HETEROGENEITY OF PROTEINASES FROM THE HYPERTHERMOPHILIC ARCHAEOBACTERIUM *PYROCOCCUS FURIOSUS.*

BY

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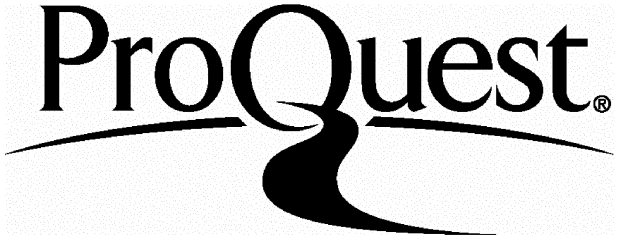

TO MY PARENTS AND SISTERS, SOFIE AND AVGES.

ABSTRACT

In order to investigate the role of proteolysis in *Pyrococcus furiosus,* cultures were grown on a variety of complex and simple carbon substrates and assayed for proteolytic activity using azocasein as the protein substrate. Growth on complex protein substrates such as peptone or tryptone induced high levels of extracellular proteinase activity, whereas growth in the presence of the oligosaccharide maltose largely repressed proteinase activity.

Gelatin SDS PAGE revealed thirteen proteolytically active bands in both cell extracts and culture supernatants, with apparent molecular weights ranging from 66kDa to 135kDa. It was concluded that these bands were not artefacts but probably discrete polypeptides. The band pattern was observed after a variety of treatments, which included different incubation times and temperature, SDS concentration, different proteolytic substrate (casein) and thiol-interchange mechanisms.

Using a variety of chromatographic techniques, only the 66kDa proteinase could be resolved. Mono Q, Hydrogen Bond cellulose, sucrose density gradient centrifugation, and gel permeation under non-denaturing conditions all produced similar band patterns suggesting that the polypeptides existed as active aggregates or as a high molecular weight complex.

The proteinase 'complex' from cell extracts was purified to near homogeneity with a yield of 35%. The molecular weight was estimated to be approximately 10^6 Daltons. The pH optimum for activity was between 6 and 8, and the "temperature optimum" was 100°C. At 95°C, the proteinase complex had a half-life of 69h. The proteinase complex was stable at 95°C in 6M urea, 10mM dithiothreitol and in 4.4M guanidinium chloride. Enhanced stability was observed in the presence of 0.1% and 1% Triton X-100, whereas the detergents SDS and CTAB produced a large destabilising effect. Organic solvents (ethanol, acetone and dimethylformamide) significantly reduced thermostability of the proteinase complex at 100°C. Enhanced stability of the complex was observed in the presence of 1M NaCl.

Inhibition studies indicated that the activity of the complex was predominantly of a serine-type. Inhibition by EDTA of a PMSF-insensitive proteinase was observed in gelatin SDS gels, suggesting the presence of at least one metalloproteinase activity in the complex.

A preliminary determination of substrate specificity indicated a preference for small basic amino acids and substrates containing phenylalanine adjacent to the leaving group.

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INTRODUCTION

1.1 PROTEINASES

1.1.1 Historical Background

Proteinases have long played a significant role in the processing of natural products. They have been used in diverse applications such as cheese making, in the leather industry and the detergent industry. For centuries, the Japanese have used fungal proteinases for the production of soy sauce, miso and tamari sauce. This process involves growing proteolytic strains of *Aspergillus oryzae* on rice or soy beans where the product formed is added to large vats containing roasted soybeans, cereal grains and salt. Proteolysis, solubilisation and extraction occur to produce the soy sauce which is separated from residual solids (Crewther and Lennox, 1950).

In the past, there have been many problems in the detergent industry in finding suitable proteinases which remained active and stable at pH 9 to 11. This problem was first highlighted in 1913 when Rohm and Haas marketed the first enzymatic pre-soak detergent. The detergent contained a mixture of soda and crude pancreatin and remained in the market in Europe for 50 years. Despite it wide usage, the problems of low initial activity and instability at high alkaline pH instigated the search for other suitable proteinases (see Ward, 1983).

Microbial proteinases were found to perform much better under a variety of environmental conditions, such as extreme pH. The alkaline proteinase Subtilisin Carlsberg from *Bacillus licheniformis* was developed by Novo Industries in 1960 and replaced the pancreatin and pancreatic trypsin which were originally used in detergent powders. The enzyme was found to be highly stable and active in the pH range 9 to 11. However, since most laundry washes are carried out at temperatures up to 100°C, the enzyme was only effective for 15-20 minutes at 60°C.

The first heat stable proteinase to be studied in detail was Thermolysin, isolated from *Bacillus thermoproteolyticus* (Endo, 1962). Over the past two decades, a large number of thermostable proteinases have been isolated and characterized from many thermophilic micro-organisms (Table 1.1).

1.1.2 Role of Proteinases

The proteinases are a highly complex group of enzymes exhibiting a wide range of physico-chemical and catalytic properties. All catalyse the hydrolysis of peptide bonds but individual enzymes differ markedly in their specificity. Proteinases are produced by all organisms intracellularly and/or extracellularly.

Extracellular proteinases are relatively limited in their functional diversity. The primary function is the hydrolysis of large polypeptide substrates into smaller molecular entities to allow absorption into the cell for nutritional requirements. A more important role for extracellular proteinases is the cleavage of membrane-bound protein precursors to release the active forms. This was observed by Aiyappa and co-workers (1977) who isolated an extracellular serine proteinase with unusual specificity from *B. licheniformis*. The proteinase converted membrane-bound penicillinase to the free extracellular form. Activation of other proteinases by extracellular proteinases from the same organism have also been described. Drapeau (1978) reported that the neutral proteinase of *Staphylococcus aureus* remained inactive unless activated by the extracellular serine proteinase from the same organism.

The multiple roles of intracellular proteinases are far more complex and less understood. Some of the roles of these proteinases in mammalian systems have been well characterized (Barrett, 1977). These include the activation of precursors, metabolic control by specific hydrolysis of other enzymes, and protein degradation and turnover. However, the physiological functions of intracellular proteinases from micro-organisms have not been as well characterized as those in mammalian systems.

Table 1.1 Some thermostable proteinases from thermophilic[a] micro-organisms

Proteinase	Micro-organism	Reference
Neutral proteinases	*Bacillus stearothermophilus*	O'Brien and Campbell (1957) Sidler and Zuber (1980)
Thermolysin	*B. thermoproteolyticus*	Endo (1962)
Aminopeptidase I	*B. stearothermophilus*	Roncari and Zuber (1969)
Thermomycolin	*Malbrancha pulchella*	Gaucher and Stevenson (1976)
Thermitase	*Thermoactinomyces vulgaris*	Hausdorf *et al.* (1980)
Caldolysin	*Thermus aquaticus* T-351	Cowan and Daniel (1982)
Serine Proteinase	*Thermus caldophilus* GK24	Taguchi *et al.* (1983)
Archaelysin	*Desulfurococcus* Tok12S1	Cowan *et al.* (1987)
Dipeptidase	*B. stearothermophilus*	Cho *et al.* (1988)
Caldolase	*Thermus* Tok3	Savarani *et al.* (1989)
Alkaline serine proteinase	*Thermomonospora fusca* YX	Kristjansson and Kinsella (1990)
Thermopsin	*Sulfolobus acidocaldarius*	Lin and Tang (1990)
Proteinase complex, "Pyrolysin"	*Pyrococcus furiosus*	Blumentals *et al.* (1990) Eggen *et al.* (1990) Connaris *et al.* (1991)

[a] 'Thermophilic' covers moderate, extremely and hyperthermophilic micro-organisms.

Intracellular microbial proteinases have been implicated in protein turnover (Cheng and Aronson, 1977). This function is essential for the adaptation of cells to new environmental conditions, particularly when the environment is devoid of amino acids, where proteolysis provides a source of amino acids for the synthesis of nascent protein. A good example is reported by Goldberg and Dice (1974) who demonstrated that *E. coli* grown in a medium deprived of nitrogen can still synthesize β-galactosidase in response to an inducer because of increased protein catabolism. This mechanism ceases when the pathway for protein degradation is blocked.

The roles of intracellular and extracellular proteinases from thermophiles are not well understood but it has been suggested that since normal molecular controls such as allosterism operate in extreme thermophiles then their functions are not too dissimilar to those of mesophilic proteinases (Amelunxen and Murdock, 1978).

1.1.3 Classification and Specificity of Microbial Proteinases.

The nomenclature used to describe microbial proteinases divides them into two categories: the exopeptidases, which cleave single amino acid residues from the C and N termini of the polypeptide chain, and the endopeptidases, which cleave the peptide bond internally. The exopeptidases are subdivided on the basis of their peptide cleavage into aminopeptidases (-N terminus) and carboxypeptidases (-C terminus). The endopeptidases are subdivided on the basis of their catalytic activity (Hartley, 1960) into serine, cysteine, aspartic acid and metallo-proteinases. These classes, usually determined by the effects of proteinases inhibitors on enzyme activity, are broadly described in Table 1.2. Morihara (1974) has also classified these proteinases according to their side-chain specificity (Table 1.2). The exopeptidases can also be differentiated according to their catalytic activity but will not be described here (see Ward, 1983). The word proteinase will therefore denote those hydrolases which attack internal peptide bonds.

Table 1.2 Classification of Microbial Proteinases (from Morihara, 1974, Ward, 1983, and Barrett and Rawlings, 1991)

Catalytic Mechanism	Family (Representative Member, Other Members)	Side-Chain Specificity	Inhibitors
Serine Proteinases (His,Asp,Ser catalytic triad at active site)	**Chymotrypsin**	Basic amino acid residues at the carboxyl side of the splitting point.	DFP, tosyl-L-lysine chloromethyl ketone, soybean trypsin inhibitor, PMSF.
	Subtilisin.	Aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point.	
	Staphylococcal proteinases	Aspartic or glutamic acid residues at carboxyl side of the splitting point.	
Cysteine Proteinases (Cysteine at active site)	Clostripain.	Basic amino acid residues at carboxyl side of splitting point.	*p*-chloromercuribenzoate tosyl-L-lysine chloromethyl ketone.
	Streptococcal proteinases	Broad.	
Aspartic Proteinases (Acid residue at active site)	**Pepsin.**	Aromatic or hydrophobic amino acid sides of splitting point.	Diazoacetyl-DL-nor-leucine methyl ester.
	Thermopsin		
Metalloproteinases (Presence of metal ion, normally zinc, at active site)	Thermolysin	Neutral proteinases: Hydrophobic or bulky amino acid residues at amino acid side of splitting point. Alkaline: Broad	EDTA, *o*-phenanthroline.

1.1.4 Catalytic Mechanisms of Microbial Proteinases.

As outlined above, it is possible to differentiate microbial proteinases on the basis of the chemical groups that are responsible for their catalytic activity. These mechanisms will be discussed below.

Serine Proteinases. The serine proteinases are the most extensively studied and best understood enzymes. This class of enzymes exhibits a set of common properties which is listed as follows (Stamato *et al.,* 1984):

(i) All of these proteinases contain a reactive serine side chain;

(ii) A general base-catalyzed nucleophilic attack of the hydroxyl oxygen atom of the reactive serine on the carbonyl carbon atom of the substrate is observed;

(iii) The active site is composed of the same catalytic triad of amino acid residues: a reactive serine, a histidine and an aspartate residue. This mechanism has also been observed with other serine-type enzymes (Brady, 1990).

There are two major families of serine proteinases, the chymotrypsin family, and the subtilisin family which appears to occur only among the Bacilli.

The conservation of structure among the chymotrypsin-related proteinases is evident in the amino acid sequences around the residues most directly involved in catalytic activity: these are His^{57}, Asp^{102}, and Ser^{195} (chymotrypsin numbering) forming the catalytic triad.

Members of the subtilisin family can also be recognised by the conservation of the amino acid sequence around the catalytic residues but with a different numbering system: Asp^{32}, His^{64}, and Ser^{221} (subtilisin numbering).

