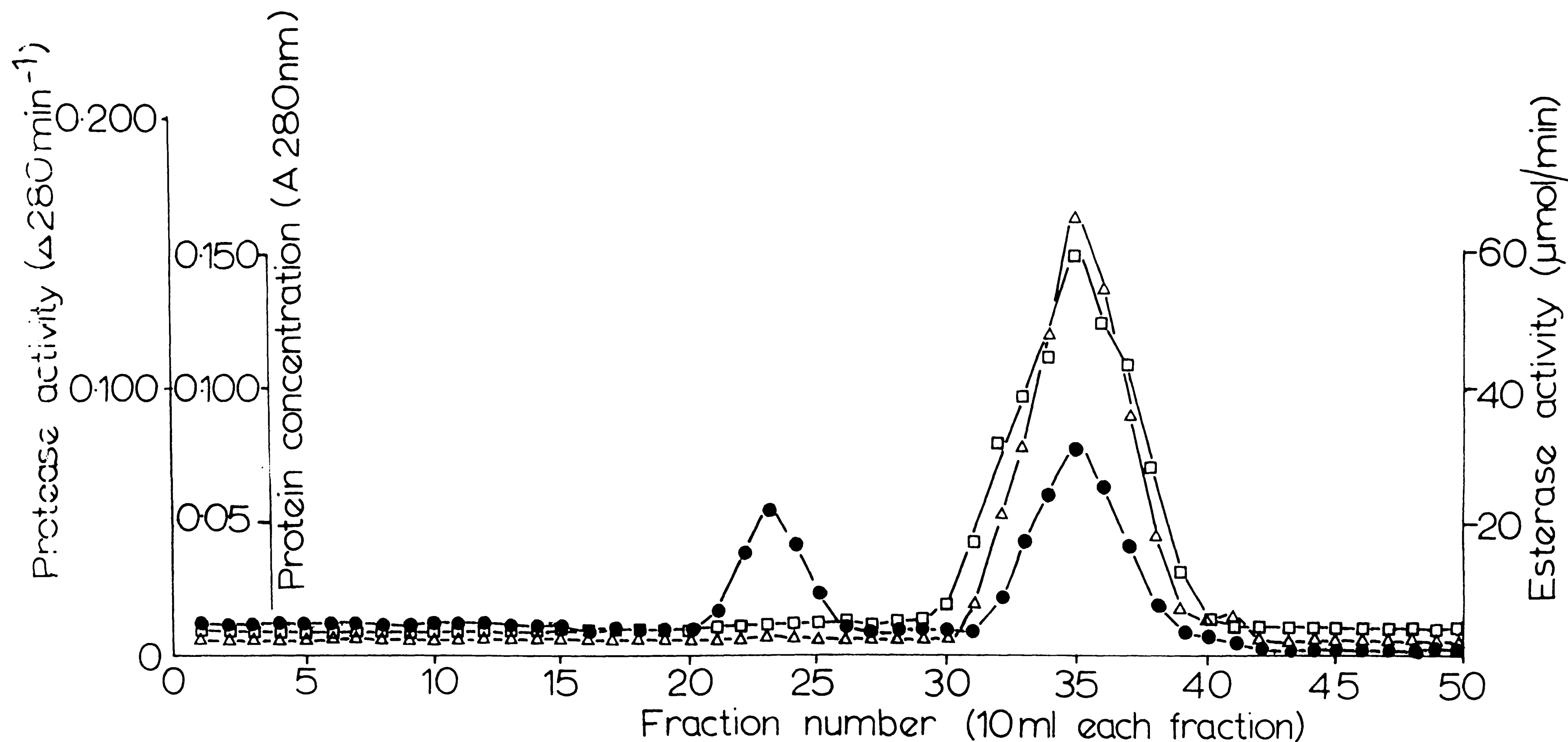


FIGURE 6.3 PURIFICATION OF CALDOLASE BY G-75 SEPHADEX CHROMATOGRAPHY FOR PROTEASE AND ESTERASE ACTIVITY COMPARISON.

4ml enzyme were applied to a sephadex G-75 column, and the enzyme was eluted with 0.1M Tris acetate pH 8.0 containing 0.5M NaCl + 10mM $CaCl_2$. 10ml fractions were collected. The protein concentration (●), protease activity (Δ) and esterase activity (□) were determined.

6.6 CLEAVAGE SITES OF BRADYKININ AND INSULIN B CHAIN

6.6.1 Bradykinin Hydrolysis

6.6.1.1 Methods

i Hydrolysis of Bradykinin by Caldolase

A reaction mixture containing Caldolase and Bradykinin (enzyme-substrate ratio ≃ 1/10,000) was incubated for time intervals of 0 seconds, 30 seconds, 1 min. and 15 min. at 50°C. The reaction was terminated by lowering the pH to ≃ 2.5 using H_2PO_4 solution (5 μl concentrated acid was added to a mixture of 110 μl enzyme-solvent solution). Controls containing enzyme only and Bradykinin only were included.

ii Acid hydrolysis and amino acid analysis.

Acid hydrolysis and amino acid analysis of each sample were carried out as described in Section 5.6

6.6.1.2 Results

The results revealed that Caldolase was not able to hydrolyse Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) during the reaction periods which were tested. This suggests that the enzyme may lack affinity towards non-polar amino acids.

6.6.2 Insulin B Chain Hydrolysis

An attempt was made to identify the cleavage site of insulin B chain by Caldolase.

6.6.2.1 Methods

i Purification of the substrate

A 100 μg insulin B chain in 50 μl solvent (20mM KH_2PO_4, pH2.5, CH_3CN) was purified by a reverse phase column using the ~~above~~ solvent. The fractions of the major insulin peak were pooled and used for hydrolysis by Caldolase.

ii Substrate hydrolysis and amino acid analysis

Hydrolysis of purified insulin B chain by Caldolase acid hydrolysis (using 6M HCl containing 7% thioglycolic acid) and amino acid analysis of all samples were carried out as described above for Bradykinin. Reaction times of 1 min., 5 min., 10 min. and 30 min. were used.

6.6.2.2 Results and Discussion.

In contrast to Bradykinin, cleavage of insulin B chain even for short reaction periods resulted in a very complex pattern, suggesting a low degree of enzyme specificity.

7. ENZYME STABILITY

7.1 THERMOSTABILITY OF CALDOLASE

7.1.1 Introduction

Most enzymes from extremely thermophilic micro-organisms remain stable at temperatures which result in the denaturation of many mesophilic enzymes. However, at high temperatures, thermophilic proteases may lose activity as a result of either autolysis or denaturation. In preliminary experiments no distinction has been made between the two effects.

7.1.2 Methods

a. The stability of Caldolase at temperatures ranging from 75° to 100°C.

Purified enzyme (24 μg ml^{-1} in 0.1M Tris acetate pH8, containing 0.5M NaCl and 10mM $CaCl_2$) was incubated in a 7 ml Kimax Hungate tube, (in one ml volume) sealed with a rubber-lined metal screw cap. Aliquots of enzyme solution were removed at intervals, and the residual protease activity was determined at 75°C by the Kunitz method.

b. The stability of Caldolase at temperatures ranging from 100° to 110°C.

Aliquots of 30 μl of enzyme (24 μg ml^{-1} in 0.1M Tris acetate buffer pH8 containing 0.5M NaCl and 10mM $CaCl_2$) were sealed in melting-point capillary tubes and heated in a polyethylene glycol 400 bath at temperatures from 100° to 110°C for known periods. Each sample tube was then immediately transferred to iced water. All incubations were carried out in duplicate or triplicate. The residual protease activity of each sample was measured at 75°C by the standard Kunitz method.

7.1.3 Results and Discussion

Stability profiles at temperatures between 75°C and 110°C are presented in Fig. 7.1 and 7.2. The half-lives of the enzyme are listed in Table 7.1 and 7.2. The half-life of Caldolase at 100°C was 3.5 minutes. Loss of enzyme activity at temperatures above 100°C was very rapid; at 106°C the half life of the enzyme was less than one minute and at 110°C 99% of enzyme activity was lost within two minutes.

A difference in volume of the enzyme sample and type of tubes used were responsible for the variation in the half lives at 100°C between Table 7.1 and 7.2 (see Section 7.1.2 Methods).

Table 7.1 Half-Lives Data for Caldolase at 80° - 100°C

Temperature (°C)	t ½ (min)
100	5
95	10
90	45
85	150
80	840

Table 7.2 Half-Lives Data for Caldolase at 100° - 110°C

Temperature (°C)	t ½ (min)
110	0.4
108	0.5
106	0.6
104	1.5
102	2.5
100	3.5

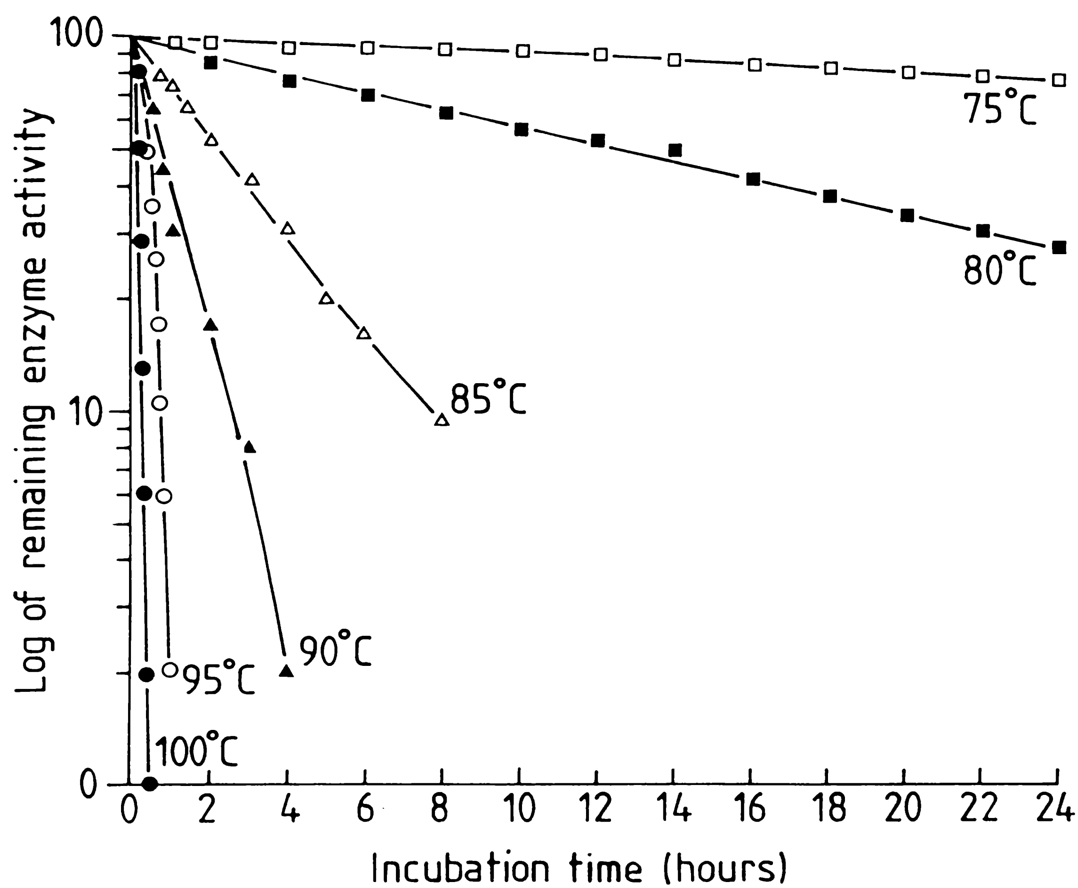


FIGURE 7.1 THE STABILITY OF CALDOLASE AT TEMPERATURES RANGING FROM 75°C to 100°C.

Purified enzyme (24 μg ml^{-1} in 0.1M Tris acetate pH8.0 containing 0.5M NaCl + 10mM $CaCl_2$) were incubated at specified temperatures. Aliquots of enzyme solution were removed at intervals, and the residual protease activity was determined at 75°C by the Kunitz method.

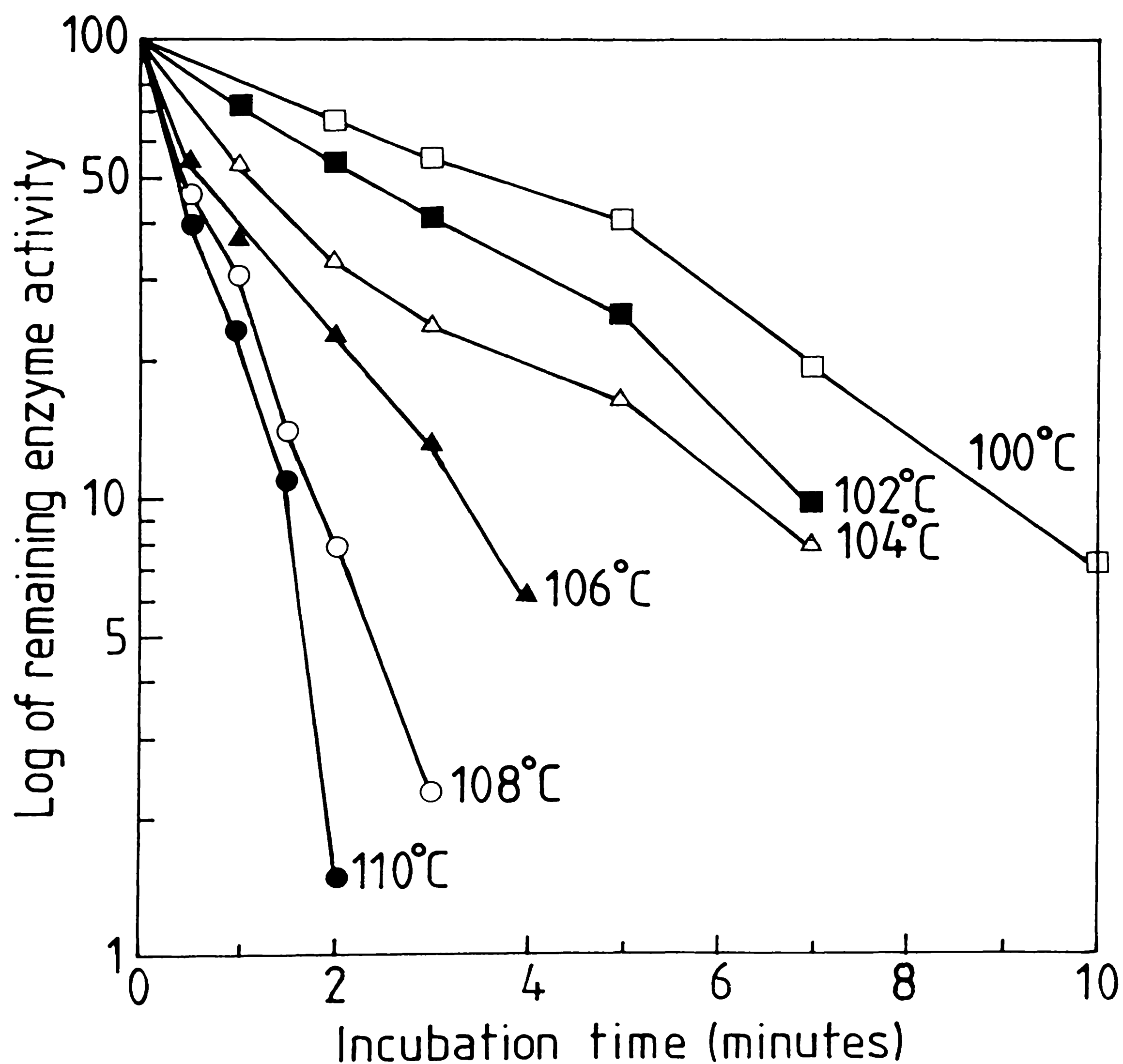


FIGURE 7.2 THE STABILITY OF CALDOLASE OVER 100°C.

Purified enzyme (24 μg ml^{-1} in 0.1M Tris acetate pH 8.0 containing 0.5M NaCl + 10mM $CaCl_2$) were incubated at specified temperatures. Aliquots of enzyme solution were removed at intervals, and the residual protease activity determined at 75°C by the Kunitz method.

Caldolase demonstrated a very high degree of thermal stability and is thus comparable with Caldolysin which had a half-life at 90°C of one hour (Cowan and Daniel, 1982a). For comparison, the half lives of some thermophilic and mesophilic proteases are listed in Table 7.3.

Table 7.3 The Thermostability of Proteases From Thermophilic and Mesophilic Organisms.

Protease	Source	T ½ (min)	Temp.	Reference
Thermolysin	*B. Thermo Proteolyticus* (T)	60	80	Ohta *et al*, (1966)
Thermomycolase	*Malbranchea Pulchella* (T)	110	73	Ong and Gaucher, (1976)
Neutral Protease	*B. Subtilis* (M)	20	60	McConn *et al*, (1964)

(T) = Thermophile
(M) = Mesophile

Caldolysin and the proteases listed in Table 7.3 are calcium stabilised. Removal of calcium ions causes a substantial loss of thermostability (Cowan and Daniel, 1982a). Calcium ions are not apparently involved in the stabilisation of Caldolase (see below).

7.2 THE EFFECT OF pH ON CALDOLASE STABILITY

7.2.1 Method

Lyophilised enzyme was dissolved in distilled water and immediately diluted tenfold in Universal buffers (Dawson *et al*, 1969) at pHs ranging from 3 - 12. The enzyme samples were then incubated for 90 minutes at room temperature (22^{0}C). The activity of each sample was determined at 75^{0}C by the Kunitz method, using 50 µl of Caldolase solution per assay.

7.2.2 Result and Discussion.

Under the conditions of this experiment, Caldolase was stable to a broad pH range. The maximum enzyme stability was at pH 7 - 10 (Fig. 7.3). No significant changes in conductivity of the Universal buffers occurred at high pHs, indicating that ionic strength had no interference in this experiment.

Chell and Sundaram (1978) have reported the disruption of external salt bridges in Malate Synthase from thermophilic bacilli at extreme pHs, resulting in the enzyme destabilisation.

Ohta (1967) has noted 19 hydrogen bonds in Thermolysin. Having found a greater decrease of enzyme stability at 80^{0}C than at 20^{0}C, he concluded that the disruption of hydrogen bonds at pHs above 9 combined with a temperature of 80^{0}C was responsible for the loss of enzyme stability.

The above findings indicate the involvement of a variety of factors in enzyme destabilisation at extreme pHs.

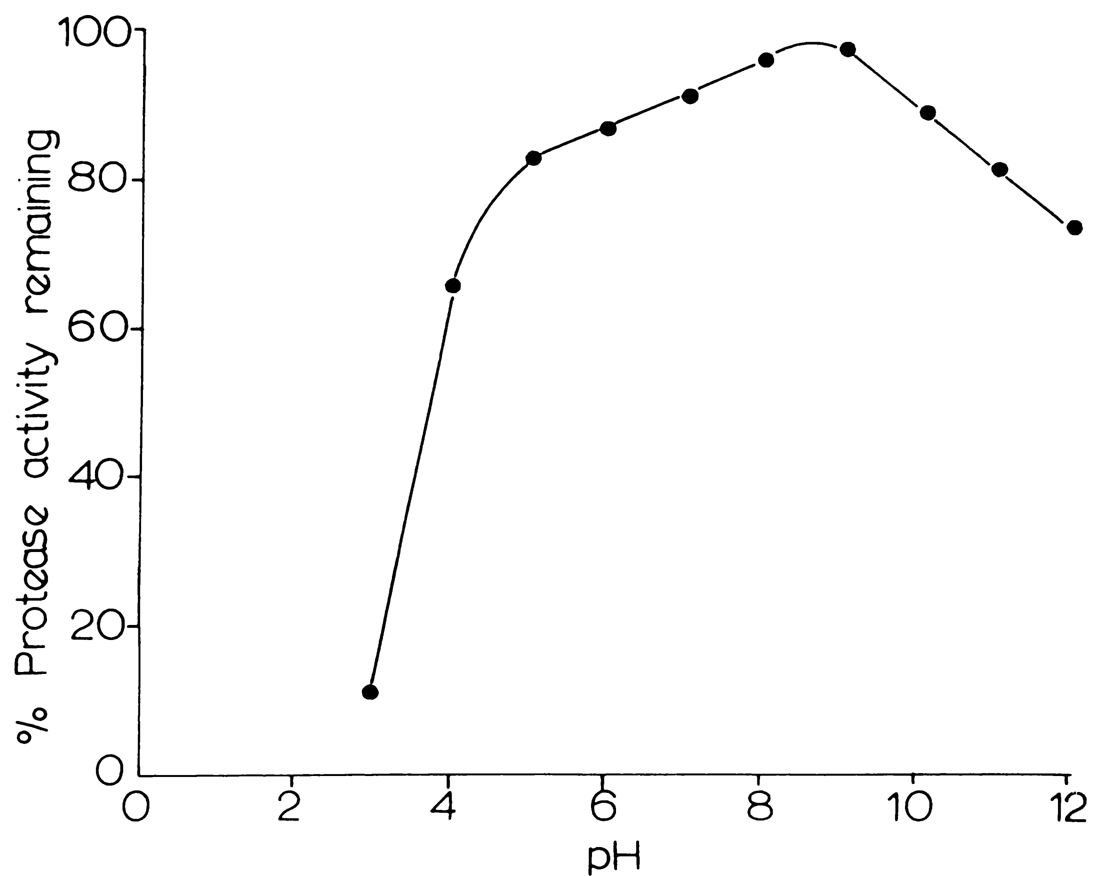


FIGURE 7.3 THE EFFECT OF pH ON CALDOLASE STABILITY

The enzyme samples in the Universal buffer were incubated at 22°C for 90 min at pHs ranging from 3 - 12. Protease activity of each enzyme sample was determined at 75°C.

7.3 THERMOSTABILITY OF CALDOLASE IN THE PRESENCE OF METAL CHELATING AGENTS.

7.3.1 Introduction

None of the metal chelators (EDTA, EGTA and O-phenanthrolin) used had any effect on the catalytic activity of Caldolase (see Section 5.5). To determine whether Ca^{2+} or Zn^{2+} ions were involved in the stabilisation of the enzyme, the following experiments were carried out. Two well-characterised proteases, Thermolysin and Caldolysin were included for comparison.

7.3.2 Methods

Each protease was incubated in 10mM O-phenanthroline at 75°C. The enzyme activity of the samples was monitored over a period of 30 minutes using 0.1% azo-casein substrate in 0.1 M Tris acetate pH8.0. The same procedure was repeated using EGTA and EDTA but with 0.2% casein as a substrate. In the case of EDTA, protease activity was monitored over a period of 40 minutes.

7.3.3 Results and Discussion.

Thermolysin was entirely inactivated shortly after the addition of any one of the above chelating agents. It is well established (e.g. Tajoma *et al*, 1976) that zinc is essential for catalytic activity, and that calcium ions are involved in the molecular stabilisation of Thermolysin. Caldolysin was also inactivated after incubation at 75°C confirming the results of Cowan & Daniel (1982a) which showed that Ca^{2+} ions were necessary for the stability of this enzyme. Neither the activity nor stability of Caldolase was affected by the chelating agents (Fig. 7.4). This result suggests

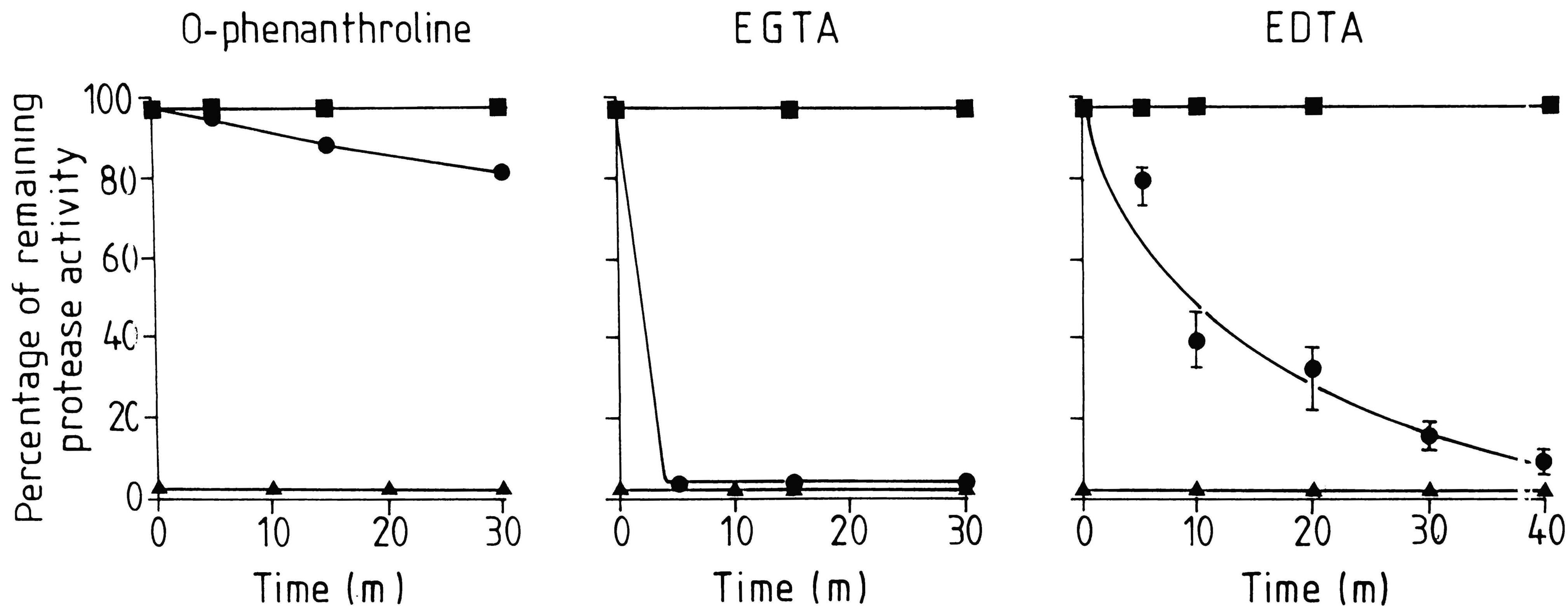


FIGURE 7.4 THERMOSTABILITY AT 75° C OF CALDOLASE, CALDOLYSIN AND THERMOLYSIN IN THE PRESENCE OF METAL CHELATORS.

The protease activity of Caldolase (■), Caldolysin (●) and Thermolysin (▲) in the presence of O-phenanthrolin, EGTA and EDTA was measured at specified times.

that neither Ca^{2+} nor Zn^{2+} ions are involved in either the active site of the enzyme or in its molecular stabilisation. The results further confirm that Caldolase is a chelator-insensitive enzyme and thus different from the other two proteases.

7.4 EFFECT OF DENATURING AND REDUCING AGENTS

7.4.1 Introduction.

Proteins show various degrees of susceptibility toward denaturing agents (Tanford, 1968). Although urea is the most widely employed denaturant, many proteins are not fully denatured at urea concentrations of up to 8M. GuHCl is a more effective protein denaturant. Detergents are unique among protein denaturants in that they are often able to produce marked structural disruption at very low concentrations (10^{-5} - 10^{-2}M) (Mahler and Cordes, 1971). This can be explained only by the existence of strong binding forces between the detergent and protein molecule. By contrast the denaturation of proteins by urea and GuHCl occurs at a very high concentration. This can be attributed to a weak binding force between the protein and these denaturants, and thus only a very minute fraction of the total denaturant present in solution can actually be bound to the protein molecule.

To assess the stability of Caldolase in the presence of these denaturants the following experiments were carried out using azo-casein and a synthetic peptide, N-Succinyl-alanyl-alanyl-L-alanine-p-nitroanilide, as substrates.

7.4.2 Methods

Caldolase (12 μgml^{-1} in 0.1M Tris acetate buffer pH8.0 containing 0.5M NaCl and 10mM $CaCl_2$) was incubated for 90 minutes at 22°C, 60 minutes at 75°C and for 30 minutes at 85°C in the following denaturing and reducing agents; SDS, urea, guanidin-HCl, urea + 1% mercaptoethanol, mercaptoethanol and dithiothreitol. The protease activity of each enzyme sample was then determined at 75°C by the Kunitz method using 0.1% azo-casein in 0.1M Tris acetate buffer pH8.0, or by using the tripeptide substrate, suc-ala_3-pNA, (see Section 6.3 for method). In the latter assay, a protease concentration of 24 μgml^{-1} was used.