This catalytic triad was described by Blow *et al* (1969) as a charge relay system linked through a hydrogen bond network. In this system, the negative charge of a buried aspartate residue is transferred to the reactive external serine residue through the imidazole ring of histidine. The process involves the transfer of two protons and constitutes the initial step of the catalytic mechanism (Figure 1.1). An alternative system proposed by Wang (1968) and

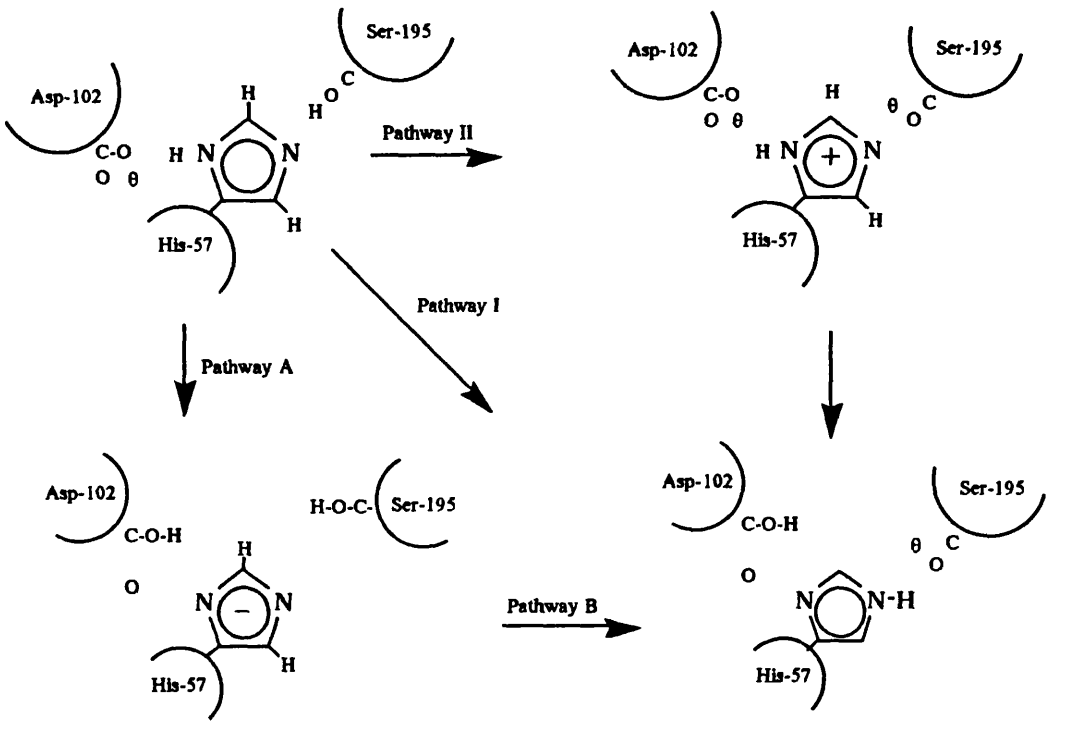


Figure 1.1 **Proposed mechanisms for proton transfer within the serine proteinases. Pathway I = A + B. Pathway I was proposed by Blow et al. (1969) pathway II, by Wang (1968) and Polgar and Bender (1969) (reproduced from Stamato *et al.*, 1984).**

Polgar and Bender (1969) suggested that the transfer of a single proton of the reactive serine to the histidine takes place instead. However, recent evidence by Stamato *et al.*, (1984) has shown that the double proton transfer is the most feasible mechanism for the initial step of the enzyme reaction. In both cases however, each mechanism results in the enhancement of the nucleophilic character of the serine oxygen Oγ atom. This attacks the substrate carbonyl carbon atom to produce an acyl enzyme. The subsequent deacylation of the acyl-enzyme produces the final hydrolysis product.

Cysteine Proteinases. Cysteine proteinases have a Cys side chain at the active site. These enzymes employ a similar reaction mechanism to the serine proteinases involving a tetrahedral intermediate and an acyl-enzyme intermediate in which the acyl group is bonded to the Cys sulphur atom.

The most studied family of cysteine proteinases are the papains which are generally non-bacterial. The catalytic site is defined by the residue Cys^{25} which forms an acyl enzyme, interacting closely with His^{159} which is believed to be analogous to the His^{57} of the serine proteinase catalytic triad. However, there is no obvious apparent analogue of Asp^{102}; the nearest acidic group is 7.5Å away.

The best studied microbial proteinase is clostripain from *Clostridium histolyticum.* It is highly specific for the cleavage of peptide bonds on both the C- and N-terminal sides of arginine and is calcium activated. Clostripain is composed of two polypeptide chains, M_r of 43kDa and 12.5kDa. It has a Cys residue at position 41 in its amino acid sequence which has been shown to be the catalytically essential residue (Gilles *et al.*, 1983). However, the amino acid sequence bears no similarity to the primary structure around the conserved cysteine in all other plant cysteine proteinases. The amino acid sequence of the bacterial cysteine proteinase, streptococcal proteinase (Tai *et al.*, 1976) also shows no homology with that of plant cysteine proteinases. This suggests that plant and bacterial cysteine proteinases represent another example of convergent functional evolution as observed with the families of the serine proteinase class.

Aspartic proteinases. Aspartic proteinases depend upon carboxyl groups for their activity and occur in all groups of organisms but are very rare in micro-organisms. There are only two known microbial groups which contain aspartic proteinases: one is thermopsin from an extremely thermophilic micro-organism *Sulfolobus acidocaldarius* (Lin and Tang, 1990). The other group of micro-organisms is that of the fungi where the best known proteolytic enzyme is pepsin, which is active in the pH region of between 1 to 5 (Fruton, 1976).

The structures of members of the pepsin family in fungi are comprised of two domains between which the active site cleft is positioned. Two Asp residues at positions 32 and 215 are within this cleft and are directly responsible for catalytic activty. They have equivalent positions within the two homologous domains which allow the use of Asp^{32} as a base to attack the acyl carbon of the substrate and Asp^{215} as an acid which allows transfer of a proton to the NH leaving group (Fruton, 1976; James 1980). Both may form covalent adducts with the two portions of the substrate.

Metalloproteinases. The best studied microbial proteinase from a thermophile is Thermolysin from *Bacillus thermoproteolyticus* (Endo, 1962). This metalloproteinase has a molecular weight of 34,000 and contain 316 amino acids in a single peptide without disulphide bridges (Titani *et al.*, 1972). The active site cleft contains a zinc atom bound by two closely spaced His side chains and a more remote glutamic acid residue (Matthews, 1988). In almost all known metalloproteinases, zinc appears to be the metal ion that is bound at the active site. In some metalloproteinases, this ion can be replaced with another metal ion such as cobalt and still retain activity.

The mechanism of action of metalloproteinases is not entirely understood, but in each case the zinc ion interacts with the carbonyl oxygen of the scissile peptide bond. The zinc ion is five-coordinated with the native enzyme to the two His side chains and the two carbonyl oxygen atoms of the glutamic acid residue and a water molecule. Upon binding of a substrate, its carbonyl oxygen replaces the water molecule. The nucleophiles that interact

with the carbonyl carbon of the scissile bond are either the carboxylate oxygen atom of Glu residues or water molecules hydrogen-bonded to them (Rees and Lipscomb, 1981).

1.2 THERMOPHILY AND THERMOSTABILITY OF ENZYMES.

1.2.1 Definition of Thermophily

Temperature ranges for growth have often been used as a means to classify groups of organisms. The most common divisions are the psychrophiles (-3 to 20°C), the mesophiles (13 to 45°C) and the thermophiles (42 to 100°C or more). Within each of these divisions, an organism is identified according to its minimal, optimal and maximal growth temperatures. However, not all organisms fall into distinct groups. For example, *Bacillus coagulans* has a temperature range for growth of 30-60°C and falls both in the mesophilic and thermophilic divisions. Due to this overlap, the bacterium is often referred to as a facultative thermophile.

Before the discovery of extreme thermophiles, thermophilic bacteria were divided into three categories (Farrell and Campbell, 1969): obligate thermophiles (growth temperature optima between 65 and 70°C), facultative thermophiles (maximal growth temperature between 55 and 65°C), and thermotolerant bacteria (maximal growth at 45 and 50°C). However, since many extreme thermophiles have growth optima above 70°C, Heinen and Heinen (1972) introduced the term "caldoactive" (caldus meaning hot) to describe extreme thermophiles. In a classification scheme proposed by Williams (1975), caldoactive bacteria have a maximum growth temperature above 70°C, optimum above 65°C, and a minimum above 40°C.

The generally accepted categorization of high temperature organisms into thermophilic and extreme thermophilic species is based rather arbitrarily on chosen cardinal temperatures with growth minima, optima and maxima within defined ranges (Table 1.3).

However, with the increasing number of organisms growing at temperatures greater than 70°C, the need for an additional category has become apparent. Thus the term "hyperthermophilic" (Zillig *et al.*, 1990) is used which refers to an organism having a growth temperature optimum of 90-100°C.

Table 1.3 Classification of Thermophiles by means of cardinal temperatures (adapted from Wiegel and Ljundahl, 1985).

	Cardinal Temperature [oC]		
	Tmin	Topt	Tmax
Thermophiles	>30	>50	>60
Extreme thermophiles	>40	>65	>70
Hyperthermophiles	not defined	≥100	>100

1.2.2 Mechanisms of Thermophily

The ability of micro-organisms to grow at elevated temperatures has held a particular fascination for biochemists, as such organisms exist at temperatures where proteins would normally be denatured. Extensive studies have shown that the phenomenon of thermophily cannot be explained by a single universal mechanism, but is the product of an array of molecular mechanisms (see below).

Historically, it has been recognised that growth temperature has an effect on the lipid composition of micro-organisms (Heilbrunn, 1924; Belehradek, 1937). It was observed from studies on a thermophilic *Bacillus* resembling *Bacillus stearothermophilus* (Daron, 1970) that fatty acids containing 16 or 17 carbons comprised more than 80% of the total, with branched chain fatty acids being at a higher level than normal fatty acids. Bauman and Simmonds (1969) also reported an increase in saturated and branched chain fatty acids containing up to 19 carbons in some extreme thermophilic organisms. Subsequent studies of fatty acid composition in thermophiles (Ray *et al.*, 1971*a* and *b*) confirmed that this phenomenon was indicative of enhanced membrane stability at higher growth temperatures.

Allen (1953) proposed that organisms growing at higher temperatures should have increased rates of metabolism which was necessary for cell maintenance. It was thought that rapid resynthesis of enzymes and other macromolecules may be essential for cell

maintenance at elevated temperatures. However, Koffler (1957) considered that it was not the rapid resynthesis of macromolecules but differences in intrinsic thermostability of macromolecules or structures which allowed growth at high temperatures. This observation was based on Arrhenius-type plots of thermophilic organisms. Brock (1967) found that slopes of an Arrhenius-type plot of a thermophile were much less than the slope based on *E. coli* grown at various temperatures. He also reported that the temperature coefficients of many mesophilic and thermophilic organisms (that is, the activation energy in the Arrhenius equation, which is proportional to the ratio of the growth rates at two different temperatures) were found to be similar, suggesting that the growth rates increased at a similar predictable rate (Table 1.4).

Table 1.4 Temperature coefficients for the growth of psychrophilic, mesophilic, and thermophilic micro-organisms (adapted from Amelunxen and Murdoch, 1978)

Organism	Temperature Coefficient (cal)
Psychrophiles	
Pseudomonas 21-3C	13,000 (14 - 25°C)
Pseudomonas P-200	24,000 (4 - 12°C)
Micrococcus cryophilus	21,000
Mesophiles	
Escherichia.coli	27,100
Pseudomonas aeruginosa	17,100
Thermophiles	
Thermus aquaticus YT1	21,200[a]
Thermus thermophilus	13,600[a]
Bacillus stearothermophilus var *nondiastaticus*	19,000

[a] These values estimated from growth rates at 10 to 15°C below optimum growth temperature.

Hence, the major reason for slower optimal growth rates of organisms growing at higher temperatures than would be predicted by the Arrhenius equation appeared to be due to genetic differences governing the catalytic processes, that is, the catalysts responded to temperature in a particular manner but their absolute rates were different. This further

suggested that rapid resynthesis cannot take place at elevated temperatures. If this mechanism was significant, then protein synthesis would be expected to increase more rapidly at elevated temperatures than would be predicted from the Arrhenius equation, that is, the temperature coefficient of thermophiles would be greater than values obtained from mesophiles.

Initial reports on the unusual thermostability of enzymes from thermophilic bacteria were made using crude cell-free extracts. Militzer *et al.*, (1949) demonstrated that malate dehydrogenase from an obligate thermophile was thermostable at 65°C for 2 hours, whereas the homologous enzyme from *Bacillus subtilis* showed rapid inactivation at this temperature. Amelunxen and Lins (1968) reported that nine out of eleven enzymes from crude cell-free extracts of *B. stearothermophilus* were significantly more thermostable than their counterparts from the mesophile *B. cereus*.

It was hypothesized that the thermostability of enzymes arose from one of two possible mechanisms. The first, described by Allen (1953) suggested that rapid resynthesis of macromolecules was responsible for cell maintenance at high temperature. The second mechanism implicated the existence of intracellular, transferable factors which would considerably increase the stability of enzymes in the cell. Although very few reports have shown that these 'transferable factors' have existed and been successfully isolated (Prasad and Maheshwan, 1978; Oshima, 1982), it has been shown that this mechanism for thermostability does not reflect the true state of most enzymes at high temperatures.

The current concept on the nature of thermostability can now be confined to two basic ideas. The first view is that enhanced thermal stability of enzymes is due to subtle differences in their molecular structure as compared to their mesophilic counterparts, and that stability at high temperatures results from either intrinsic molecular forces or extrinsic influences.

The second is the increased stability of enzymes due to the participation of other components in the cell in supramolecular structure (such as lipids, proteins,

polysaccharides). As a result of this natural immobilisation, the native, catalytically active centre may be fixed, and the enzyme may be less likely to be denatured than in a mesophilic cell (Alexandrov, 1978).

1.2.3 Molecular Mechanisms of Thermostability

Current research has shown that a variety of both extrinsic and intrinsic stabilising effects, such as hydrogen and ionic bonding, hydrophobic interactions, disulphide bonds, metal binding, single amino acid substitutions and so on, can contribute to protein stability at high temperatures. In addition to stability at high temperatures, most thermostable enzymes have been shown to be more resistant to most common protein denaturants than their mesophilic counterparts (Veronese *et al.*, 1984; Owusu and Cowan, 1987).

1.2.3.1 Extrinsic Molecular Mechanisms

Metal ion binding. *In vitro* studies have shown that additional thermostability of enzymes *in vivo* may come from a variety of interactions with other naturally occurring cellular components (Table 1.5). For example, some thermophilic proteinases exhibit enhanced stability in the presence of calcium ions (Tajima *et al.*, 1976; Cowan and Daniel, 1982; Khoo *et al.*, 1984). The mechanism of metal ion stabilisation appears to be via the binding of cations to the labile parts of the protein structure, particularly at the bends of the polypeptide chain (Matthew *et al.*, 1974) making the overall structure of the protein more compact and rigid (Dalgleist *et al.*, 1976; Hachimori *et al.*, 1979). In the presence of metal chelators such as EDTA or other complexing agents, the dissociation of metal cations has been shown to considerably decrease stability, in some cases, partial or complete inactivation of the enzyme can also occur (Ohta, 1967; Heinen and Lauwers, 1976).

Substrates, Prosthetic Groups and Other Low Molecular Weight Substances. Studies have shown (Table 1.5) that like most other enzymes, some thermophilic enzymes are stabilised in the presence of substrates or other low molecular weight effectors of enzymatic activity (Wedler and Hoffmann, 1974; Hachimori *et al.*, 1974; Hibino *et al.*, 1974; Bendzko and Hintsche, 1978). For instance, this mechanism of

Table 1.5 ***In Vitro*** **Thermostability of Some Thermophilic Enzymes**

Organism and Enzyme	Temperature of thermostability (°C)		References
	Intrinsic	+ Stabilisers	
Bacillus caldolyticus			
α-amylase	45	70 (Ca^{2+})	Heinen and Lauwer (1976)
B. stearothermophilus			
Enolase	40	60 (Mg^{2+})	Boccu *et al* (1976)
NADP Isocitrate Dehydrogenase	55	<70 (substrate)	Hibino *et al* (1974)
Glutamine synthetase	<60	70 (cofactors and substrate)	Hachimori *et al* (1974) Wedler and Hoffmann (1974)
α–amylase	50	<70 (Ca^{2+})	Ogasahara *et al* (1970)
B. thermoproteolyticus			
Thermolysin	<50	80 (Ca^{2+})	Dahlqhist *et al* (1975) Ohta (1967)
Thermus aquaticus			
Enolase	88	100 (Mg^{2+})	Stellwagen and Barnes (1976)
Caldolysin		(Ca^{2+})	Cowan and Daniel (1982)
Thermotoga maritama			
Lactate dehydrogenase	80	90 (NAD+ and substrate)	Wrba *et al* (1990)

Note: Stability data obtained at their optimal operating conditions.

stabilisation accounts for the enhanced stability of thermophilic ferrodoxin and cytochrome C_{552}. Their structures differ only slightly from those of the respective mesophilic proteins, with the exception being that the Fe-S clusters and the haem are more firmly attached to the protein moiety. This mechanism of stabilisation is also common in many enzymes from mesophiles (Schellman, 1976).

Protein-Protein Contacts. Protein-protein interactions have been reported to increase the stability of some thermophilic enzymes. These may involve the rearrangement of subunit structure to form more stable oligomeric forms such as that observed with glutamine synthetase from *B. stearothermophilus* and *B. caldolyticus* (Wedler and Hoffmann, 1974; Wedler *et al.,* 1976). In addition, Hamori *et al.*. (1979) found that intersubunit interactions were necessary for the stability of thermophilic inorganic pyrophosphatase and ATPase.

1.2.3.2 Intrinsic Molecular Mechanisms

Electrostatic Interactions. Stabilisation via electrostatic interactions has been found to occur in many thermophilic enzymes. Many researchers have reported that the addition of only one or two salt bridges in the interior of the protein can significantly enhance the thermostability of an enzyme. The addition of these bonds can result in a 5-7kcal.mol^{-1} decrease in free energy of a thermophilic protein compared to its mesophilic counterpart, thereby stabilising the protein structure (Schulz and Schirmer, 1979; Perutz, 1978; Kagawa *et al.,* 1979; Iijima *et al.,* 1980; Sundaram *et al.,* 1980).

This mechanism however, is different in halophilic bacteria (organisms that grow in high salt environments). At low ionic strength, purified halophilic enzymes tend to denature rapidly (Keradjopoulos and Wulf, 1974; Crabb *et al.,* 1977). It has been shown that the structures of these enzymes differ from those of mesophilic proteins in having a greater number of glutamic and aspartic acid residues on the surface of the protein. This mechanism may involve screening the excess negative charges on the surface of the protein

by counterions, thereby rendering the enzyme more rigid and stable than common mesophilic proteins (Crabb *et al.*, 1977; Rao and Argos, 1981).

Intramolecular Disulphide Bonds. Disulphide bonds are a common feature of many extracellular enzymes, in contrast to many intracellular enzymes which have no S-S bridges due the internal reducing environment. A good example is the thermophilic subtilisin-type protease, aqualysin I from *Thermus aquaticus* YT-I (Matsuzama *et al.*, 1988). This enzyme contains two disulphide bonds at Cys 67/Cys 99 and Cys 163/Cys 194. The authors report that these disulphide bonds appear to be responsible for the thermostability of the protease.

The addition of intramolecular disulphide bonds to enhance the thermostability of an existing thermostable enzyme has been shown by Takagi *et al* (1990). Using site-directed mutagenesis, they have demonstrated that the introduction of a disulphide bond to the cysteine-free serine protease, subtilisin E from *B. subtilis*, increased the melting temperature of this enzyme by 4.5°C compared with the wild type, without affecting its catalytic efficiency.

It has been reported that some mesophilic enzymes exhibited enhanced thermostability by the introduction of a disulphide bond. From thermodynamic data, Wetzel *et al* (1988) demonstrated that an engineered 3-97 disulphide bond, in the disulphide-free enzyme T4 lysozyme (Perry and Wetzel, 1984), stabilises the molecule against reversible thermal unfolding.

In general, the stabilising effect of this type of cross link in the protein structure has been attributed predominantly to the reduction in chain entropy of the unfolded state (Chan and Dill, 1989). However, contrary to this suggestion, there are many thermostable extracellular enzymes which lack S-S bonds (Amelunxen and Murdock, 1978). In these cases, the important regulating factor for the presence of disulphide bonds may not be the requirement for stability but the conditions in which they are synthesized. Since cysteine is a highly reactive residue (subject to oxidation), it is likely that it may be subjected to a

variety of undesirable side reactions prior to disulphide formation at temperatures up to 100°C (Volkin and Klibanov, 1987).

Hydrophobic Interactions. Hydrophobic interactions make a significant contribution to the maintenance and stability of the native structure at elevated temperatures. Reports that hydrophobic interactions were related to the thermostability of thermolysin (Ohta, 1967), independent studies have been carried out on known thermophilic proteins to determine whether a correlation exists between the stability of the protein and the hydrophobic amino acid content.

Physical studies on thermophilic pyrophosphatase (Hachimori *et al.*, 1979), and glyceraldehyde-3-phosphate dehydrogenase (Suzuki,1973) revealed that the aromatic amino acid content had a more nonpolar microenvironment than their respective mesophilic enzymes. Yutani *et al.*(1977) studied the effect of single amino acid substitutions and other changes, such as hydrogen bonds on the stability of the α-subunit of tryptophan synthetase from *E. coli* . They found that the thermostability of the enzyme increased significantly when a single glutamine residue was substituted with a more hydrophobic residue such as methionine. This point mutation raised the energy of stabilisation of the enzyme molecule by only 3.14kJ.mol-1. From this evidence, it was suggested that enhanced thermostability of the protein correlated with amino acid hydrophobicity. Since these reports were published, investigators have shown that enhanced stability of many thermophilic proteins and some mesophilic enzymes correlated with their high hydrophobicity (Matsumura *et al.*, 1988).

In some cases, the correlation between hydrophobicity and thermostability is not always evident. For example, Wedler *et al.* (1976) reported that thermophilic glutamine synthetase was found to be less hydrophobic than the less stable mesophilic enzyme. The difference in this case was the localisation of particular amino acids within the structure and not necessarily its hydrophobicity.

It has been reported that in some thermophilic proteins, a rise in the content of aliphatic amino acids in the interior parallels a decrease in the number of aromatic amino acids (Singleton *et al.*, 1969; Ljundahl *et al.*, 1976). These two trends can compensate for each other, so that the overall hydrophobicity on the Tanford scale may remain unchanged or even decrease. Even so, such proteins should be more stable than their mesophilic analogues.

Hydrogen Bonds. The presence of additional hydrogen bonds has been known to make a contribution to protein conformation and stability. This is generally done via single amino acid substitutions (Yutani *et al.*, 1977) by site directed mutagenesis, which provide limited numbers of additional hydrogen bonds or modifying the environment in the vicinity of critical H-bonds, to produce sufficient stabilisation to account for differences in mesophilic and thermophilic proteins.

Amino Acid Composition. Apart from the presence of additional covalent and non-covalent interactions that differentiate thermophilic proteins from their mesophilic counterparts, it has been reported that significant differences in the composition of the amino acid sequence of both mesophiles and thermophiles can affect protein stability.

Studies have shown that there are either few or no cysteine residues in thermophilic proteins (Kagawa *et al.*, 1976; Chell *et al.*, 1978). In contrast, there are many cysteine residues in the mesophilic counterparts. The relationship between the stability of enzymes and the content of cysteine residues is apparently due to the fact that inactivation often results from the oxidation of SH-groups (Katchalski-Katzir and Freeman, 1982). It has been shown that the content of cysteine residues may be similar with some thermophilic proteins and their mesophilic counterparts. However, it has also been reported that the localisation and accessibility of these residues actually determine the stability of the thermophilic protein compared to its mesophilic analogues. For instance, the alcohol dehydrogenase from *B. stearothermophilus* has all of its cysteine residues situated within the interior of the protein making them less accessible to the solvent (Bridgen *et al.*, 1973).