7.4.3 Results and Discussion.

Caldolase activity was little affected after incubation in urea and guanidine-HCl [these reagents are known to disrupt intramolecular hydrogen bonds and hydrophobic interactions (Cockle and Epand, 1978)], at low temperature, but reduced significantly after incubation at high temperatures (85°C). (Table 7.4).

Mercaptoethanol and dithiothreitol (reducing agents active against disulphide bonds) had no significant effect on enzyme stability at either low or high temperatures (Table 7.4). This suggests that either disulphide bonds are in the interior of the molecule and thus well protected from the solvent, or they are not involved in the stability of Caldolase. The enzyme showed a degree of susceptibility toward SDS, a detergent known to dissociate hydrophobic bonds (Yonath *et al*, 1977), both at low and high temperatures (Table 7.4). This supports the involvement of hydrophobic interactions in protease stability.

It is noteworthy that above a crucial concentration (9.16 x 10^{-3}M at 55^{0}C) detergent can form micelles (Mukerjee & Mysels, 1971). When this occurs, the enzyme may possibly become enveloped in these globular structures and thus inaccessible to the substrate. Micelle formation is a reversible process and dilution of the mixture would release the enzyme. In this study the concentration of SDS in the enzyme solution was either 0.034M or 0.017M, and was further diluted in the assay mixture by a factor of 21, well below the critical micelle concentration therefore micelle formation phenomena should not interfere significantly in the above experiments.

Caldolysin was unstable at 75^{0}C in the presence of mercaptoethanol and dithiothreitol (Cowan, 1980), but revealed significant resistence toward SDS at 18^{0}C (Cowan and Daniel, 1982a). Like Caldolase, it was very stable in the presence of guanidine-HCl and urea. (Cowan and Daniel, 1982a).

7.4.4 The Effect of Denaturing Agents on the Protein Substrates.

It has been shown that the addition of denaturing agents (GuHCl. Tween 80 or Triton x-100) to the casein substrate increased the apparent enzyme activity of Caldolysin considerably (Cowan and Daniel, 1982b). This increase may have been due to denaturation of the protein substrate, making available a greater number of susceptible cleavage sites. Therefore in addition to azo-casein substrate a low molecular weight synthetic peptide, succinyl ala-ala-ala-p-nitroanilide, was used as a comparison. Having no tertiary structure, it is unlikely that this substrate would be affected by denaturants. The similarity of the results (Table 7.5) suggests

that the effect of denaturants on the casein substrate was not significant, due possibly to the fact that the final concentration of these agents in the assay mixture was very low (e.g. the final concentration of urea in the assay mixture was 0.38M).

It is possible that although the enzyme might unfold in a denaturing agent, subsequent dilution into the substrate could cause renaturation.

Table 7.4 The Effect of Denaturing and Reducing Agents on Caldolase (Casein Substrate).

	% Protease activity remaining		
	22 °C (90 min.)	75 °C (60 min.)	85 °C (30 min.)
Control (buffer only)	100	99	91
8M urea	102	31	6
4M urea	100	82	39
6M GuHCl	99	20	3
3M GuHCl	100	71	21
8M urea + 1% mercaptoethanol	101	44	11
1% SDS	49	41	35
0.5% SDS	70	66	54
1% mercaptoethanol	99	97	88
1mM dithiothreitol	100	100	93
5mM "	100	98	94
10mM "	99	91	88

Table 7.5 The Effect of Denaturing and Reducing Agents on Caldolase (Suc.ala-ala-ala-pNA Substrate)

	% Protease activity remaining		
	22 °C (90 min.)	75 °C (60 min.)	85 °C (30 min.)
Control (buffer only)	100	100	92
8M urea	103	35	4
6M GuHCl	93	16	3
1% mercaptoethanol	99	97	90

7.5 THE EFFECT OF ORGANIC SOLVENTS ON CALDOLASE STABILITY

7.5.1 Introduction

Many protease inhibitors (PMSF, Di-phenyl carbamyl chloride), synthetic peptides and ester substrates (see Chapter 6 for further description) are insoluble in water. These inhibitors or substrates were first dissolved in an organic solvent and the buffer was subsequently added to give the desired concentration.

The aim of this experiment was to determine the effect of various organic solvents on enzyme stability in order to select the most suitable solvents in which to dissolve the appropriate protease inhibitors or substrates.

7.5.2 Method.

Organic solvents were added to aqueous solutions of protease to give final concentrations of 50 and 80% solvent. The mixtures were then incubated at room temperature for one hour. Protease activity of each enzyme sample was determined by Kunitz method, using 0.1% azocasein in 0.1M Tris acetate pH 8.0.

7.5.3 Results and Discussion.

No significant effect on Caldolase activity was observed when using di-methyl sulphoxide, ethanol and propanol. Enzyme activity was increased in the presence of 2-methoxy ethanol, 1,4 dioxan and acetone (Table 7.6). This is probably due to the effect of these solvents on the substrates. It is well documented that denatured protein is more susceptible to proteolysis (Daniel *et al*, 1982). The final concentrations of each solvent in the assay mixture were 2.38% and 3.8%. These results further confirm the stability of Caldolase at room temperature.

Table 7.6 The Effect of Organic Solvents on Protease Stability.

Organic Solvent	Percentage	Substrate	% Enzyme Activity Remaining
di-methyl sulphoxide	50	0.2% Casein	96
	80	"	91
2-methoxy ethanol	50	"	117
	80	"	125
1,4-dioxan	50	"	113
	80	"	108
control	0	"	100
acetone	50	0.1% azo-casein	89
	80	"	113
ethanol	50	"	106
propanol	50	"	99
control	0	"	100

7.6 THE EFFECT OF IONIC STRENGTH ON ENZYME STABILITY.

(a) The Effect of NaCl

7.6.1 Introduction

When lyophilised protease containing a very low salt concentration was dissolved in distilled water or 0.1M Tris acetate pH8, enzyme activity was significantly reduced. Further experiments using various buffers with different salt concentrations were carried out to determine the effect of ionic strength on enzyme stability.

7.6.2 Method.

The lyophilised protease was dissolved in various salt concentrations (0.1M Tris acetate buffer pH 8.0 containing 0.1M to 0.7M NaCl) and incubated at 22°C or 75°C for 60 minutes. The protease activity of each sample was determined at 75°C using the Kunitz method.

(b) Buffer Effects

7.6.3 Method.

10ml of Caldolase (35 $\mu g ml^{-1}$ in 0.5M sodium acetate pH 7.5) were dialysed against distilled water for 24 hours at 4°C. The enzyme sample was diluted five-fold with various buffers. The samples were then incubated at 75°C for 60 minutes and protease activity of each enzyme sample was assayed at 75°C by Kunitz method.

7.7 THE EFFECT OF VARIOUS METALS ON STABILITY OF CALDOLASE.

7.7.1 Introduction

The importance of ionic strength in protease activity and stability has already been demonstrated (see Tables, 7.7; 7.8). To establish whether ionic strength was a major determining factor in the activity or stability of Caldolase or whether the presence of any specific metal was involved, the following experiment was conducted.

7.7.2 Method.

Caldolase was purified by TSK gel filtration chromatography using 0.5M sodium acetate pH 7.5 as eluent. The enzyme solution was then dialysed against distilled water at 4°C and freeze-dried. The lyophilised enzyme was dissolved in 0.1M Tris acetate pH 8.0, and diluted 10-fold in various salt solutions (10mM of each salt was dissolved in 0.1M Tris acetate buffer pH 8.0). The mixtures were then incubated at either 22°C or 75°C for 60 minutes or at 85°C for 30 minutes. Protease activity of each sample was determined at 75°C using 0.2% casein or 0.1% azo-casein in 0.1M Tris acetate pH 8.0.

7.8 RESULTS AND DISCUSSION

The results suggest that Caldolase was unstable at low salt concentration (Fig. 7.5) and that enzyme stability increased considerably in the presence of various salts (Table 7.7 and 7.8). It is concluded that ionic strength plays a major role in structural stabilisation, possibly by protecting hydrogen bonds and strengthening hydrophobic interactions (see Section 7.4.3 for further discussion).

An interesting point is that enzyme stability appears to be dependent to some extent upon the specific ion, e.g. protease activity and stability are somewhat higher in the presence of salts with divalent cations ($CaCl_2$ and $MgCl_2$) and cobalt nitrate than with other salts tested. (Table 7.9). Enzyme activation by various salts has been reported by other authors. Shannon *et al*, (1982) have shown that an intracellular protease from *Rhodococcus erythropolis* was able to be activated two - threefold in the presence of NaCl and KCl at an ionic strength of 0.2 - 0.5, fourfold by $MgCl_2$ at an ionic strength of 0.05 - 0.15 and 1.5-fold by $CaCl_2$ at an ionic strength of 0.05.

The activation and stabilisation of several other enzymes by various salts has been reported. Griffiths and Sundaram (1973) showed that isocitrate lyase was activated by a variety of salts. The enzyme was also thermostable in the presence of KCl at concentrations of up to 0.4M. Hengartner and Zuber (1973) showed that 0.2M NaCl significantly enhanced the thermostability of thermophilic glucokinase from *Bacillus stearothermophilus*. Crabb *et al*, (1977) reported that ionic strength of 1.8 using NaCl is able to

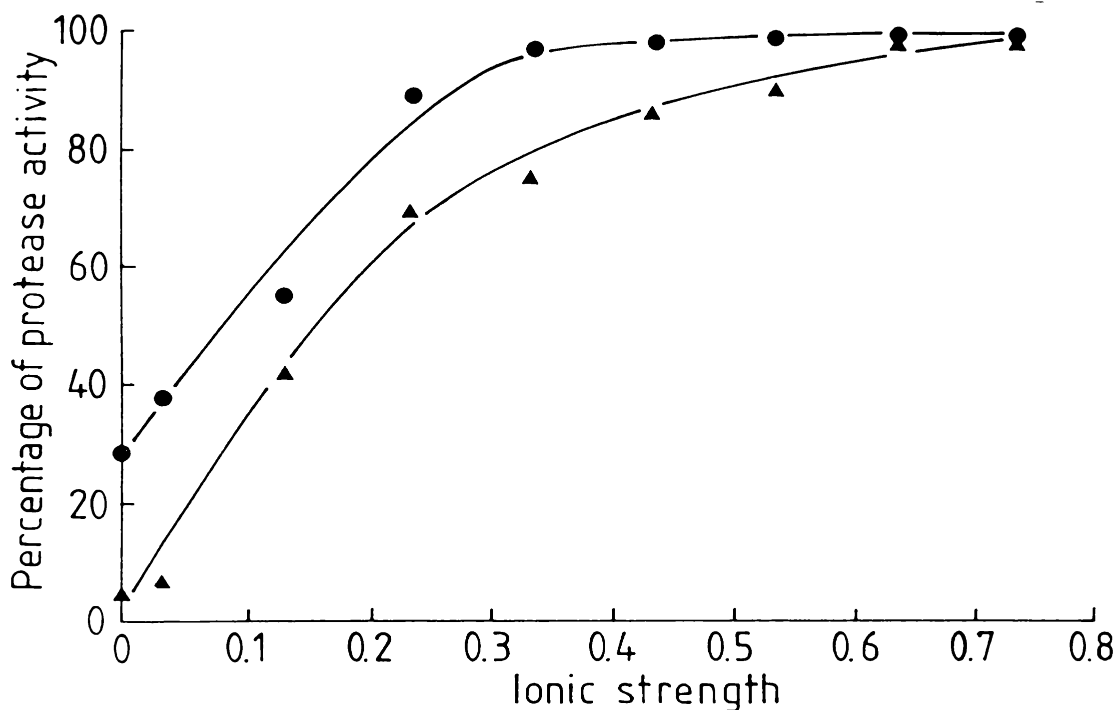


FIGURE 7.5 THE EFFECT OF IONIC STRENGTH ON ENZYME ACTIVITY USING NaCl

The salt-free Caldolase was dissolved in various salt concentrations (0.1M Tris acetate pH8.0 containing 0.1M to 0.7M NaCl) and incubated at 22°C (●) or 75°C (▲) for 60 min. The protease activity of each sample was determined at 75°C using the Kunitz method.

The ionic strength shown above is that calculated for each concentration according to the formula $I = \frac{1}{2} \Sigma m_i Z_i^2$ where Σ = the sum, m_i = molarity of the ion, and Z_i = net charge of the ion. The conductivity of Tris was measured by conductivity meter and converted to ionic strength using NaCl as a standard curve.

protect *Bacillus coagulans* glyceraldehyde-3-phosphate dehydrogenase against thermal denaturation. They proposed that the enhanced thermostability of this enzyme in the presence of high ionic strength might be related to an increase in hydrophobic interactions. They also suggested that a high level of acidic residues in one part of the molecule could destabilise the enzyme by electrostatic repulsion. Increased ionic strength could suppress this repulsion.

Table 7.7 The Effect of Ionic Strength on Enzyme Activity Using NaCl.

Salt	ionic strength (ml^{-1})	% enzyme activity remaining	
		22 °C	75 °C
Distilled water	0	28	4
Tris acetate pH 8	0.03	38	6
Tris acetate + 0.1M NaCl	0.13	55	42
" " + 0.2M NaCl	0.23	91	70
" " + 0.3M NaCl	0.33	98	76
" " + 0.4M NaCl	0.43	99	87
" " + 0.5M NaCl	0.53	100	91
" " + 0.6M NaCl	0.63	101	100
" " + 0.6M NaCl	0.73	101	100

Table 7.8 The Effect of Ionic Strength on Enzyme Stability Using Various Buffers.

Buffer	Ionic Strength	% Enzyme Activity Remaining
distilled water	0	16
0.1M Trisacetate pH8.0	0.03	22
0.1M Tris + 0.1M NaCl	0.13	51
" + 0.2M NaCl	0.23	78
" + 0.4M NaCl	0.43	100
" + 0.1M $CaCl_2$	0.33	148
" + 0.2M $CaCl_2$	0.53	150
" + 0.1M $MgCl_2$	0.33	140
" + 0.2M $MgCl_2$	0.53	153
" + 0.1M KCl	0.13	55
" + 0.2M KCl	0.23	90
" + 0.4M KCl	0.43	103
0.1M Bicine pH8.0	0.023	27
0.2M Bicine	0.046	51
0.4M Bicine	0.092	76
0.1M Hepes	0.024	40
0.2M Hepes	0.028	72
0.4M Hepes	0.096	82
0.1M Sodium Phosphate pH8.0	0.55	58
0.2M " "	1.1	81
0.4M " "	2.2	90
0.1M Ammonium Carbonate pH8.0	0.3	27
0.2M " "	0.6	54
0.4M " "	1.2	79

Table 7.9 The Effect of Various Metals on Protease Stability.