There is often a high content of arginine residues and a lower content of lysine residues in thermophilic proteins (Barnes and Stellwagen, 1973; Crabb *et al.*, 1977). A major proportion of these residues is located on the surface of the protein where contact with water makes them thermodynamically unfavourable (lysine being more unfavourable than arginine). The replacement of lysine by arginine has been shown to enhance the stability of a large number of proteins by making the area of contact less unfavourable and due to differences in hydrocarbon moieties (Argos *et al.*, 1979).

1.2.4 Rigidity and Catalytic Efficiency of Thermophilic Enzymes

Conformational flexibility of proteins at high temperatures must be possible if catalytic reactions are to take place. Brock (1967) speculated that the thermostability of thermophilic proteins was due to an inflexible conformation which was thought to account for thermophilic survival. This might imply a reduction of catalytic efficiency and metabolic control. By using proteolytic enzymes as probes of structure (Daniel *et al.*, 1982; Suzuki and Imai, 1982) it has been shown that thermostable proteins are in fact quite rigid molecules at room temperature with respect to their mesophilic counterparts. This is due to the clamping effect of the protein structure brought about by interactions and forces stabilising thermophilic proteins.

Many researchers have also shown that the rigidity of thermophilic enzymes has an adverse effect on their catalytic efficiency since the appropriate degree of flexibility is required for enzyme catalysis (Careri *et al.*, 1975; Gurd *et al.*, 1979; Williams, 1979; McCammon and Karplus, 1983; Ringe and Petsko, 1985). Considering that the rates of enzyme reactions follow the Arrhenius law (that is, doubling in rate for every 10 degree rise in temperature), it would be expected therefore that catalytic rates of thermophilic enzyme reactions might be higher at high temperatures compared to their mesophilic counterparts. In contrast, it has been observed that the specific activities of thermophilic enzymes are much less than that predicted by simple extrapolation of the specific activities of their mesophilic analogues (Ljungdahl and Sherod, 1976; Zuber, 1981). However, extremely thermophilic proteinases have been shown to have higher specific activities than their mesophilic counterparts

(Cowan *et al.,* 1987). It was discovered that the higher specific activities of these enzymes were not due to any specialised functional characteristic of the enzyme but may possibly result from increased frequency of reversibly denatured substrated conformers present at high temperatures.

Consequently, since a thermophilic enzyme is less effective at lower temperatures, then an increase in thermostability (for example, by site directed mutagenesis of a mesophilic enzyme) might bring about a reduction in catalytic efficiency. This inverse correlation was observed in the case of enolase from mesophilic and thermophilic sources (Stellwagen and Barnes, 1976) and with four mutants of kanamycin nucleotidyltransferase (Matsumura *et al.*, 1984). This reduced efficiency would therefore be compensated by the higher temperature of their habitat so that similar specific activities would be observed for thermophilic and mesophilic enzymes at their respective temperature optima.

1.3 THE ARCHAEA

1.3.1 Introduction

Until the late 1970's, all living organisms were divided into two primary kingdoms, Procaryotae and Eucaryotae (Chatton, 1937; Allsopp, 1969; Murray, 1974). However, a number of discoveries in the field of microbiology, in particular, the isolation of unique organisms growing in extreme environments, led Carl Woese and collaborators to propose a concept which reclassified all living organisms into three primary kingdoms: the eukaryotes, eubacteria and the archaeobacteria (Woese *et al.*, 1978; Woese and Fox, 1977; Fox *et al.*, 1980). This concept was based primarily on the homologies of partial sequences of 16S and 18S ribosomal ribonucleic acid (rRNA) of a large number of species. These molecular comparisons were found to reflect the true phylogenetic position of a species in a particular kingdom.

1.3.2 Origins of the Archaebacteria

Before Woese's concept was proposed, there had been many species of bacteria which had been studied in detail and recognised as being different from typical bacteria. These include *Thermoplasma* (Darland, 1970), the extremely thermoacidophilic bacterium *Sulfolobus* (Brock *et al.*, 1972), members of the *Halobacterioceae* (Larsen, 1962), and the methanogens (Barker, 1956)).

Thermoplasma. *Thermoplasma acidophilum* was first isolated by Darland *et al.* (1970) from a spontaneously burning refuse pile. This organism was found to have an optimum growth temperature of 59°C and to inhabit environments of very low pH (1 to 2). The organism was originally classed with the Mycoplasmas because of the morphological resemblance to these organisms, but was later classified with the Archaeobacteria, on the basis of molecular similarities with the halophiles, methanogens and *Sulfolobus* (Woese *et al.*, 1978). Even more interesting was the discovery of a histone-like protein (HTa) from *Thermoplasma* associated with the host DNA and observed to stabilise the DNA against thermal denaturation (Searcy and Stein, 1980). The size and amino acid composition of this

small basic protein resembled that of eukaryotic histones. (Searcy and Delange, 1980). It was postulated therefore, that *Thermoplasma* was not related to prokaryotes.

Sulfolobus. In the early 1970's, Brock *et al.* (1972) isolated a sulphur-oxidising bacterium from solfataric areas, which was able to grow at high temperatures and low pH. This unique organism was assigned the genus name of *Sulfolobus*, and was shown to have an optimum growth temperature of 70-75°C and a pH optimum of 2-3. Its cell wall was found to be devoid of diaminopimelic acid and murein, and to contain unusual membrane lipids of C_{40} tetraethers, instead of ester lipids (Langworthy *et al.*, 1974). Later studies using 16S rRNA sequences and other molecular features proved this species to be typically archaeobacterial.

The Halobacteria. Bacteria isolated from highly saline environments (>2.5M sodium chloride) were originally classified with the eubacterial gram-negative aerobic rods *Pseudomonas* and *Acetobacter* (Larsen, 1962). Although closely related, differences in their ionic requirements, cell wall compostition, lipid content of C_{20} isoprenoid glycerol diethers (Sehgal, 1962; Kushner *et al.*, 1964), DNA components and the presence of a red, orange or pink pigment distinguished these halophiles from other gram negative aerobic rods, providing evidence for the concept of separate phylogenetic groupings well before Woese's proposal was published.

The Methanogens. The methane-producing bacteria have long been recognised as a coherent taxonomic group of strict anaerobes due to their unique mode of methanogenic energy metabolism (Barker, 1956). The methanogenic bacteria were initially dispersed among the better characterised bacterial groups on the basis of their morphologies, and were clustered into a single family known as the *Methanobacteriaceae* (Barker, 1956). However, the grouping based on morphological criteria provided little insight into relationships between the various species. Fox *et al.* (1977) applied comparative cataloguing of 16S rRNA to a variety of methanogenic bacteria. The results showed the methanogens to be a unique biological group, phylogenetically distant from the typical

bacteria. Subsequent studies on DNA structure, intermediary metabolism, lipid composition and cell wall composition placed this group of organisms among the halobacteria and the extreme thermoacidophiles *Sulfolobus* and *Thermoplasma* to form the third kingdom known as the Archaeobacteria (Woese *et al.*, 1978).

1.3.3 Universal Phylogeny.

Definition of a Chronometer. A molecular chronometer is a molecule whose sequence changes randomly in time. The amount of sequence change it accumulates (formally a distance) is the product of a rate (at which mutations become fixed) multiplied by a time (over which changes have occurred). This change cannot be measured by comparison of some original state to a final state as the original state (ancestral pattern) is not accessible. Therefore, the change is measured from the fact that two (or more) versions of a given sequence that occur in extant representatives of two (or more) lineages have ultimately come from the same common ancestral pattern, and so measures the sequence differences between the two (or more) extant versions.

The essentials of a good chronometer consist of the following (Sogin *et al.*, 1972):
(a) ubiquitous occurrence of equivalent macromolecules throughout the living world,
(b) ease of isolation,
(c) technical feasibility of obtaining substantial primary sequence information.

In 1965, Zuckerkandl and Pauling first recognised the importance of molecular chronometers when they discovered that the primary structures of macromolecules held a trace of evolutionary history which could be exploited to define phylogenetic relationships. At the time, it was demonstrated that cytochrome c was an excellent molecular chronometer for eukaryotic phylogeny (Fitch and Margoliash, 1967). Sequence comparisons of this chronometer have been used very successfully to time key events in eukaryotic evolution (in sequence distance terms) and to determine molecular genealogies (Fitch and Margoliash, 1967). However, the disadvantage of using cytochrome c as a molecular chronometer is that its effective range is restricted to the subdivision level of the eubacteria.

Since then, there has been a search for other molecules to serve as molecular chronometers. These include glyceraldehyde-3-phosphate dehydrogenases (Hensel *et al.*, 1989) and antigenic determinants such as Ef Tu factors (Cammarano *et al.*, 1989). The most useful and functionally constant molecular chronometer is rRNA.

Oligonucleotide Cataloguing. Until recently, partial oligonucleotide sequences were used to determine the phylogenetic position of a given species. Before complete sequences were constructed, classification of organisms was based on the oligonucleotide cataloging method (Fox *et al.*, 1977). Short oligonucleotides of lengths of up to 20 or so bases are produced by digestion of 16S rRNAs with the enzyme ribonuclease T_1. These partial sequences were then grouped together producing a complex pattern characteristic for that species. This in turn was used for comparative studies among other species from different lineages permitting phylogenetic grouping at various taxonomic levels by constructing trees reflecting the evolutionary origins of various strains. This was done on the basis of association coefficients (S_{AB}) :

$$S_{AB} = 2N_{AB} / (N_A + N_B),$$

where N_A, N_B, and N_{AB} are the total number of sequences and the identical sequences in two sequence catalogues to be compared (Fox *et al.*, 1977). These values are only related to true similarity in an unknown, nonlinear fashion, and if used in tree construction, branch lengths produced would be meaningless. The only approach in using S_{AB} would be to perform cluster analysis (see later).

The cataloguing method is sufficient to define most of the major bacterial phyla but generally fails to resolve the branching orders among them or their subdivisions, as well as the branching order of rapidly evolving lines of descent. However, the use of complete sequences of 16S rRNA largely overcomes these problems as it greatly increases the resolving power of the rRNA chronometer.

Analysis of Sequence Alignments

Once a sequence alignment of rRNAs is constructed for a species, it is necessary to analyse this to extract the most phylogenetically useful information in determining the position of a species on a phylogenetic tree. This is done by using one of the following computational methods: distance matrix treeing, maximum parsimony analysis, or cluster analysis.

Distance Matrix Treeing Distance matrix treeing uses only the sequence distances between pairs of sequences, that is, the fraction of positions in which the two sequences differ. This distance is actually an underestimate of the true evolutionary distance between sequences. Although most of the differences between two sequences reflect single mutational events at any given position in the sequence alignment, some of them represent multiple events. These multiple changes remain a major problem in tree construction whatever the method. Also, this method assumes that evolutionary distances conform to a tree topology (Felsenstein, 1982).

Maximum parsimony analysis Unlike the distance matrix method, maximum parsimony analysis does not reduce the differences among sequences to a single number or distance. It treats the positions individually. This method assumes that the correct phylogenetic tree is the most parsimonious one, that is, the one for which the smallest overall number of mutational changes have to be postulated to arrange the set of considered sequences upon it. As with distance matrix treeing, maximum parsimony analysis looks at all possible tree branching arrangements and chooses the most parsimonious one. As with distance matrix analysis, problems of finding the correct tree can be computationally intense and requires devising computer algorithms (Felsenstein, 1982).

Cluster analysis This method groups sequences on the basis of how similar they are to one another or to other groups of sequences. Although cluster analysis is the least computationally intense of the other methods of analysis, it is also the least accurate (Sneath and Sokal, 1973).

The main difficulty with all analysis of sequence data is that different lineages and different positions in a sequence can evolve at significantly different rates. Parsimony analysis tends not to position rapidly evolving lineages correctly and is confused by rapidly changing positions, more so than distance matrix treeing. Cluster analysis is sensitive to these problems in that rapidly evolving lineages are positioned too deeply in trees so constructed. In other words, analyses of sequence data are far from optimal. Improvements in methods can basically be the result of empirical approaches. Given a large enough sequence data base, it will become possible to describe the pattern of change at given positions in a rRNA molecule and design specific analyses based upon these descriptions. What is required is an analysis that corrects more accurately for multiple changes at sites and utilise only the positions in the molecule appropriate to the phylogenetic range being measured.

Using these methods in the analysis of complete 16S/18S rRNA sequences, detailed phylogenetic trees indicating relationships between the archaeobacteria, the eukaryotes and the eubacteria have been derived.

1.3.4 Universal Phylogenetic Tree

The molecular evolutionary studies on the origin and evolution of the primary kingdoms carried out by the construction of phylogenetic trees are generally based on comparison of a single molecule (i.e. a chronometer) from a variety of extant species.

The phylogenetic tree is essentially an evolutionary distance-matrix tree calculated from inter- and intra-aligned sequences of organisms. This is based on the assumption that all positions in the sequence change at the same rate in any given lines of descent, taking into account the evolutionary rate of more rapidly lines of descent. The phylogenetic tree can be rooted or unrooted depending on the method of computational analysis in determining the position of a particular species.

One of the first representations of an unrooted phylogenetic tree which encompasses all extant life (Fig. 1.2) was based on a matrix of evolutionary distances calculated from an

alignment of homologous 16S rRNA sequences from each of the three urkingdoms (Mc Carroll *et al.*, 1983; Woese and Olsen, 1986). However, from the comparisons of nucleic acid sequences, it was not possible to derive the exact evolutionary relationship among the urkingdoms because the root of the tree was unknown.

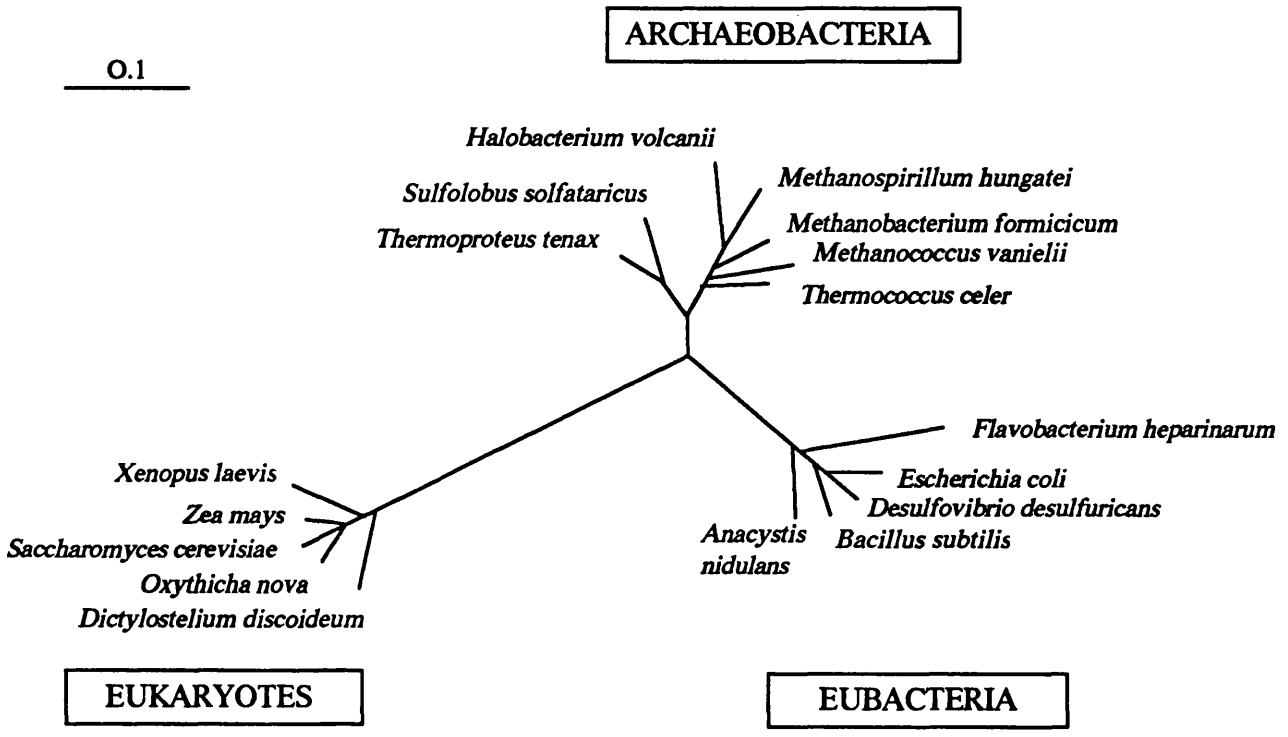


Figure 1.2 The unrooted phylogenetic tree of the three primary kingdoms (reproduced from Woese and Olsen, 1986)

The distance measure bar corresponds to 0.1 mutational events per sequence position.

Iwabe *et al.* (1989) proposed that it was possible to derive this evolutionary relationship by comparing a pair of duplicated genes such as elongation factors Tu and G, and the α and β subunits of ATPase. Both groups are thought to have diverged by gene duplication before divergence of the primary kingdoms. By inferring composite phylogenetic trees from each protein pair, it was revealed that the archaeobacteria were more closely related to the eukaryotes than to the eubacteria. Furthermore, simultaneous comparisons of another pair

of proteins, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were also found to produce a similar composite phylogenetic tree.

The topology of this universal phylogenetic tree has been favoured by many evolutionists in that the use of several "evolutionary markers" such as 5S rRNA (Wolters and Erdmann, 1986) and RNA polymerases (Zillig *et al.*, 1982) from archaeobacteria, show greater sequence similarity to eukaryotes than to eubacteria. However, this topology has been disputed in the phylogenetic derivation ("eocyte" tree) proposed by Lake (1988). On the basis of rate invariant analysis of RNA sequences using *evolutionary parsimony* (Lake, 1987), a parkaryote-karyote classification was proposed where all extant life was organised into two monophyletic superkingdoms - the parkaryotes (bacteria) and the karyotes (the proto-eukaryotic group). The kingdoms further subdivide into five groups: the eubacteria, the halobacteria, the eocytes (the extremely thermophilic sulphur-metabolising anucleate cells), the methanogens and the eukaryotes. The eocyte topology was constructed using evolutionary parsimony which estimates the length and statistical significance of the central branch of the four taxa trees. Unlike distance and maximum parsimony, evolutionary parsimony was found to be the algorithm least sensitive to unequal rate effects of taxa evolution (Holmquist *et al.*, 1988; Felsenstein, 1984; Cavender, 1989). In addition, Lakes' preference of the eocyte tree over that of the archaeobacteria-eubacteria-eukaryote tree has been described by sequence similarities of both small and large rRNAs. It has been reported (Woese and Fox, 1977; Woese and Olsen,1986; Woese, 1987; Gouy and Li, 1989) that sequence homology was higher between archaeobacteria and eubacteria rather then between the archaeobacteria and the eukaryotes. Iwabe *et al.* (1989) who favour the archaeobacterial-eubacteria-eukaryote tree, suggested that mutational changes may have accumulated more rapidly in the eukaryotic rRNA than in the archaeobacterial and eubacterial rRNA shortly after the separation of the archaeobacteria and eukaryotes.

1.3.5 Reclassification of the Kingdoms.

Woese *et al.* (1990) has recently proposed a reclassification of all organisms in a phylogenetic system which recognises the primacy of the three groupings. Woese and

colleagues proposed that the name archaeobacteria should be replaced by 'archaea' to avoid any confusion with the true bacteria. It was also proposed that the organisation of organisms in a kingdom be assigned to a new lower level taxon and be replaced with a taxon of the highest rank known as a domain.

The three domains thus suggested would be as follows (Fig. 1.3): The Bacteria (ie. the eubacteria) which is based upon a traditional name for the group; the Eucarya, which is derived from the group's common name and allows certain cytological features to be defined such as the presence of a nucleus, and the Archaea (ie the archaeobacteria) which is used to denote the primitive nature of these organisms. The names assigned to these domains should therefore be simple enough to serve in common usage such that "Bacteria" cannot include the Archaea since the term archaeobacteria suggests a specific relationship between the Archaea and the Bacteria.

According to Woese *et al.* (1990), these domains should preserve the kingdoms such as Plantae, Animalia and Fungi and that the reclassification allowed the preservation of the phylogenetic structure at the kingdom level.

The Archaea fall into two distinct phylogenetic groups (Woese, 1987), the methanogens and their relatives, which include a diverse mixture of all the Archaea phenotypes, and the sulphur-dependent extremely thermophilic archaea, comprising a closely related and phenotypically homogeneous collection of thermophilic sulphur dependent species (Woese, 1987; Achenbach-Richter *et al.*, 1987). Woese *et al.* (1990) proposed that these two groups should adopt the formal names of Euryarchaeota (or euryotes) for the methanogenic group, and Crenarchaeota (or crenotes) for the other group, respectively (Fig. 1.4).

The reclassification of organisms may possibly provide a natural system at the highest levels and allow a fully natural classification of microorganisms (eukaryotic as well as prokaryotic). In addition, it may recognise that the lineages of Bacteria and the Archaea are

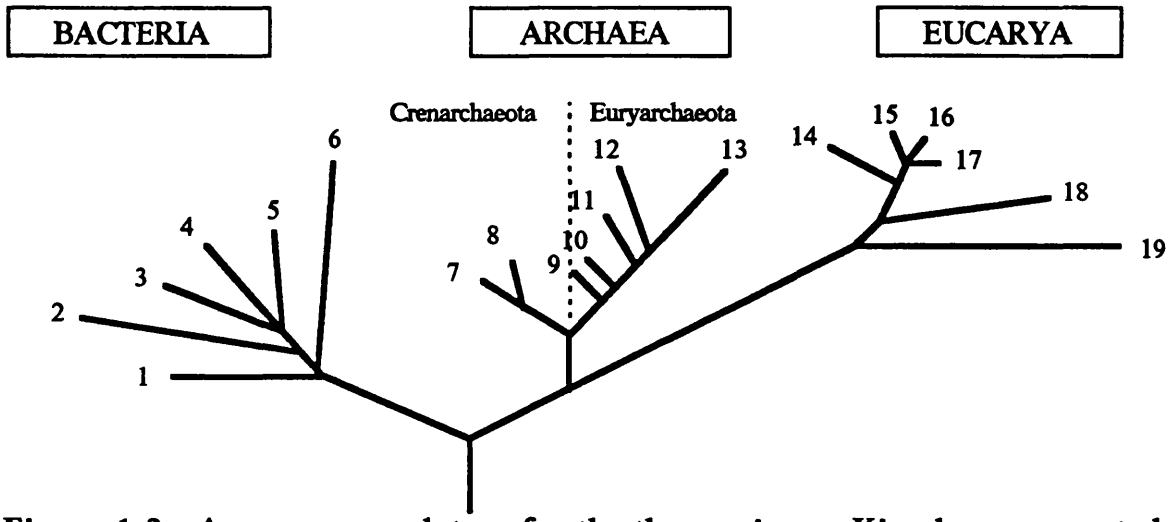


Figure 1.3 A new nomenclature for the three primary Kingdoms presented on the rooted phylogenetic tree. (reproduced from Woese *et al.*, 1990)

Numbered groups refer to 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; 5, the Gram positive bacteria; 6, the green non-sulphur bacteria; 7, *Pyrodictium* species; 8, *Thermoproteus* species; 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacteriales; 12, the Methanomicrobiales; 13, the extreme halophiles; 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates, and 19, the microsporidia.