Buffer	Substrate	% Enzyme activity remaining after incubation at:		
		22°C	75°C	85°C
0.1M Tris acetate pH8.0 + 0.5M NaCl + 10mM $CaCl_2$	0.2 % casein	100	100	84
0.1M Tris acetate pH8.0	"	63	14	6
$CaCl_2$ (10mM)	"	75	32	28
$MgCl_2$ (10mM)	"	71	26	20
$ZnCl_2$ (10mM)	"	77	24	18
KCl (10mM)	"	61	14	8
Sodium acetate (10mM)	"	63	8	6
0.1M Tris acetate pH8.0 + 0.5M NaCl + 10mM $CaCl_2$	0.1 % azo-casein	100	100	86
Vanadium chloride (10mM)	"	62	14	12
Cabalt nitrate (10mM)	"	98	70	38

7.9 DETERMINATION OF THE REVERSIBILITY OF PROTEASE INACTIVATION AT LOW SALT CONCENTRATION.

7.9.1 Introduction

As shown in Sections 7.7 and 7.8 Caldolase loses activity at low ionic strength. An investigation was carried out to determine whether or not activity loss at low salt concentration was reversible.

7.9.2 Method.

A solution of Caldolase (38 $\mu g ml^{-1}$) was dialysed against distilled water. The dialysed enzyme was then incubated at 85°C for 15 minutes, at 75°C for 30 minutes, and at 22°C for 60 minutes. The control enzyme solution was incubated on ice-water for 60 minutes. When the salt concentration of the treated enzyme samples was increased (up to 0.4M NaCl + 10mM $CaCl_2$), the following results were obtained (Table 7.10)

7.9.3 Results and Discussion

The results suggest that at 85°C the reactivation of Caldolase is insignificant while the enzyme recovery at 75°C and 22°C is considerable. This suggests that at 22°C in the presence of low ionic strength the enzyme undergoes reversible denaturation. At high temperatures (85°C) the denatured enzyme will probably undergo considerable autolysis and thus lose the capacity for renaturation. (Table 7.10 and Fig. 7.6)

Table 7.10 Determination of the Reversibility of Protease Inactivation at Low Salt Concentration.

Temperature (°C)	Incubation time (minute)	Percentage of enzyme activity	
		Before NaCl	After Addition of NaCl
0 - 2	0	96	100
0 - 2	60	95	100
22	60	14	81
75	30	12	42
85	15	2	8

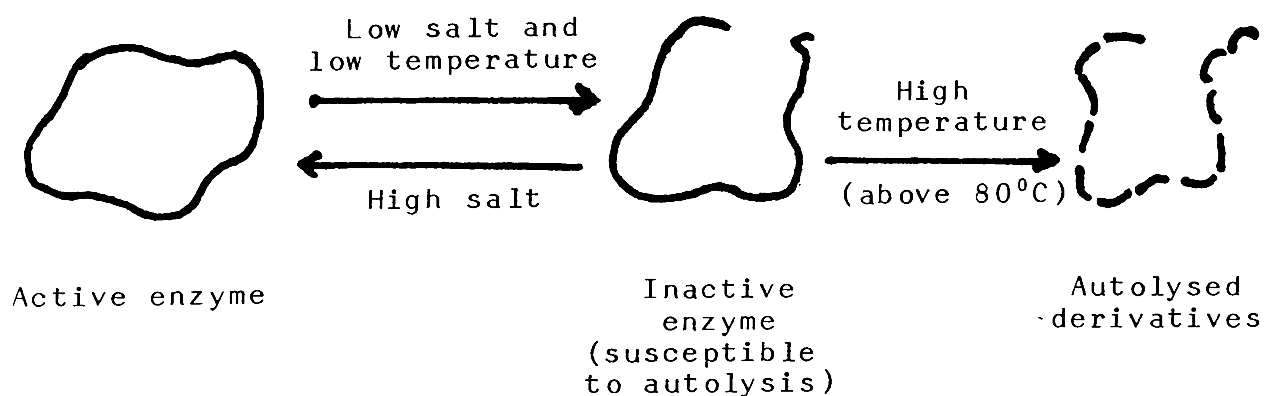


FIGURE 7.6 REVERSIBLE DENATURATION AND AUTOLYSIS OF CALDOLASE

7.10 DENATURATION AND AUTOLYSIS

7.10.1 Introduction

It has been demonstrated that the stability of Caldolase can be maintained in the presence of a variety of salts (Tables 7.7 and 7.8). A more detailed investigation of the denaturation and autolysis of the enzyme in the presence of various salt concentrations and at temperatures ranging from 75° to 95°C was carried out. For this purpose two different procedures were selected (see below).

7.10.2 Methods

High-Performance Liquid Chromatography Analysis and Kinetics Study.

In this study the molecular weight distribution of protease autolysis products were evaluated, using high-performance liquid chromatography (HPLC) (Regnier, 1983) with a column of TSK G3000 SW (developed by Toyo Soda manufacturing Co. Ltd, Japan). Highly purified Caldolase was desalted using Sephadex G-25 gel filtration chromatography at 4°C. The conductivity of the enzyme sample was measured to check that the enzyme was salt-free. The enzyme was then freeze-dried in volumes of 5ml. The contents of each vial was dissolved in one of the following buffers: 0.1M Tris acetate pH8.0, 0.1M Tris acetate pH8.0 containing 0.1M NCl + 10mM $CaCl_2$, 0.1M Tris acetate pH8.0 containing 0.3M NaCl + 10mM $CaCl_2$ and 0.1M Tris acetate pH8.0 containing 0.5M NaCl + 10mM $CaCl_2$. Each enzyme sample (40 $\mu g ml^{-1}$) was then incubated in a Kimax Hungate tube at a chosen temperature (75°, 80°, 85°, 90° or 95°C). Aliquots of enzyme were removed periodically in order to measure the protease activity (20 μl enzyme

solution per assay) and to determine the molecular weight distribution of the components using high-speed gel filtration column chromatography (100 μl enzyme was injected into the column).

The activity data obtained were used in an analysis of the kinetics of enzyme denaturation and autolysis. The reciprocal of the concentration of the remaining active enzyme was plotted against time. Linearity of the plots indicates second-order kinetics (autolysis) (Voorduow and Roche, 1975a). When log (remaining active enzyme)/(total enzyme) vs time is plotted, linearity of the plots implies first-order kinetics (thermal denaturation) (Cowan and Daniel, 1982a; Moore, 1962b).

7.10.3 Results and Discussion.

At high ionic strengths and temperatures up to 85°C, second-order kinetics were predominant, while at 90° and 95°C first-order kinetics were significant (denaturation was dominant). At low ionic strengths second-order kinetics were predominant up to 95°C (Table 7.11 and Figs. 7.7 - 7.10). The results of TSK gel filtration column chromatography demonstrated that either by increasing the incubation time, raising the temperature level or a combination of both, autolysis products are increased (Figs. 7.11 - 7.14).

The above study emphasises that an environment of high ionic strength protects Caldolase against both autolysis and denaturation.

The fact that ionic species protect some proteases against autolysis and thermal or chemical denaturation is well documented. The effect of Ca^{2+} ions on the stability of a number

of proteases has been demonstrated: e g Trypsin (Epstein *et al*, 1974), Chymotrypsin (Chervenka, 1959), Thermolysin (Feder *et al*, 1971, Voorduow and Roche, 1975a), and Caldolysin (Cowan and Daniel, 1982a).

7.11 ANALYSIS OF AUTOLYSED DERIVATIVES OF CALDOLASE

7.11.1 Introduction.

An analysis of Caldolase denaturation and autolysis showed that at least some of the autolysed derivatives of Caldolase still possessed catalytic activity (see Section 10). Further experiments were carried out to determine the extent of autolysis at lower temperatures (22^0 and 60^{0}C) and the catalytic ability of the fragments resulting from the auto-digestion of the enzyme.

7.11.2 Methods

Caldolase was purified by affinity chromatography, then further purified by preparative TSK gel filtration column chromatography. The active fractions were pooled and dialysed against distilled water. This was followed by freeze-drying the salt-free enzyme solution. The lyophilised enzyme was then dissolved in 0.1M Tris acetate buffer pH8.0 and divided into three samples: the first kept on ice for 180 minutes, the second incubated at 22^{0}C for 180 minutes and the third at 60^{0}C for 150 minutes. Each enzyme solution was chromatographed on an analytical TSK column, and fractions of one ml collected. The enzyme activity of all fractions was assayed by the Kunitz method and the relative protein concentration

TABLE 7.11 KINETICS AND TSK GEL FILTRATION COLUMN CHROMATOGRAPHY ANALYSIS OF DATA

Salt Concentration	Technique	Incubation Temperature (°C)				
		75	80	85	90	95
0.1M Tris-acetate pH8.0	Kinetics	2nd order (a)	2nd order (a)	2nd order (a)	2nd order (b)	Enzyme inactivation
	TSK (autolysis)	+	++	+++	+++	++++
0.1M Tris + 0.1M NaCl + 10mM $CaCl_2$	Kinetics	2nd order	2nd order	2nd order	1st order	1st order
	TSK (autolysis)	+	+	++	++++	++++
0.1M Tris + 0.3M NaCl + 10mM $CaCl_2$	Kinetics	2nd order	2nd order	2nd order	1st order	1st order
	TSK (autolysis)	+	?	+++	?	++++
0.1M Tris + 0.5M NaCl + 10mM $CaCl_2$	Kinetics	No activity loss	No activity loss	2nd order	1st order	1st order
	TSK (autolysis)	Insignificant	Insignificant	++	++	++++

(a) - Second-order Kinetics (autolysis is significant)

(b) - First-order Kinetics (denaturation is predominant)

+ = 5 - 10% degradation products

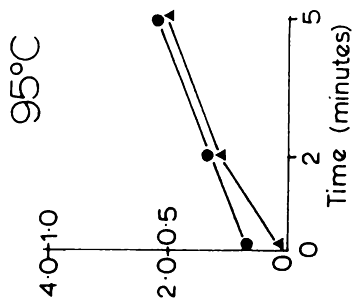
++ = 20 - 40% " "

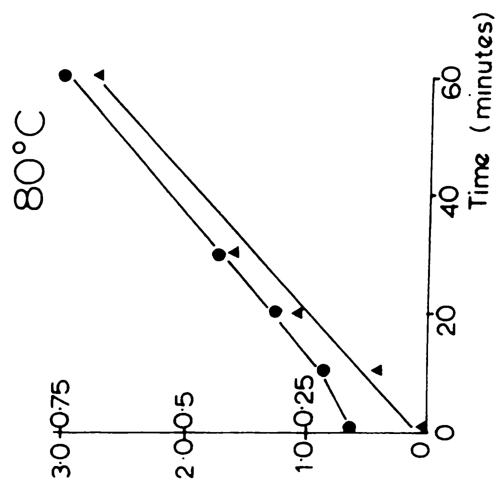
+++ = 50 - 70% " "

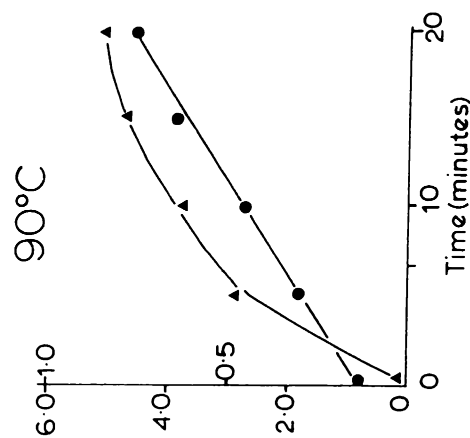
++++ = 80 - 90% " "

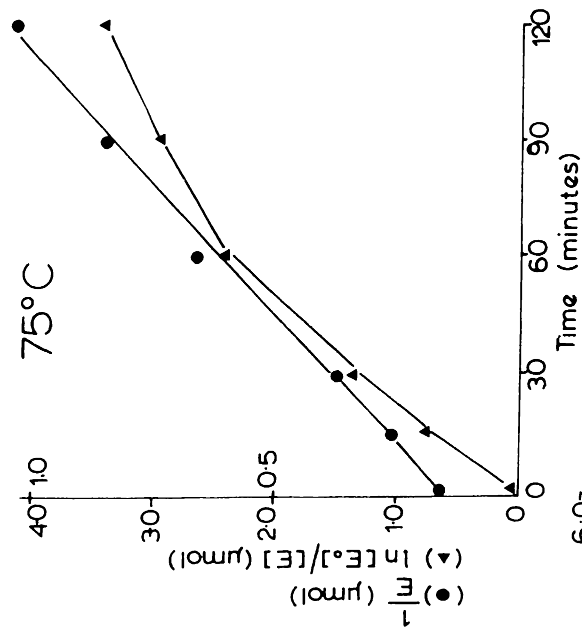
FIGURE 7.7 KINETIC ANALYSIS OF CALDOLASE DENATURATION AND AUTOLYSIS

Thermostability profiles of Caldolase (40 μg ml^{-1} in 0.1M Tris acetate pH8) were obtained at 75° - 95°C. Linearity of plots of $^1/$[residual enzyme] vs. time and ln [residual enzyme]/[total enzyme] vs. time implies first-order kinetics (thermal denaturation) and second-order kinetics (autolysis), respectively.


95°C
Time (minutes)
0
2
5
4·0
2·0
1·0
0·5


80°C
Time (minutes)
0
20
40
60
3·0
2·0
1·0
0·75
0·5
0·25


90°C
Time (minutes)
0
10
20
6·0
4·0
2·0
1·0
0·5


75°C
Time (minutes)
0
30
60
90
120
(●) 1/E (µmol)
(▲) ln[E₀]/[E] (µmol)
40
30
2·0
1·0
1·0
0·5

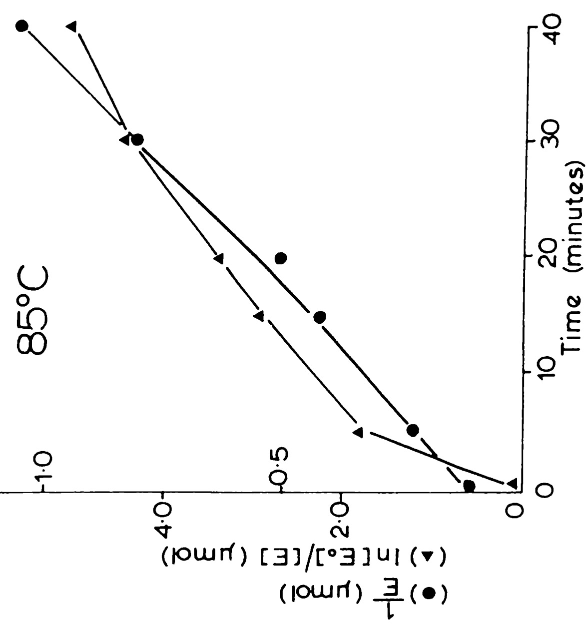
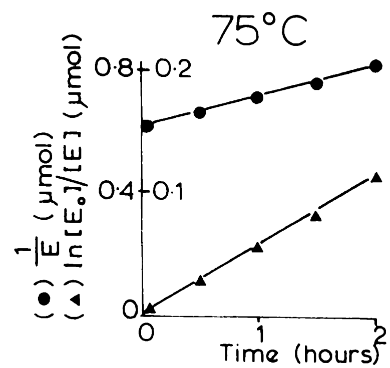
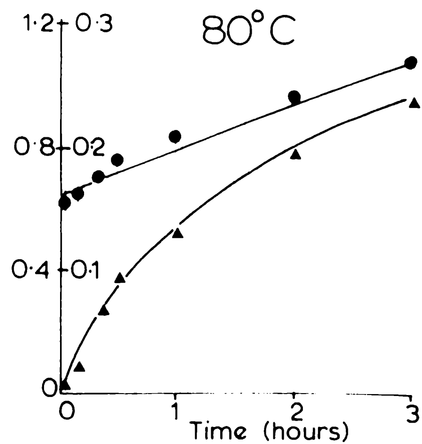
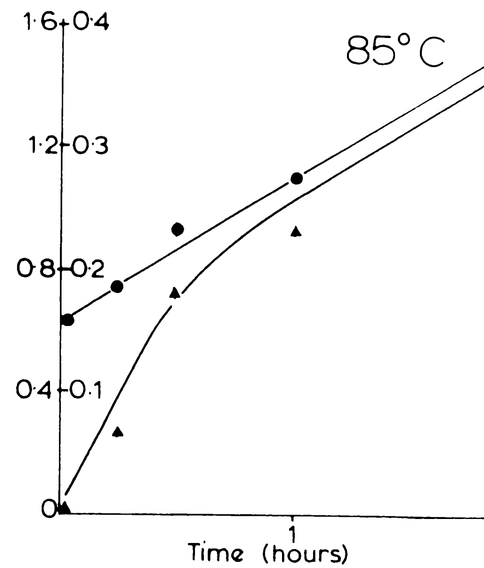

85°C
Time (minutes)
0
10
20
30
40
(●) 1/E (µmol)
(▲) ln[E₀]/[E] (µmol)
6·0
4·0
2·0
1·0
0·5

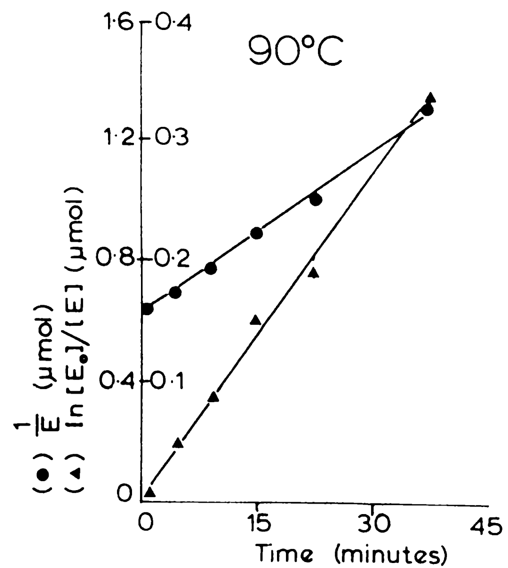
FIGURE 7.8 KINETICS ANALYSIS OF CALDOLASE DENATURATION AND AUTOLYSIS

Thermostability profiles of Caldolase (40 $\mu g\ ml^{-1}$ in 0.1M Tris acetate pH8 containing 0.1M NaCl + 10mM $CaCl_2$) were obtained at $75^0 - 90^0C$. Linearity of plots of $^1/$[residual enzyme] vs. time and ln [residual enzyme]/[total enzyme] vs. time implies first-order kinetics (thermal denaturation) and second-order kinetics (autolysis), respectively.


75°C
(●) 1/E (μmol)
(▲) ln [E_o]/[E] (μmol)
0·8
0·2
0·4
0·1
0
0
1
2
Time (hours)


80°C
1·2
0·3
0·8
0·2
0·4
0·1
0
0
1
2
3
Time (hours)


85° C
1·6
0·4
1·2
0·3
0·8
0·2
0·4
0·1
0
1
Time (hours)


90°C
(●) 1/E (μmol)
(▲) ln [E_o]/[E] (μmol)
1·6
0·4
1·2
0·3
0·8
0·2
0·4
0·1
0
0
15
30
45
Time (minutes)

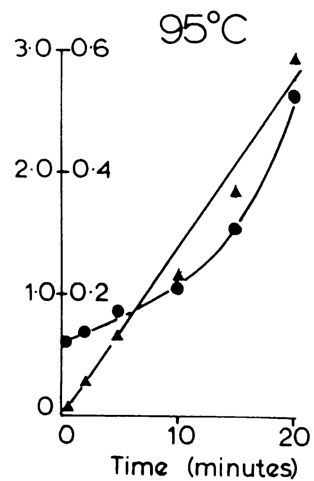
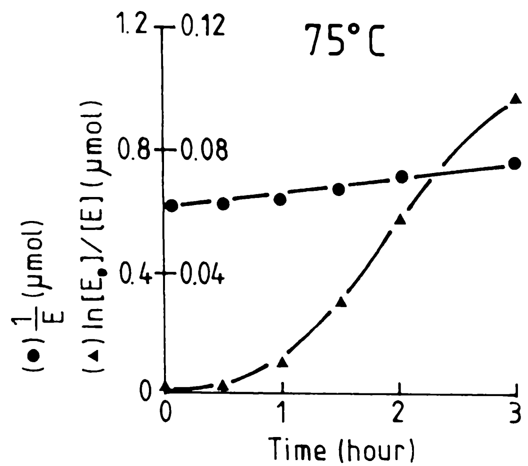
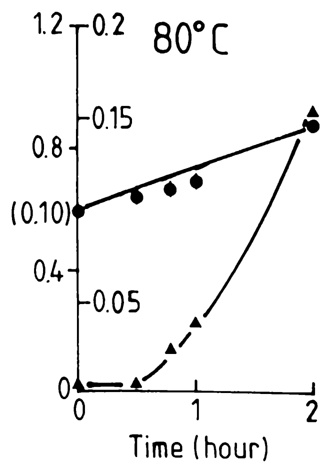

95°C
3·0
0·6
2·0
0·4
1·0
0·2
0
0
10
20
Time (minutes)

FIGURE 7.9 KINETICS ANALYSIS OF CALDOLASE DENATURATION AND AUTOLYSIS

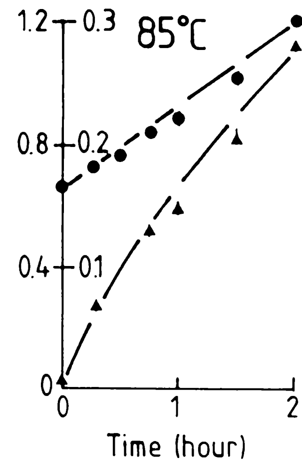
Thermostability profiles of Caldolase (40 μg ml^{-1} in 0.1M Tris acetate pH8 containing 0.3M NaCl + 10mM $CaCl_2$) were obtained at 75° - 95°C. Linearity of plots of $^1/$ [residual enzyme] vs. time and ln [residual enzyme] / [total enzyme] vs time implies first-order kinetics (thermal denaturation) and second-order kinetics (autolysis), respectively.



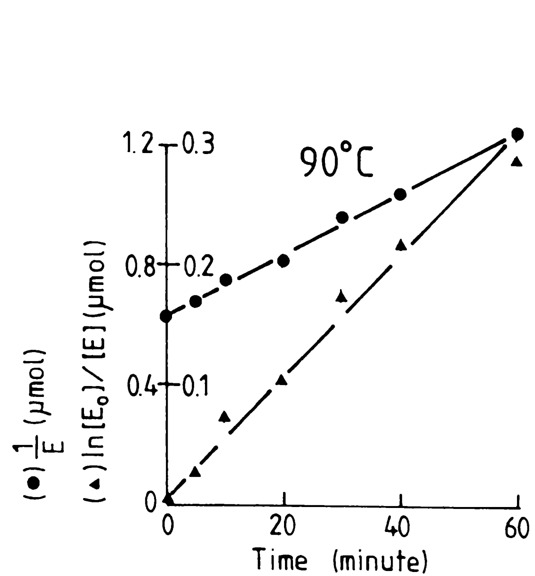
75°C
(●) 1/E (μmol)
(▲) ln[E₀]/[E] (μmol)
1.2 0.12
0.8 0.08
0.4 0.04
0
0 1 2 3
Time (hour)



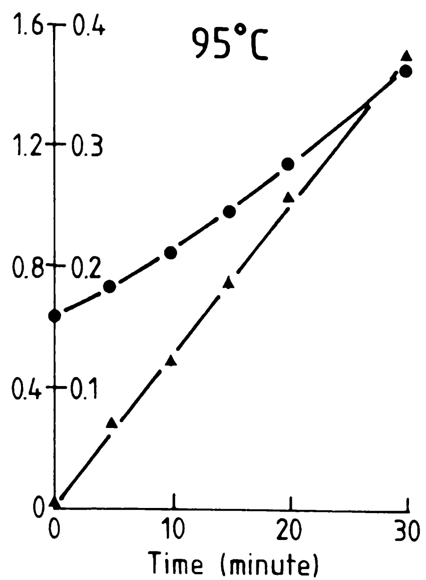
80°C
1.2 0.2
0.15
0.8
(0.10)
0.4
0.05
0
0 1 2
Time (hour)



85°C
1.2 0.3
0.8 0.2
0.4 0.1
0
0 1 2
Time (hour)



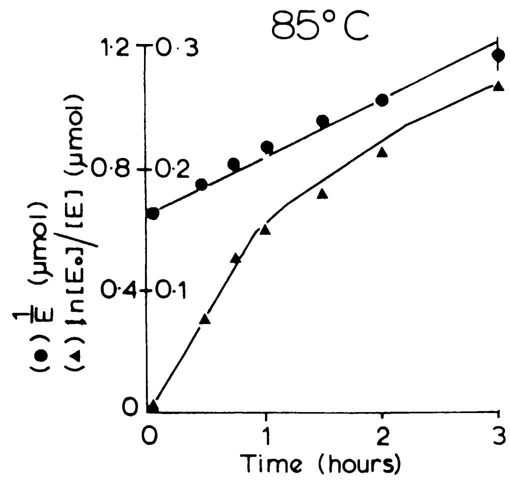
90°C
(●) 1/E (μmol)
(▲) ln[E₀]/[E] (μmol)
1.2 0.3
0.8 0.2
0.4 0.1
0
0 20 40 60
Time (minute)

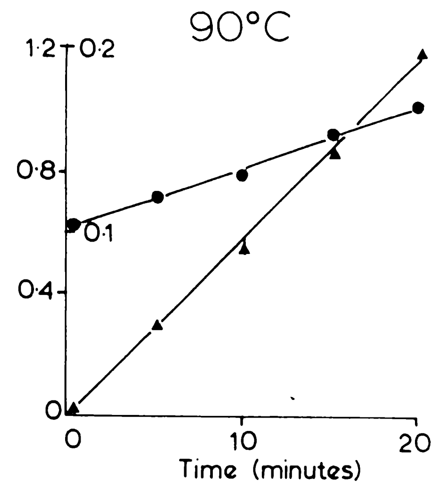


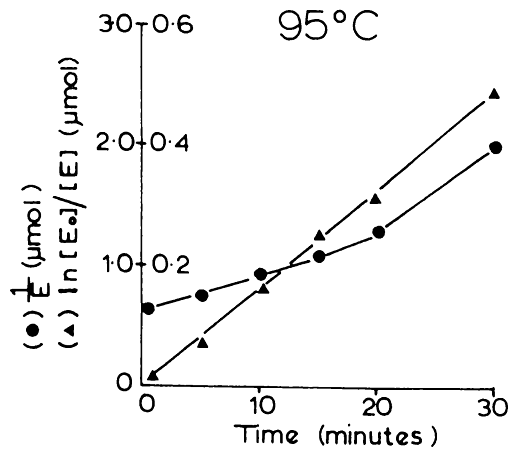
95°C
1.6 0.4
1.2 0.3
0.8 0.2
0.4 0.1
0
0 10 20 30
Time (minute)

FIGURE 7.10 KINETICS ANALYSIS OF CALDOLASE DENATURATION AND AUTOLYSIS

Thermostability profiles of Caldolase (40 g ml^{-} in 0.1M Tris acetate pH8 containing 0.5M NaCl + 10mM $CaCl_2$) were obtained at 80° - 95°C. Linearity of plots of $^1/$[residual enzyme] vs. time and ln [residual enzyme]/[total enzyme] vs. time implies first-order kinetics (thermal denaturation) and second-order kinetics (autolysis), respectively.