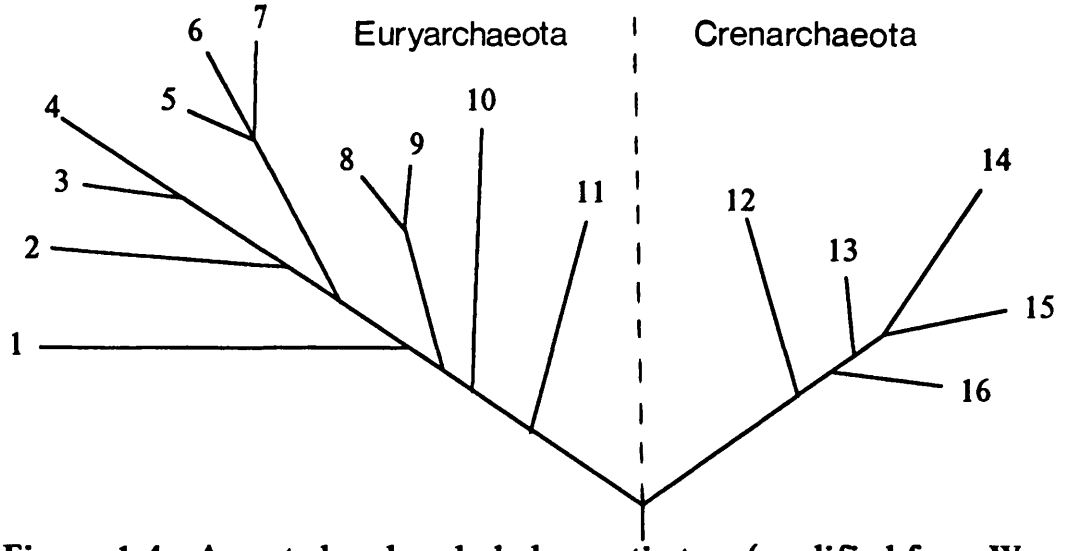


Figure 1.4 A rooted archaeal phylogenetic tree (modified from Woese, 1987).

Phylogenetic data is based on 16S rRNA sequence comparisons. The root was imposed by the use of outgroup concensus sequence. numbered groups refer to: 1, *Thermoplasma*; 2, *Methanospirillum*; 3, *Methanosarcina*; 4, FS-1; 5, *Halobacterium volcani*; 6, *Halococcus morrhuae*; 7, *Halobacterium cutirubrum*; 8, *Methanobacterium thermoautotrophicum*; 9, *Methanobacterium formicicum*; 10, *Methanococcus vanielii*; 11, *Thermococcus celer*; 12, *Thermoproteus tenax*; 13, *Pyrodictium occultum*; 14, *Sulfolobus solfataricus*; 15, *Desulfurococcus mobilis*, and 16, *Hyperthermus butyliticum*.

independent of each other, and that the original classification of organisms as animals and plants should not strictly be seen as the most important kingdoms of the classification system.

1.3.6 Diagnostic properties of the Archaea

The use of partial and complete 16S/18S rRNA sequences to distinguish the archaea from the other two domains has generally been successful due to the extremely conserved structure and function of these molecular chronometers. In addition to their 16S rRNA sequences, there are many other features that clearly set the archaea apart from the bacteria (Table 1.6). For instance, different types of cell envelopes usually consisting of protein, glycoprotein, polysaccharide, or a non-murein peptidoglycan are observed in archaeal species instead of the murein sacculus normally present in the bacteria (Kandler, 1982). Isoprenoid ether lipids that have not been found in bacteria are typical constituents of archaeal membranes (Kates, 1972; Langworthy *et al.*, 1974).

There are several other features that the archaea and the eukaryotes have in common. An elongation factor in protein biosynthesis, for instance, is inactivated by diptheria toxin as in eukaryotes (Kessel and Klink, 1980). A relationship between archaeaobacteria and eukaryotes has been suggested by the structure of the DNA-dependent RNA polymerase (Zillig et al., 1978) and the inhibition of replication by aphidicolin *in vivo* (Forterre *et al.*, 1984) and *in vitro* (Zabel *et al.*, 1985). Introns in tRNA genes are quite common in the archaea (Kaine *et al.*, 1983) and an intervening sequence was also found in an archaeobacterial 23S rRNA gene (Kjems and Garrett, 1985).

Like the bacteria and eukaryotes, the archaea can also serve as hosts for viruses. Phages have been found in all archaeal phenotypes (Reiter *et al.*, 1988). In addition, a virus like particle, SSV1 has been characterised in *Sulfolobus* (Martin *et al.*, 1984) which was originally thought to be a plasmid but was subsequently found to be a UV-inducible prophage. Hexagonal viruslike particle has also been reported in the culture medium of *Pyrococcus woesei* (Zillig *et al.*, 1987).

Table 1.6 Some molecular characteristics of the Archaea.

	Molecular Characteristics	Reference
Archaea	Phytanyl ether-linked lipids instead of fatty esters in cell membranes	Langworthy and Pond (1986)
	Replacement of ribothymidine by 1-methylpseudouridine or uridine in the common arm of their tRNA's	Woese (1983)
	Different types of cell envelopes instead of murein	Kandler (1982)
	Resistant to cell wall antibiotics such as penicillin and vancomycin	Hilpert *et al.* (1981)
'Eucaryal' features of the Archaea	Presence of intervening sequences (introns) in genes coding for tRNAs, rRNAs and possibly proteins	Kaine *et al.* (1983)
	RNA polymerases immunogically related to eucaryotic counterparts	Zillig *et al.* (1978)
'Bacterial' features of the Archaea	Translation system is sensitive to some antibiotics such as chloramphenicol and streptomycin	Pecher and Bock (1981)
	Existence of restriction endonucleases	Schmid *et al.* (1984)
	Infection by bacteriophage	Schnabel et al. (1982)
	Occurence of 70S ribosomes	Woese (1982)
	Presence of plasmids	Zillig *et al.* (1986)

Although morphological features of the archaea strongly resemble those species which belong to the bacterial domain, high sequence homology between the archaea and the eucarya, rather than the archaea with bacteria, and similar molecular characteristics suggests these two domains to be closely related. Even so, no conclusive evolutionary evidence has

proved the exact relationship between these domains, but whatever the controversy, this problem can only be solved at the molecular level.

1.4 THE GENUS *PYROCOCCUS*

The genus *Pyrococcus* belongs to the family *Thermococcaceae* which is the only known family of the order *Thermococcales.* Species from this order are mainly characterised by their cell morphology and metabolism but are distinct from other species of the archaeobacteria on the basis of their cross-reactivity among RNA polymerases within this order (Zillig *et al.*, 1987).

The genus *Pyrococcus* currently contains only two species: *Pyrococcus furiosus* (Fiala and Stetter, 1986) and *Pyrococcus woesei* (Zillig *et al.*, 1987). Both species were isolated from marine solfataras at Vulcano, Italy, and are very similar in morphology but metabolically different. Comparisons of restriction patterns of the DNA from both organisms reveal striking similarities as well as significant differences. On this basis and their slight differences in their metabolism, the organisms were designated as different species (Zillig *et al.*, 1987).

1.4.1 Physiology and Biochemistry

P. furiosus belongs to the group of hyperthermophilic anaerobic archaeobacteria that grow at temperatures higher than 100°C (Stetter *et al.*, 1990). It is an obligate heterotroph capable of growing on a variety of complex substrates such as peptone and yeast extract, as well as utilising less complex substrates such as maltose (Fiala and Stetter, 1986). *P. furiosus* can modify its metabolism depending on whether it is grown with or without elemental sulphur (Fiala and Stetter, 1986; Kelly and Deming, 1988). In the absence of sulphur, *P. furiosus* produces CO_2 and H_2 with the latter eventually becoming inhibitory for growth. In the presence of sulphur, only trace amounts of H_2 can be detected and H_2S is produced in a growth associated fashion (Malik *et al.*, 1989).

The apparent metabolic shift in response to sulphur showed a significant phenotypic response when elemental sulphur was available. Using gel electrophoresis, analysis of both the soluble and complete fractions of *P. furiosus* grown in the presence and absence of sulphur, distinctly showed several new proteins synthesized by this hyperthermophile

when grown in the presence of sulphur. There are also distinct bands in the complete fraction which were absent in the soluble fraction suggesting that there may be several membrane-associated proteins involved in the metabolism of elemental sulphur (Kelly and Deming, 1988).

The ability of *P. furiosus* to produce hydrogen led researchers to isolate and purify a thermostable hydrogenase from cells grown under strictly anaerobic reducing conditions (Bryant and Adams, 1989). The native enzyme was found to be a hexamer with an M_r of approximately 185,000 and lacked Ni-S (has only 1 Ni atom/mol) and Fe-S catalytic sites generally observed with its mesophilic analogues. The hydrogenase was found to function in the presence of a novel thermostable reduced ferrodoxin which was shown to be the physiological electron donor to the enzyme (Aono *et al.*, 1989). This protein was found to have a molcular weight of 12,000 Da and contains 8 iron atoms and 8 cysteine residues/mol. From spectroscopic studies, the *P. furiosus* ferrodoxin was found to be distinct from other ferrodoxins in that the major proportion of iron in its structure was not present as Fe-S clusters, thus possibly representing a new type of evolutionary electron carrier.

During preliminary growth studies with *P. furiosus*, Bryant and Adams (1989) discovered that the addition of tungsten to the medium significantly stimulated cell growth. Although the hydrogenase did not contain tungsten, it was shown that a tungsten-iron-sulphur protein was produced during growth which contained iron and acid-labile sulphur in addition to tungsten. The discovery of this protein created was particularly notable in that it was only the second tungsten-containing protein ever characterised (Mukund and Adams, 1990), the first being formate dehydrogenase from *Clostridium thermoaceticum* (Ljungdahl *et al.*, 1983). In addition, this protein was found to be present in very high concentrations in *P. furiosus* cell-free extracts and used ferrodoxin as its electron acceptor. It has been shown to be extremely thermostable and forms part of a metal cluster different to that found in the tungsten centre of formate dehydrogenase. Further studies by Mukund and Adams

(1991) showed that this novel tungsten-iron-sulphur protein was an aldehyde ferrodoxin oxidoreductase, possibly participating in a unique glycolytic pathway.

In all the archaea studied, evidence of a unique glycolytic pathway, which appears to be a simplified modification of the Entner-Doudoroff pathway (Danson, 1988) has been demonstrated. This was supported by the absence of substrate-level phosphorylation, the low net yield of ATP and the optional use of either NAD^+ or $NADP^+$ as electron carriers (Mukund and Adams, 1991).

The existence of a unique glycolytic pathway was further supported by the ability of *P. furiosus* to grow on pyruvate as an energy and carbon source resulting in the production of acetate, CO_2 and H_2, rather than the complete oxidation to CO_2 (Schafer and Schonheit, 1991). Acetate formation from acetyl-CoA and generation of ATP in this mode of fermentation was found to be catalysed by a single enzyme, an ADP-forming acetyl CoA synthetase, rather than by phosphate acetyl transferase and acetate kinase usually involved in acetate formation by chemolithotrophs.

The identification and purification of several other enzymes involved with carbohydrate metabolism has also been reported from this hyperthermophile. In the presence of carbohydrate substrates such as starch, pullulan, glycogen and maltose, the production of several amylolytic enzymes were identified. These include an α-glucosidase located in the cell cytoplasm, with optimum activity at pH 5-6 over a temperature range of 105°C to 115°C. The half-life of this enzyme was reported to be approximately 48h at 98°C. The enzyme was specific for maltose and glucopyranoside substrates (Constantino *et al.*, 1990).

P. furiosus also exhibited α-amylase activity (Koch *et al.*, 1990) and pullulanase activity (Brown *et al.*, 1990), both in intracellular and extracellular fractions. These amylolytic enzymes were found to have temperature optima of at least 100°C and exhibited remarkable thermostabilities (amylase, 120°C for 2h; pullulanase, 100°C for 1.5h at pH 5.5).

Few studies have been carried out on nitrogen metabolism in the archaea. Only *Sulfolobus* and *Pyrococcus* have demonstrated an arginine biosynthetic pathway (Van de Castelle, 1990). *Pyrococcus* utilises a similar version of this pathway as seen in mesophilic bacteria, but possesses only the enzymes required for the last three steps (Fig. 1.5). The involvement of carbamoyl phosphate raises the interesting question on how this extremely unstable metabolite is protected. It has been shown that the half-life *in vitro* of this metabolite at 100°C is less than 1 second (Van de Castelle, 1990). The high levels of ornithine carbamoyltransferase activity reported in *P. furiosus* cell extracts might have evolved as a mechanism to minimise carbamoyl phosphate pool sizes. The recent isolation of a thermostable glutamate dehydrogenase suggests the enzyme could be involved in the first step of nitrogen metabolism, catalysing the conversion of 2-oxoglutarate and ammonia to glutamate (Consalvi *et al.*, 1991).