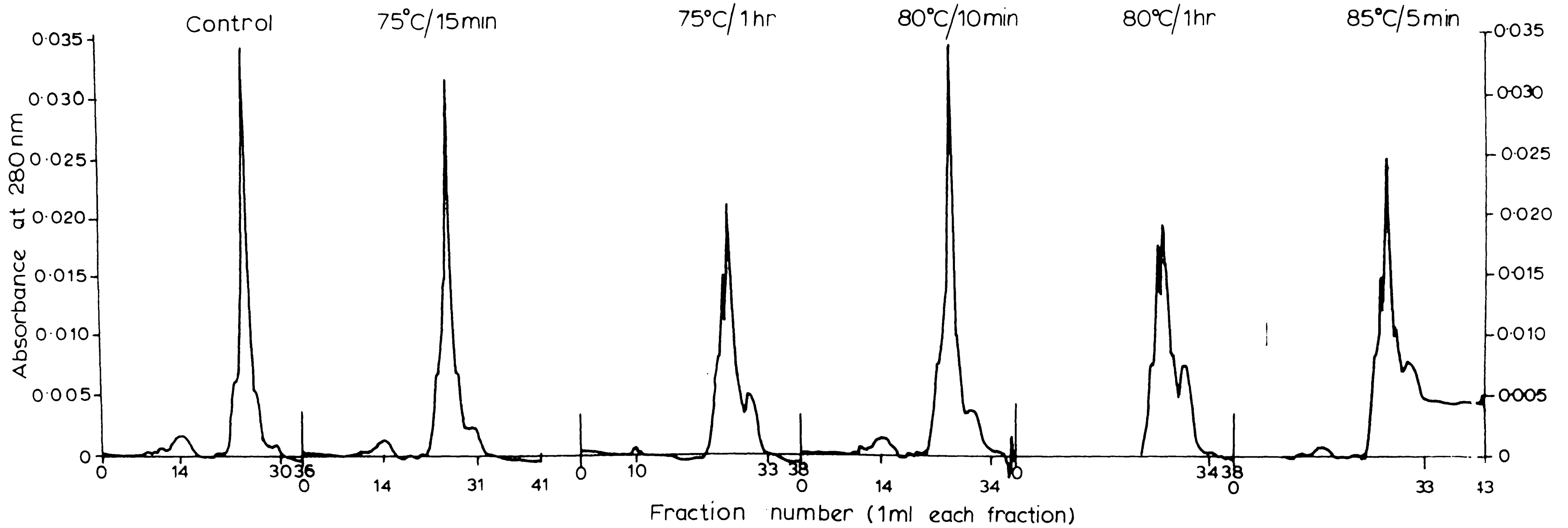


E = The concentration of active enzyme remaining.

E_0 = The enzyme concentration at zero time.

FIGURE 7.11 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF TREATED CALDOLASE

The salt-free lyophilised Caldolase was dissolved in 0.1M Tris acetate pH 8.0 and incubated at the temperatures indicated. Aliquots of enzyme were removed periodically to determine the autolysed derivatives of the enzyme using HPLC with a column of TSK G3000 SW as outlined in section 7.10.2.


Control
75°C/15min
75°C/1hr
80°C/10min
80°C/1hr
85°C/5min
Absorbance at 280nm
0·035
0·030
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

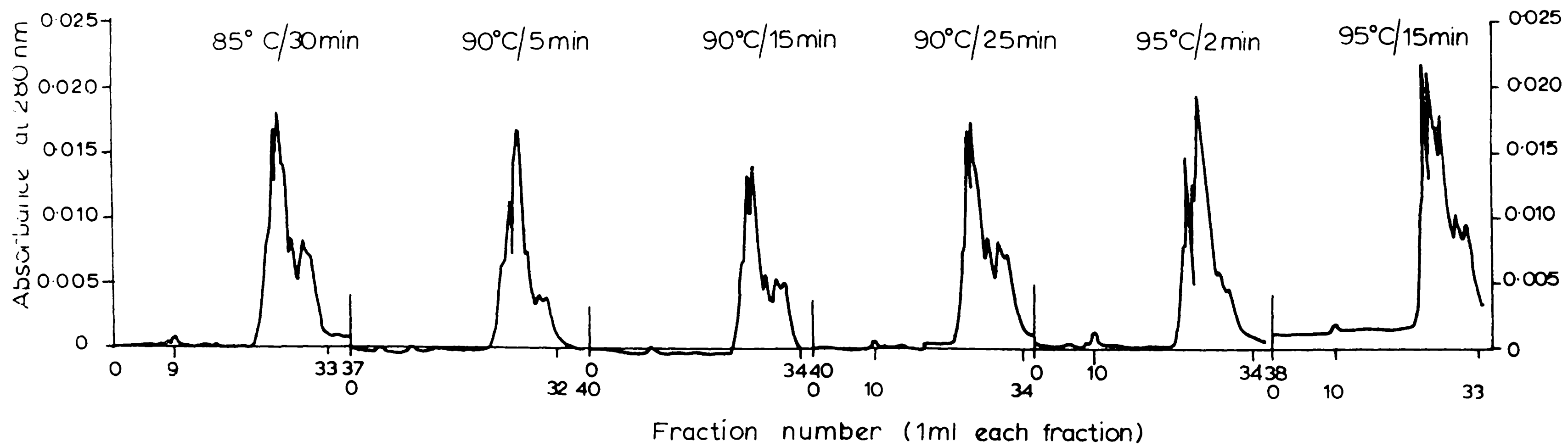
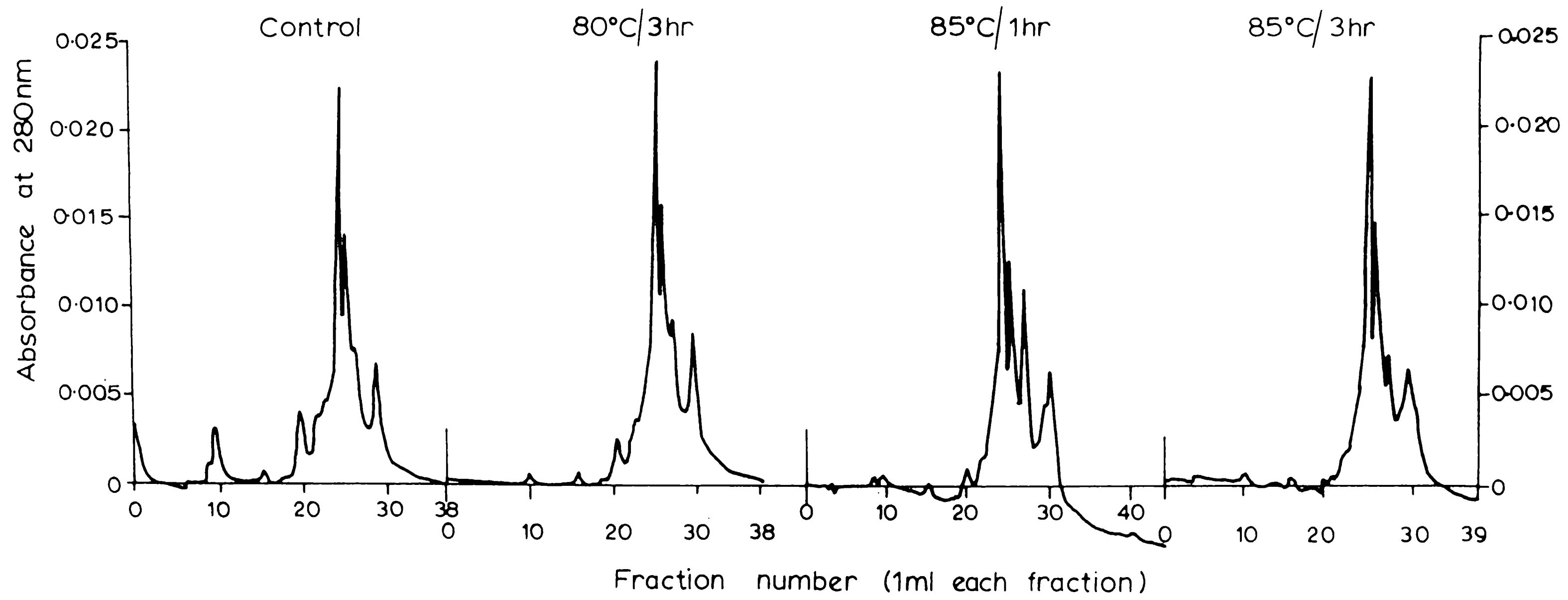

85° C/30min
90°C/5min
90°C/15min
90°C/25min
95°C/2min
95°C/15min
Absorbance at 280nm
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

FIGURE 7.12 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF TREATED CALDOLASE

The salt-free lyophilised Caldolase was dissolved in 0.1M Tris acetate pH 8.0 containing 0.1M NaCl + 10mM $CaCl_2$ and incubated at the temperatures indicated. Aliquots of enzyme were removed periodically to determine the autolysed derivatives of the enzyme using HPLC with a column of TSK G3000 SW as outlined in section 7.10.2.


Control
80°C/3hr
85°C/1hr
85°C/3hr
Absorbance at 280nm
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

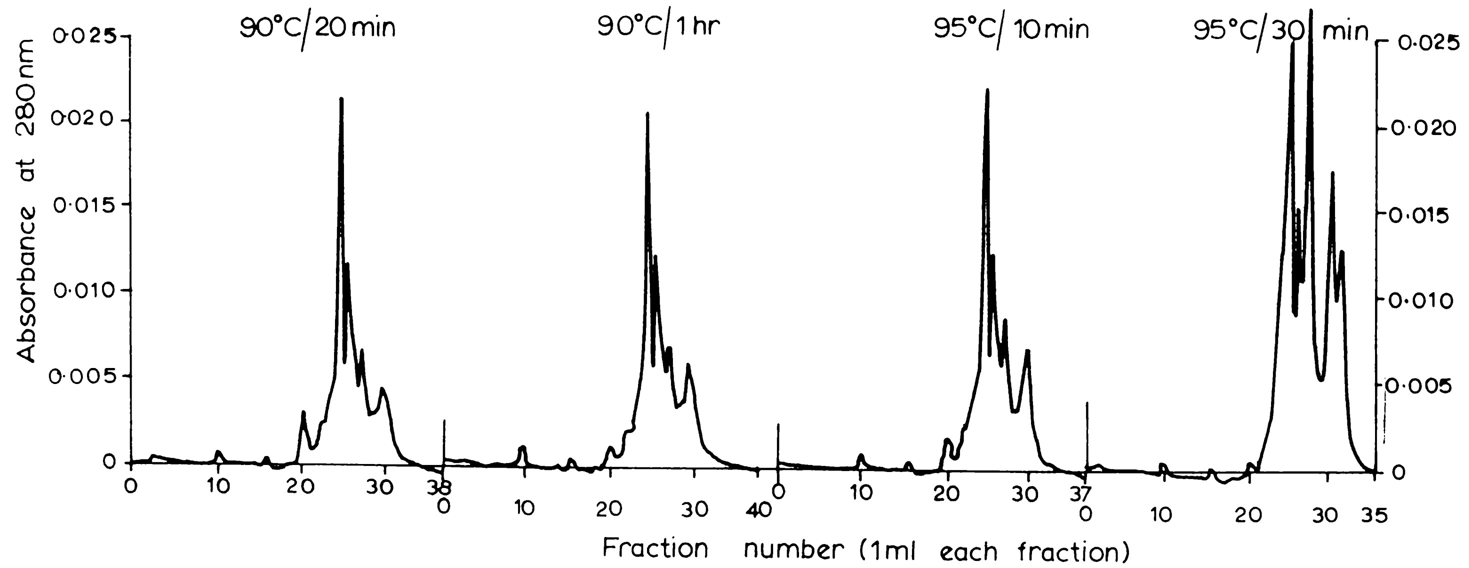
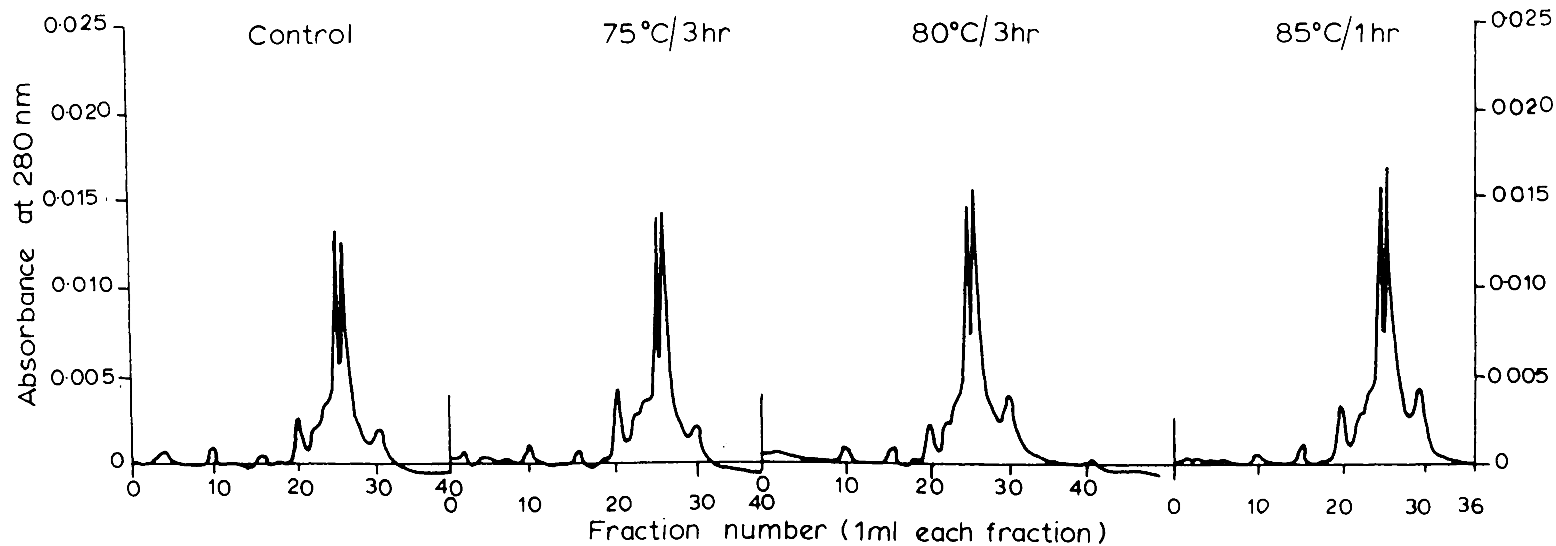

90°C/20min
90°C/1hr
95°C/10min
95°C/30 min
Absorbance at 280nm
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

FIGURE 7.13 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF TREATED CALDOLASE

The salt-free lyophilised Caldolase was dissolved in 0.1M Tris acetate pH 8.0 containing 0.3M NaCl + 10mM $CaCl_2$ and incubated at the temperatures indicated. Aliquots of enzyme were removed periodically to determine the autolysed derivatives of the enzyme using HPLC with a column of TSK G3000 SW as outlined in section 7.10.2.


Control
75°C/3hr
80°C/3hr
85°C/1hr
Absorbance at 280 nm
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

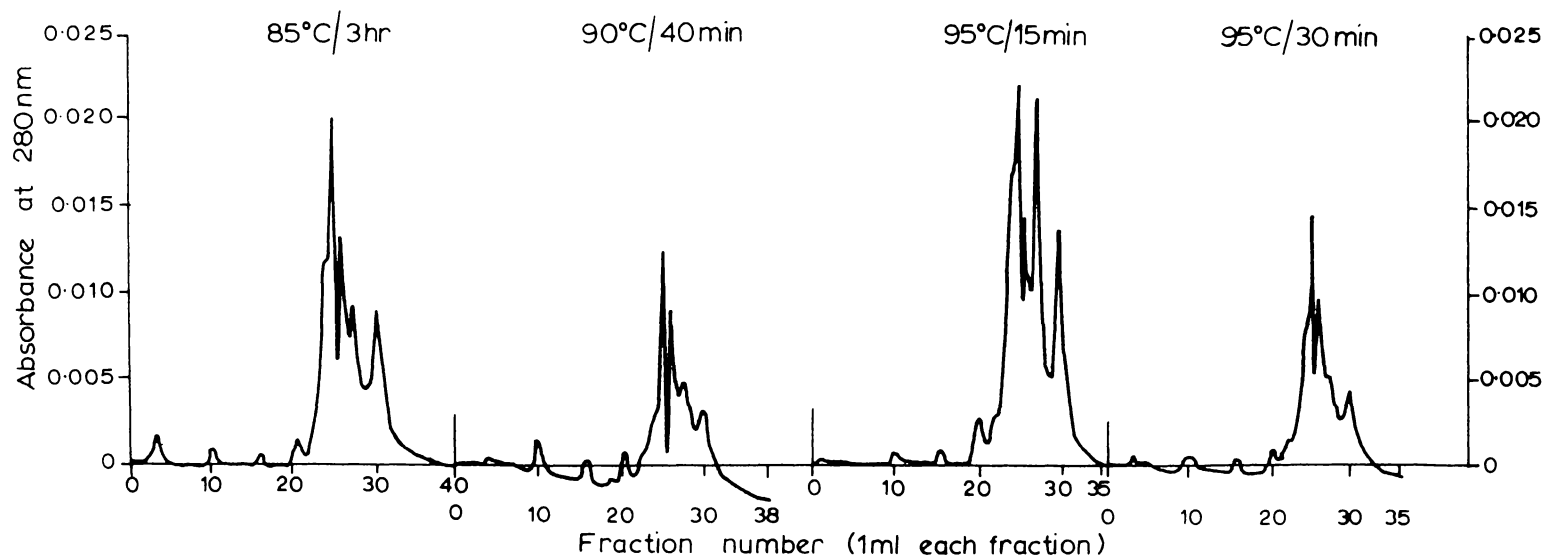
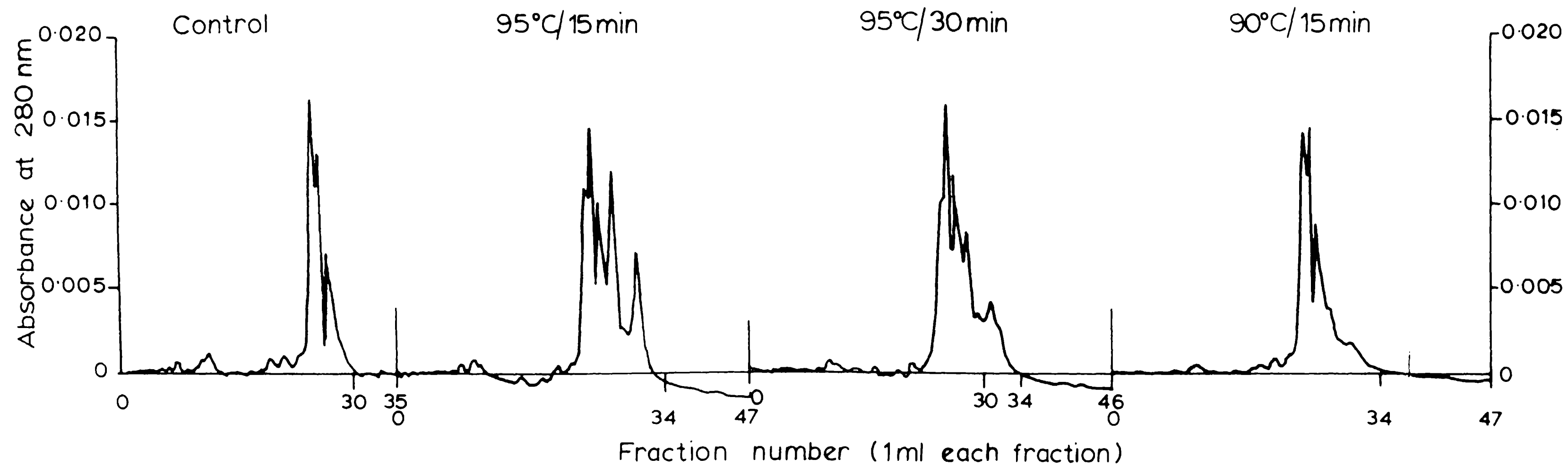
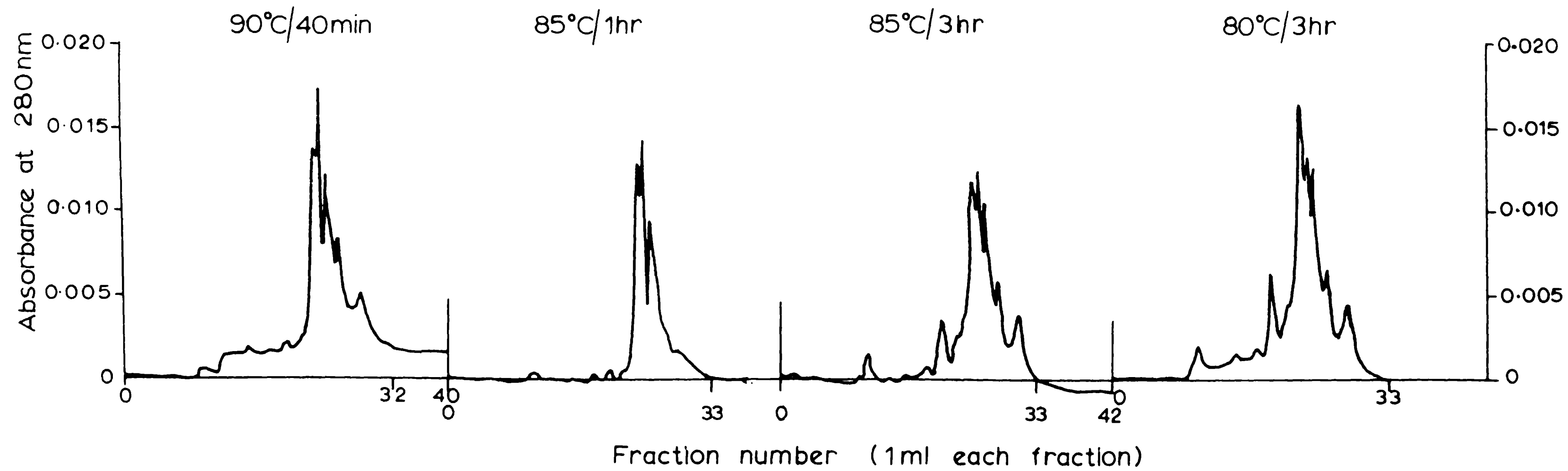

85°C/3hr
90°C/40min
95°C/15min
95°C/30min
Absorbance at 280nm
0·025
0·020
0·015
0·010
0·005
0
Fraction number (1ml each fraction)

FIGURE 7.14 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF TREATED CALDOLASE

The salt-free lyophilised Caldolase was dissolved in 0.1M Tris acetate pH 8.0 containing 0.5M NaCl + 10mM $CaCl_2$ and incubated at the temperatures indicated. Aliquots of enzyme were removed periodically to determine the autolysed derivatives of the enzyme using HPLC with a column of TSK G3000 SW as outlined in section 7.10.2.


Control
95°C/15min
95°C/30min
90°C/15min
Absorbance at 280 nm
0·020
0·015
0·010
0·005
0
0
30
35
0
34
47
0
30
34
46
0
34
47
Fraction number (1ml each fraction)


90°C/40min
85°C/1hr
85°C/3hr
80°C/3hr
Absorbance at 280nm
0·020
0·015
0·010
0·005
0
0
32
40
0
33
0
33
42
0
33
Fraction number (1ml each fraction)

was determined at 280 nm.

7.11.3 Results and Discussion.

TSK gel filtration column chromatography revealed the appearance of new fragments in the enzyme samples incubated at 22° and 60°C (Fig. 7.15). Some of these fragments were catalytically active. This suggests that the integrity of the whole enzyme molecule might not be necessary for catalytic activity as long as the unique structure of the active site remained intact.

Autolysed derivatives of some proteases have been characterised. For example, cleavage of either ARG_{105} - Val_{106} or Lys_{131} - Ser_{132} in bovine trypsin did not result in activity loss, although inactive products were obtained after hydrolysis of either the Lys_{49} - Ser_{50} or Lys_{176} - Asp_{177} bonds (Maroux & Desnuelle, 1969; Smith & Shaw, 1969).

7.12 CONCLUSION.

There is general agreement among those involved in work on proteins from thermophilic micro-organisms that no single mechanism can account for the stability of all thermophilic proteins.

It is now accepted that a small change in protein structure can bring about remarkable changes in thermostability. In a recent study, Walker *et al* (1980) compared the thermostability of D-glyceraldehyde-3-phosphate dehydrogenases (DPD) from *Bacillus stearothermophilus* and *Thermus aquaticus* with the similar enzyme from lobster muscle. They found additional hydrophobic interactions, intersubunit hydrogen bonds and ion-pairs in the thermophilic DPD, and attributed the additional

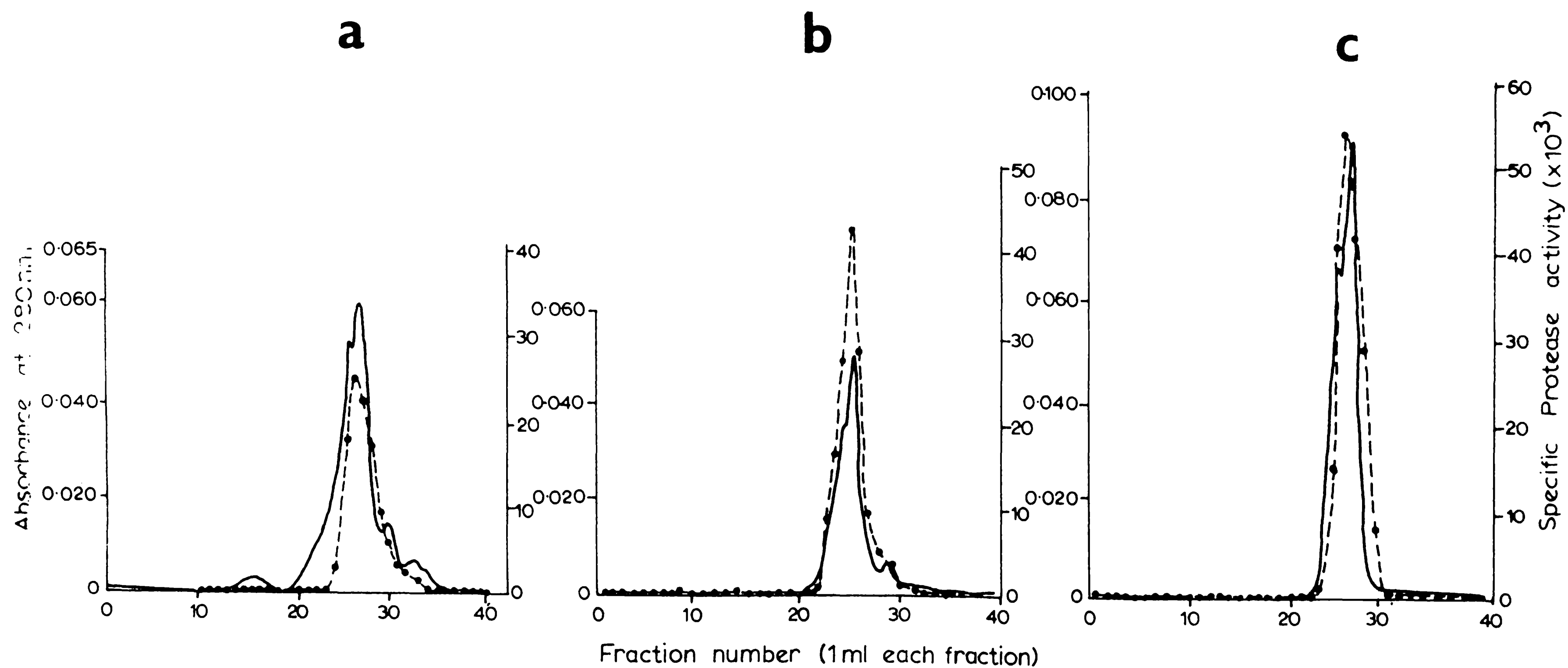


FIGURE 7.15 ANALYSIS OF AUTOLYSED DERIVATIVES OF CALDOLASE.

The highly purified Caldolase sample in o.1M Tris acetate pH 8.0 were incubated (a) at 60 C for 150min, (b) at 22 C for 180min. and (c) on ice for 180min. Each enzyme solution was chromatographed on an analytical TSK G3000 SW column. The protein concentration (—) and protease activity (--) were determined as outlined in section 7.11.2.

stability of the thermophilic enzymes to these changes.

The results of this study suggest that hydrophobic interactions, hydrogen bonds and disulphide bonds are involved in the thermostabilisation of Caldolase (see Section 7.4.3 for further discussion). The contribution of various amino acid groups (e.g. aliphatic amino-acids etc.) to the stability of Caldolase also has been considered (see Section 5.11 for further discussion).