The role of proteolysis, which is important for cell maintenance, has not been examined to any extent in *Pyrococcus*. Studies on this organism have shown a number of intracellular and extracellular proteinases but their physiological functions are still unknown (Blumentals *et al.*, 1990; Eggen *et al.*, 1990).

1.4.2 Genetics

Few studies have been carried out on the molecular biology of *Pyrococcus*. To date, amino acid sequence information of *Pyrococcus* proteins expressed in a mesophilic host, *E. coli* has been limited to two hyperthermophilic enzymes. The structural genes encoding glyceraldehyde-3-phosphate dehydrogenase from *P. woesei* (Zwickl *et al.*, 1990) and DNA polymerase from *P. furiosus* (Mathur *et al.*, 1991) have been cloned and expressed in *E. coli*. When compared with enzyme homologues from mesophilic and thermophilic archaeobacteria, *P. woesei* glyceraldehyde-3-phosphate dehydrogenase exhibited a very high proportion of aromatic acid residues and a low frequency of sulphur-containing residues. It suggested that these "changes" were linked to the stabilisation of protein conformation and thermoadaptation at temperatures around 100°C.

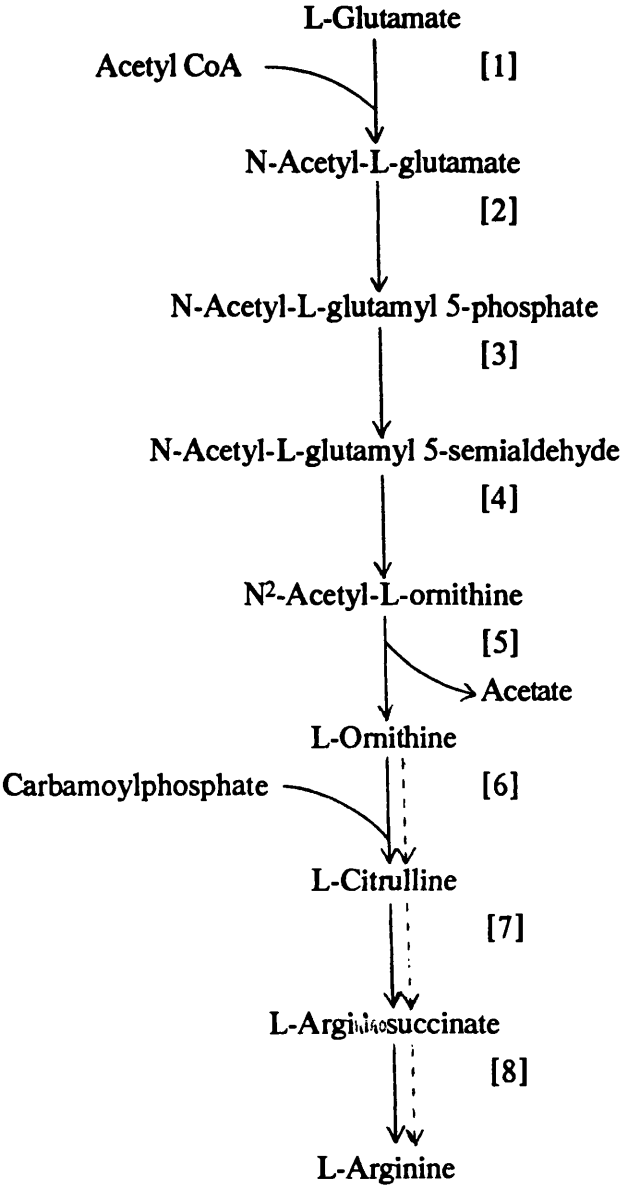


Figure 1.5 Arginine biosynthesis pathway in *S. solfataricus* (——) and *P. furiosus* (- - -).

The enzymes identified as involved are 1, N-acetylglutamate synthetase; 2, N-acetylglutamate 5-phosphotransferase; 3, N-acetylglutamate 5-semialdehyde dehydrogenase; 4, N-acetyl ornithine 5-aminotransferase; 5, acetylornithase; 6, ornithine carbamoyl transferase; 7, argininosuccinate synthetase, and 8, argininosuccinase.

It has been shown that DNA polymerases give sequences that are well suited for evolutionary analyses due to conserved regions within the gene. Mathur *et al.* (1991) used the sequence information obtained for the *P. furiosus* DNA polymerase to deduce sequence similarities with species from the eubacteria and eucaryotic domains. It was discovered that the *P. furiosus* gene had considerable homology (37 of 57 residues) with highly conserved regions in the α-like DNA polymerases, which include the α-human DNA polymerase, α-yeast, δ-yeast and REV3 cellular eucaryotic DNA polymerases. In contrast, sequence similarities between Pol1-like DNA polymerases in the same regions were considerably less (20 of 57 residues). The α-like DNA polymerases exhibited 24 of 57 conserved residues with Pol1-like DNA polymerases within the same regions.

Eubacterial and eucaryotic DNA polymerases are thought to have evolved from a common ancestral 'Klenow-like' core (Reiter *et al.*, 1988). The sequence homology of *P. furiosus* DNA polymerase not only supports the archaeaobacterial-eucaryotic relationship, but may closely resemble the ancestral form of the polymerase enzyme (Mathur *et al.*, 1991).

1.5 AIMS AND OBJECTIVES

The aims of this research are to investigate the role of proteolysis in *P. furiosus* and to determine the control of proteinase production by establishing procedures for the culturing of the hyperthermophile on different growth substrates.

The isolation, purification and characterisation of the proteinases involved in proteolysis will also be attempted, to gain some insight into the possible intrinsic or extrinsic mechanisms involved in the stabilisation of these proteinases at high temperature.

CHAPTER TWO
METHODS AND MATERIALS

2.1 MATERIALS

2.1.1 Chemicals

Bacto-peptone, yeast extract, casitone, tryptose, proteose peptone No. 3, soya peptone, tryptone, neopeptone, proteose peptone, casamino acids (T), casein, trypticase peptone, casamino acids and casein (enzyme hydrolysate) were obtained from Difco Laboratories.

Chromatography media was supplied as follows: CBZ-L-Phenylalanine-TETA-Sepharose, Pierce Biochemicals, Chester, Cheshire; Hydrogen Bond cellulose, Whatman Biosystems Ltd, Maidstone, Kent.

Titanium III chloride was purchased from Aldrich Chemical Co Ltd., The Old Brickyard, New Road, Gillingham, Dorset.

Oxygen-free nitrogen gas was obtained from The BOC Group Ltd., Guildford, Surrey.

Acrylamide, N, N'-methylene-bis-acrylamide and Folin-Ciocalteau phenol reagent were obtained from BDH (Merck), freshwater Road, Dagenham, Essex.

All other chemicals (AnalaR grade), including peptides and low molecular weight substrates, were obtained from Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset.

2.1.2 Cultures

Pyrococcus furiosus DSM 3638 was obtained from Deutsche Sammlung von Mikroorganismen, Federal Republic of Germany.

2.2 Preparation of Buffers and Solutions

2.2.1 Standard Sodium Phosphate (Na_2HPO_4-NaH_2PO_4) buffer, (0.1M stock)

$Na_2HPO_4.2H_2O$, mw 178.05; 0.2M solution contains 35.61g/l.

$NaH_2PO_4.2H_2O$, mw 156.03; 0.2M solution contains 31.21g/l

*x*ml 0.2M Na_2HPO_4, *y*ml 0.2M NaH_2PO_4, diluted to 100ml with distilled water.

pH, 25°C	*x* ml 0.2M Na_2HPO_4	*y* ml 0.2M NaH_2PO_4
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

2.2.2 HEPES(N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid) Buffer (0.1M)

23.8g of HEPES was dissolved in one litre of distilled water, and the pH was adjusted to 8.0 (20°C) with 0.1M NaOH.

2.2.3 Protein Solubilisation Solution

The solution consisted of 4% (w/v) SDS (sodium dodecyl sulphate), 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue in 0.125M Tris-HCl buffer, pH 6.8.

2.3 Growth of *Pyrococcus furiosus*

2.3.1 Closed bottle system

Pyrococcus furiosus was grown anaerobically in static culture using sealed serum or Duran bottles containing a sulphur basal salts medium (Stetter *et al.*, 1983). The basal salts medium (Table 2.1) was supplemented with Bacto-peptone and yeast extract to give final concentrations of 0.5% and 0.1% respectively. The medium was prepared under a gas phase of H_2:CO_2:N_2 (10%:10%:80%) and boiled before being reduced with a sterile neutral solution of 5% (w/v) sodium sulphide to give a final concentration of 0.05%. A redox indicator, resazurin, was also added at a final concentration of 1mg/l. The final pH of the medium was adjusted to 6.3.

Before inoculation, the culture medium was cooled to the growth temperature of 98°C (unless otherwise indicated). To initiate growth, a starter culture (1%) of *P. furiosus* was injected into the pre-heated culture medium bottles. Cultures were then incubated for 17h at 98°C.

2.3.2. Open fermenter system

For small scale fermentation studies, fermenter vessels of 3L or more were used for batch and 1L vessels for continuous culture work. All fermentations were carried out at 95°C using the medium described in Section 2.3.1. unless otherwise indicated.

a) Batch culture

An LH2000/i3000 series fermenter was used for batch culture fermentations of *P. furiosus*. The vessels, made from either glass (3L to 8L capacity) or stainless steel (25L capacity, New Brunswick Scientific), were linked to a microprocessor unit used to input physical and chemical parameters such as pH, temperature and acid/alkali additions. The medium used for batch culture was prepared as in Section 2.3.1. For sulphur-free cultures, sulphur was omitted and sodium sulphide was replaced by 0.5% (v/v) titanium III chloride. The medium was steam sterilised *in situ* before being reduced. Anaerobic status was maintained by sparging with a continuous stream of O_2-free nitrogen with slow agitation (100rpm).

Table 2.1 Composition of *Pyrococcus furiosus* complex growth medium.

Composition	Amount (per litre of distilled water)
(a) *Basal Salts Medium*	
NaCl	13.85g
$MgS0_4.7H_2O$	3.5g
$MgCl_2.6H_2O$	2.75g
KCl	0.325g
NaBr	0.05g
H_3BO_3	0.015g
$SrCl_2.6H_2O$	7.5mg
$(NH_4)_2SO_4$	10.0mg
Citric acid	5.0mg
KI	0.05mg
$CaCl_2.2H_2O$	1.0mg
KH_2PO_4	0.5g
$NiCl_2.6H_2O$	2.0mg
Trace Element solution (see below)	10.0ml
Resazurin	1.0mg
Bactopeptone	5.0g
Yeast Extract	1.0g
Sulphur	30.0g
$Na_2S.9H_2O$	0.5g
(b) *Trace Element Solution*	g/litre of distilled water
Nitrilotriacetic acid	1.5
$MgSO_4.7H_2O$	3.0
$MnSO_4.2H_2O$	0.5
NaCl	1.0
$FeSO_4.7H_2O$	0.1
$CoSO_4$ or $CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnSO_4$	0.1
$CuSO_4.5H_2O$	0.01
$KAl(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01
$NiCl_2.6H_2O$	0.025

The growth medium was then cooled to a temperature of 95°C and adjusted to pH 6.3 before inoculation (5-10% by volume).

b) Continuous culture

Continuous culture of *P. furiosus* was carried out using the LH fermentation 500 series fermentation system (Fig.2.1). The culture vessel was a 1 litre glass chemostat with a working volume of 800ml, fitted with an external jacket. The temperature of the culture medium was maintained by pumping paraffin oil at the desired working temperature through the jacket, and the temperature was monitored using a thermocouple. The contents of the vessel were kept anaerobic by continually sparging with either O_2-free nitrogen or argon. A stirrer speed of 100rpm was used.

The medium for continuous culture experiments was that as described in Section 2.3.2a unless otherwise indicated. It was stored (10L) under anaerobic conditions by purging with nitrogen gas. The culture medium was pumped into the chemostat to maintain a working volume of approximately 800mls. Sulphur was omitted and sodium sulphide was replaced with 5% titanium III chloride. The vessel was inoculated with a mid-to-late log phase culture of the organism. When the inoculum reached a cell density level of about 1×10^8 cells/ml, the medium feed was initiated. The dilution rate used originally was $0.2h^{-1}$ but was varied as described in text. Samples were collected via the sampling port and remaining product was collected in a product reservoir via a dip tube (Figure 2.1).