Chell and Sundaram (1978) suggested that an increase in ionic strength or pH (above pH9.0) would disrupt external salt bridges. External ionic interactions do not seem to play any significant role in the stabilisation of Caldolase since an increase in the ionic strength of the solvent up to 0.7M NaCl does not reduce protease stability (Fig. 7.5). A pH increase up to 11 also has no significant effect on the enzyme stability (Fig. 7.3). Nevertheless, the possibility of the involvement of shielded salt bridges in enzyme stability cannot be excluded. No specific catalytic or stabilising metal ion was found in Caldolase (Table 7.9 and Fig. 7.4).

It is possible that a strongly ionic environment strengthens various intramolecular interactions (hydrophobic interactions and hydrogen bonds), resulting in the restriction of water diffusion and preventing the unfolding of the enzyme molecule. However, at low salt concentrations these forces may diminish causing the enzyme to unfold and thus becoming susceptible to autolysis.

Any analysis of protease stability presents particular problems due to the fact that the unfolded form of enzyme is itself a good substrate for the remaining active enzyme. As

a result it is not easy to separate the effects of denatura-
tion from autolysis since both phenomena are probably present
simultaneously.

8. GENERAL DISCUSSION

Serine alkaline proteases are among the most widely distributed group of proteolytic enzymes. They are produced by an extensive range of bacteria, moulds and yeast (Morihara, 1974; Renko *et al*, 1981; Ogrydziak and Scharf, 1982; Zlotnik *et al*, 1984). These enzymes are distinguished by their sensitivity to organophosphorus reagents such as DFP and by their optimum activity at alkaline pH's (Matsubara and Feder, 1971). Caldolase, an extracellular protease produced by *Thermus* strain Tok_3, was inhibited completely by the serine inhibitors, DFP and PMSF, and displayed optimum activity at pH 9.5, indicating that it belongs to the group of serine alkaline proteases.

Caldolase revealed some similarities to Caldolysin, a serine protease from *Thermus aquaticus* strain 351 (Cowan and Daniel, 1982a), to the Subtilisins (Ottesen and Svendsen, 1970; Markland Jr., and Smith, 1971), and to a number of other serine proteases (Table 8.1).

Caldolase was not inhibited by TLCK and TPCK (known to inhibit trypsin and chymotrypsin respectively by reacting with active site histidine residue). In general, microbial serine proteases are not inhibited by these reagents with the exception of the trypsin-like enzyme from *Streptomyces fradiae* which is inactivated by TLCK (Matsubara and Feder, 1971). Although Subtilisins show resistance to TLCK and TPCK, these proteases are inactivated by related compounds such as benzyloxy carbonyl-L-phenylalanine bromomethyl ketone (Shaw and Rusica, 1968; Markland *et al*, 1968) and benzyloxy carbonyl-L-alanyl-glycyl-L-phenylalanine chloromethyl ketone

Table 8.1 Comparison of Properties of Proteases Most Closely Resembling Caldolase

Property	Caldolase	Caldolysin (a)	Subtilisin Carlsberg (b)	Thermomycolase (c)	α-chymotrypsin (d)	Trypsin (e)
	ENZYME					
M.W	≃ 25,000	20,000	27,287 (b)	?	25,000	24,000
pI	8.9	8.5	9.4	6	9.5	10.1
Active site residue	Serine	Serine	Serine	Serine	Serine	Serine
pH optimum	9.5	8.5	10-11	8.5	8	7 - 7.5 (e)
Cystine residue	4	6	0	?	5 (f)	6 (e)
Size limit in active site	4	4	-	-	-	-
% Carbohydrate content	10	13	0	?	0	0
Metal ion requirement (Ca^{2+}, Zn^{2+})	-	Ca^{2+} (6)	-	Ca^{2+} (1)	Ca^{2+} (1)	Ca^{2+}
Esterase activity	+	+	+	+	+	+
Elastase activity	+	+	+	?	?	-
Serine inhibitors	DFP, PMSF	DFP, PMSF	DFP, PMSF	DFP, PMSF	DFP,PMSF	DFP,PM[illegible]
TPCK	-	-	-	?	+	-
TLCK	-	-	-	?	-	+
Ionic strength requirement	+	-	-	-	-	-
Cysteine content	-	-	-	?		

(a) Cowan and Daniel (1982a)

(b) Ottesen and Svendsen (1970)

(c) Ong and Gaucher (1976)

(d) Hess (1971)

(e) Walsh (1970)

(f) Mahler and Cordes (1971b)

(+) Yes

(-) No

(?) Not known

(Morihara *et al*, 1971; Morihara and Oka, 1970).

Caldolase was strongly inactivated by Hg^{2+} (known to form a covalent bond to protein -SH, -Co^{2+}, histidine) and it is possible that an essential histidine residue is present at the active site of Caldolase as is the case for Subtilisins. The presence of an essential histidine residue at the active site of many proteases has been reported (Burstein *et al*, 1974; Hess, 1971; Walsh, 1970).

Caldolase apparently possesses no free sulphydryl group, although the possibility of buried SH groups cannot be ruled out. Bridgen *et al*, (1973) have studied alcohol dehydrogenase from *B. stearothermophilus* which, although possessing as many sulphydryl groups as its mesophilic counterparts, does not react with thiol group reagents. This suggests that sulphydryl groups in the thermophilic enzyme are buried within the interior of the protein molecule and thus are less accessible to solvent. However, Harris (1978) proposed that the presence of cysteine could be a potential source of instability (due to oxidation or disulphide exchange reactions), and concluded that thermophilic enzymes should contain fewer sulphydryl groups than their mesophilic counterparts.

Although 4 disulphide bonds per molecule of Caldolase were found, neither mercaptoethanol nor dithiothereitol had any significant effect on enzyme stability. This indicates that either the disulphide bonds are well protected from the reagents, or that these bonds are not involved in the molecular stabilisation of Caldolase.

Disulphide bonds in Caldolysin played a significant role in enzyme stability (Cowan and Daniel, 1982a). Some extracellular proteases e.g. Thermolysin (Matsubara, 1970) and

Subtilisins (Ottesen and Svendsen, 1970) possess neither sulphydryl groups nor disulphide bonds.

Caldolase retained full activity and stability in the presence of metal chelators (EDTA, EGTA, O-phenanthroline), indicating that no essential metal was present in the enzyme molecule.

Metal ions have not been reported to be involved in the active site of alkaline serine proteases (Dixon & Webb, 1979b). However, the stabilising influence of calcium ions on some serine proteases has been shown (Cowan and Daniel, 1982a; Ogrydziak and Scharf, 1982; Frömmel and Höhne, 1981; Tobe *et al*, 1976; Ong and Gaucher, 1976).

Caldolase possesses approximately 10% carbohydrate content. A large number of proteins from a diversity of sources are known to contain covalently-linked carbohydrate (Spiro, 1973). Several glycoproteins from yeast and viruses have been reported (Meachum *et al*, 1971; Neumann and Lampen, 1967; Luria *et al*, 1978; Wilson *et al*, 1982) including an alkaline extracellular protease (Ogrydziak and Scharf, 1982). To date only a limited number of bacterial enzymes have been investigated for the presence of carbohydrate, including Caldolysin, a serine protease from *Thermus aquaticus* 351 which contains 13% carbohydrate (Cowan and Daniel, 1982a). In addition a glycoprotein serine protease from *Bacillus subtilis* strain 168 has been reported (Roitsch and Hageman, 1983), and the diphosphophyridine nucleosidase (DPNase) and the specific DPNase inhibitor from *Bacillus subtilis* contain about 56% and 71% carbohydrate respectively (Everse and Kaplan, 1968).

It has been suggested that the carbohydrate moieties protect glycoenzymes against proteolysis by covering potential

proteolytic cleavage sites (Barker and Gray, 1983) and stabilise the three-dimentional structure of glycoproteins (Pazur *et al*, 1970; Nakamura and Hayashi, 1974).

No pronounced differences were observed when the amino acid composition of Caldolase was compared with a number of thermophilic and mesophilic proteases (Table 5.2). Several authors have tried to find a correlation between thermostability and the amino acid content and sequence of proteins (Frank *et al*, 1976; Biffen and Williams, 1976; Zuber, 1978). It has been noted that thermophilic enzymes have a higher ratio of arginine to lysine than their mesophilic counterparts (Frank *et al*, 1976; Kagawa *et al*, 1976). Caldolase also possesses these features. Current evidence suggests that the higher thermostability of thermophilic proteins is due to small changes in the amino acid sequence (Argos *et al*, 1979; Grütter *et al*, 1979), such changes being able to affect stability without any obvious structural alteration and resulting in a relatively small number of additional intra-molecular interactions (Perutz and Raidt, 1975; Rüegg *et al*, 1982).

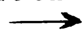
From the thermodynamic point of view this is not unexpected since the free energy of thermophilic protein stabilisation is estimated to be only 20-40 KJ mol^{-1} (Nojima *et al*, 1977). For example, comparison of the thermal stabilities of rabbit muscle and *B. Stearothermophilus* triose phosphate isomerase has shown that a thirty-fold difference in stability (as measured by the half-life) at 60°C results from the additional free stabilisation energy of only about 9 KJ mol^{-1} (Fahey *et al*, 1971; Hocking and Harris, 1976). Similarly, the additional free energy of stabilisation provided by extra salt bridges in the thermophilic ferredoxins is about 10 KJ mol^{-1}

Examination of data on lack of hydrolysis of amino acid amides (Table 6.3) implies that low molecular weight products could be inhibitors, but this is not supported by the ready hydrolysis of amino acid esters (Table 6.4).

(Perutz and Raidt, 1975).

The Arrhenius plots for both casein and peptide substrates were curved, but without any clearly marked discontinuity. It is concluded that the effect of temperature on the enzyme conformation is continuous rather than occurring at a particular temperature. No significant differences were observed in Km values at various temperatures (45^0 - 85^0C).

Kinetic data for Caldolase revealed strong substrate inhibition in the hydrolysis of both casein and azo-albumin. Dixon and Webb (1979c) have suggested that a high substrate concentration may cause two or more substrate molecules to combine with different portions of the active site, $ES + S \rightarrow ES_2$ (competitive inhibition). There is also the possibility that a binding site other than the active site could produce a similar effect (non-competitive inactivation). No substrate inhibition was observed for low molecular weight synthetic peptide or ester substrates. This suggests that substrate inhibition in Caldolase using casein and azo-albumin substrates may be due to steric interference rather than binding to the active site of the enzyme.



Caldolase showed remarkable stability toward treatment by heat, denaturing agents (urea, GUHCl), organic solvents (dimethyl sulphoxide, methoxy ethanol, propanol, etc.) and alkaline pH. Caldolase was more susceptible to SDS than was Caldolysin (Cowan and Daniel, 1982a). Caldolase was also unstable at low salt concentrations with enzyme stability increasing considerably in the presence of various salts (this presented some difficulties in purifying and handling the enzyme). It is concluded that ionic strength plays a major role in the structural stabilisation of the enzyme, possibly by protecting

the hydrogen bonds and strengthening hydrophobic interactions. Ionic strength is shown to have a significant effect upon the activity and stability of a number of enzymes (Shannon *et al*, 1982; Griffiths and Sundaram, 1973; Hengartner and Zuber, 1973). Crabb *et al* (1977) have proposed that the enhanced thermostability of glyceraldehyde-3-phosphate dehydrogenase from *Bacillus Coagulans* resulting from ionic strength could be related to an increase in hydrophobic interactions. It is also possible that two or more acidic residues in the same region of the molecule could destabilise the enzyme by electrostatic repulsion, whereas an increase in ionic strength would overcome this repulsion.

It was possible to recover Caldolase activity lost at low ionic strengths by increasing the salt concentration. It was demonstrated that recovery of the enzyme incubated at lower temperatures (22°C) was substantial compared with the enzyme sample which had been incubated at higher temperature (85°C). It is suggested that at low ionic strengths the enzyme undergoes reversible denaturation. However, at high temperatures the reversibly denatured enzyme undergoes autolysis, and recovery of activity is then not possible.

Study of kinetics and TSK gel filtration chromatography of Caldolase revealed that at high ionic strengths up to 85°C, second-order kinetics were obeyed (autolysis was dominant), while at 90° and 95°C first-order kinetics were significant (thermal denaturation predominated). A low ionic strength of the enzyme solution caused second-order kinetics to predominate up to 95°C.

Some of the autolysed derivatives of Caldolase were catalytically active, so the integrity of the whole enzyme

molecule is apparently not necessary for catalytic activity as long as the unique structure of the active site remains intact. This finding corroborates reports for other serine proteases. For example, cleavage of either Arg_{105} - Val_{106} or Lys_{131} - Ser_{132} in bovine trypsin did not result in activity loss, although inactive products were obtained after hydrolysis of either the Lys_{49} - Ser_{50} or Lys_{176} - Asp_{177} bonds (Maroux and Desnuelle, 1969; Smith and Shaw, 1967).

Caldolase was able to hydrolyse both peptide and ester bonds, and a number of proteins and dye-linked proteins including elastin, collagen and fibrin. Caldolase showed a very weak activity toward elastin. By contrast the metallo-protease Thermolysin has very strong activity toward elastin (Morihara, 1970; Morihara and Tsuzuki, 1966). Caldolase was not able to hydrolyse small peptides such as Benzoyl-D-arginine-p-nitroanilide and succinyl-L-phenylalanine-p-nitroanilide. This suggests that for hydrolysis of a synthetic peptide substrate, the minimum active site requirement of Caldolase is at least four groups (amino-acid residues and terminal blocking group). A similar requirement is exhibited by Caldolysin (Cowan and Daniel, 1982a) and by *Myxobacter* protease (Jackson and Wolfe, 1968). However, no such size requirement was observed for Caldolase when synthetic ester substrates were used. This may be due to the fact that ester substrates, unlike peptides, do not require the enzyme to possess and exhibit a large or well-organised active centre.

Caldolase was not capable of hydrolysing Bradykinin (Arg-Pro-Pro-Gly-Ser, Pro-phe-Arg). This indicates that the enzyme lacks affinity towards non-polar aminoacids. In contrast, Caldolase hydrolysis of insulin B chain resulted in

a complex pattern, suggesting of low degree of specificity

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