2.4 Monitoring cell growth

2.4.1 Direct cell counts

Growth was determined by total cell counts using a Thoma chamber (depth 0.02mm), using phase contrast microscopy (x40 objective). Aliquots (0.1ml) of sample from cultures were pipetted onto the centre of the chamber grid and the total cell count was obtained by using the following equation:

$$\text{Cells/ml} = \frac{\text{Number of cells in 4 large squares}}{64} \times 2 \times 10^7$$

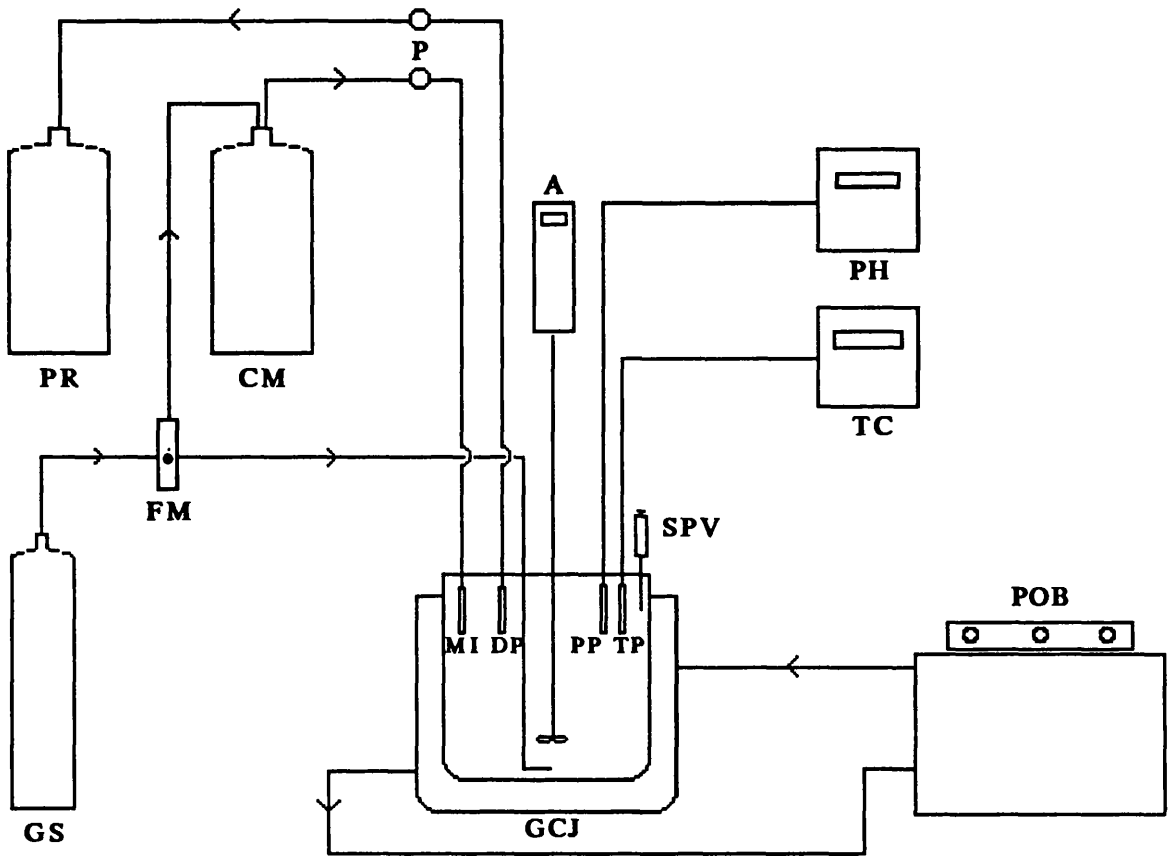


Figure 2.1

Schematic diagram of the apparatus used for continuous culture of *Pyrococcus furiosus*

Abbreviations are as follows: PR, product reservoir; CM, culture medium storage; P, pump; A, agitator-stirrer speed control; PH, pH meter; TC, temperature meter control; POB, paraffin oil bath; SPV, sample port valve; PP, pH probe; TP, temperature probe; DP, dip tube for product reservoir; MI, medium inlet valve; FM, gas flow meter; GS, anaerobic gas supply; GCJ, glass chemostat (1L) with jacket.

2.4.2 Protein content

The protein content of cells was determined by a modified Lowry assay (Peterson, 1977). Samples of culture (1ml duplicates) were centrifuged at high speed for 3 minutes in an MSE Microcentaur centrifuge. The supernatant was decanted and the pellet was washed twice with 1ml volumes of distilled water. The washed pellet was resuspended in 1ml distilled water and 1ml of a stock solution containing equal volumes of 10% SDS, 0.8N sodium hydroxide, water and CTC (0.1% copper sulphate-0.2% potassium tartrate-10% sodium carbonate) was added. After mixing, the assay was allowed to stand at room temperature for 10 minutes. 0.5ml of Folin-Ciocalteau phenol reagent (1 in 6 dilution) was then added to each tubes, mixed and the absorbance read at 750nm after 30 minutes incubation at room temperature. Bovine serum albumin standards (Sigma, between 0-0.2mg/ml) were included along with the unknowns.

2.4.3 Optical density

Optical density readings of cultures were taken at 600nm where appropriate.

2.5 Maintenance and storage of *P. furiosus*

Cultures of *P. furiosus* were maintained and stored by either subculturing or freezing. After a 17h growth incubation at 98°C, direct cell counts were obtained and cells were either inoculated directly into fresh medium or harvested by centrifugation (20,000*g* for 15min). Aliquots of cell pellets were suspended in growth medium containing either sterile 5% (v/v) dimethyl sulphoxide (DMSO) or 10% (v/v) glycerol to give a final cell density of approximately 10^9 cells/ml. Cell suspensions (100µl aliquots) were then dispensed anaerobically and sealed in glass capillary tubes as described by Hippe (1984). The capillaries were stored at either -80°C or in liquid nitrogen vapour phase.

2.6 Preparation of cell extracts and cell-free culture supernatants

2.6.1 Cell extracts

Cells were harvested after a 17h incubation at 98°C and cell pellets were collected by centrifugation (50,000*g* for 15 min). The pellets were washed and resuspended in 10mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5. Cell extracts were then prepared by one of the following methods (as indicated):

Sonication Cell extracts were prepared by sonication (power rating 10) for 3 x 1 minute bursts with 30s intervals using an MSE Soniprep sonicator. The cell lysate was centrifuged for 30 minutes at 30,000*g* to remove cell debris. The supernatant was used immediately or stored at 4°C.

Cell lysis by SDS Aliquots of culture (1ml) were centrifuged at high speed in an MSE Microcentaur centrifuge for 5 minutes. Cell pellets were washed, resuspended in 100µl of sodium phosphate buffer (as above) and lysed by the addition of an equal volume of protein solubilisation solution (section 2.2).

2.6.2 Cell-free culture supernatants

Cell-free culture supernatants were obtained after harvesting cells and used directly.

2.7 Proteinase assays

2.7.1 Azocasein assay

Proteinase activity was determined by the hydrolysis of azocasein as described by Cowan *et al.* (1987). 900µl volumes of 0.5% azocasein dissolved in 0.1M HEPES buffer, pH 8.0 (20°C), were pipetted into 1.5ml Eppendorf tubes and pre-incubated at 85°C or 95°C (as indicated). The reaction was initiated by the addition of 100µl of enzyme solution and terminated after a measured incubation period by the addition of 500µl of aqueous 15% (w/v) trichloroacetic acid. The assay tubes (duplicate including non-enzymatic controls) were allowed to cool for 10 minutes before centrifuging at high speed for 3 minutes in an MSE Microcentaur bench top centrifuge. The absorbance of the supernatant was monitored

at 400nm. One unit of proteinase activity was defined as the volume of enzyme solution giving an absorbance change of 1.0 per minute under the specified assay conditions.

2.7.2 Gelatin PAGE protease assay

Proteinase samples were analysed using a modified gelatin PAGE protease assay (Heusson and Dowdle, 1980). Unknown protein samples and standard markers (100µl SDS mixture [3mg/ml] of myosin, β-galactosidase, phosphorylase β, bovine serum albumin, egg albumin and carbonic anhydrase) were boiled for 5-10 minutes in an equal volume of protein solubilization solution (section 2.2). The samples were then loaded on to an SDS polyacrylamide slab gel (Table 2.2; Laemmli, 1970), where the separation gel (10%, unless indicated otherwise) contained copolymerised gelatin at a final concentration of 0.01% w/v. Electrophoresis was performed at 45mA constant current per gel for 2-3h at room temperature.

After electrophoresis, the gels were gently shaken for 2h at room temperature in two changes of aqueous 2.5% (w/v) Triton X-100 (Horie *et al.*, 1984) then incubated for 1h (unless otherwise indicated) at 90°C in 0.1M HEPES buffer, pH 7.5 (90°C). After incubation, the gels were immediately fixed with 50% (w/v) trichloroacetic acid for 30-60 minutes before staining with Coomassie Brilliant Blue G250 (2.5g per litre of 45% v/v methanol - 9% v/v acetic acid). Gels were then destained overnight in methanol:acetic acid:water (3:2:35) by volume. Proteolytic activity was visible as clear (unstained) zones against a blue background.

2.8 Protein assay

The protein concentration of samples was determined by the Bradford micro-assay (Bradford, 1976). To 100µl of protein sample, 1ml of Bradford reagent (diluted 1 in 6) was added. After 5 minutes at room temperature, the absorbance was read at 595nm. Bovine serum albumin was used as the protein standard to generate standard curves between 10-100µg of protein/ml.

Table 2.2 SDS Polyacrylamide Gel Constituents

	Separating gel			
% Acrylamide	7.5	10	12	15
Volume of solution(ml)				
Acrylamide[a]	11.25	15	18	22.5
2% Running buffer[b]	22.5	22.5	22.5	22.5
Demineralised water	11.25	7.5	4.5	-
Ammonium persulphate[c]	0.5	0.5	0.5	0.5
TEMED	0.02	0.02	0.02	0.02
	Stacking gel			
Acrylamide	2	2	2	2
2% Stacking buffer[d]	6	6	6	6
Demineralised water	4	4	4	4
Ammonium persulphate	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01

[a] Acrylamide solution: 30% (w/v) Acrylamide, 0.8% (w/v) N,N'-Methylenebisacrylamide

[b] 2% Running gel buffer: 0.75M Tris-HCl, pH 8.8, 0.2% (w/v) SDS

[c] Ammonium persulphate: 10% (w/v) ammonium persulphate (fresh)

[d] 2% Stacking gel buffer: 0.25M Tris-HCl, pH 6.8, 0.2% (w/v) SDS

2.9 Sucrose gradient centrifugation

Sucrose gradients [6-30% (w/v) sucrose in distilled water] were prepared as described by Martin and Ames (1961). Cell extract and cell-free culture supernatant samples (500µl) were layered on top of the gradients together with known molecular weight markers (0.25mg of β-amylase and bovine serum albumin in 50µl of distilled water). The samples were centrifuged at 208,000g for 24h at 20°C in an L-7 Beckman Ultracentrifuge. The contents were removed by inserting a capillary tube to the base of each ultracentrifuge tube and using a peristaltic pump to withdraw the contents. Fractions (0.5ml) were collected and assayed as in Section 2.7 and 2.8. Relative molecular masses were calculated using the equation

$$M_r = s_1/s_2 \times (mm_1/mm_2)^{2/3}$$

where s_1/s_2 is the partial specific volume, mm_1 is the molecular mass of the unknown protein and mm_2 is the molecular mass of the known standard (Martin and Ames, 1961).

2.10 Gel Permeation Chromatography

2.10.1 Low pressure gel permeation chromatography (non-denaturing)

Proteinase samples (2ml, unless indicated otherwise) were applied to a Pharmacia K16 column (16cm by 100cm) containing Sephacryl S300-HR (Sigma Chemical Co., Poole, Dorset). The column was equilibrated with 20mM Tris-HCl buffer, pH7.5 before loading samples mixed with standard markers (Blue Dextran 2000, bovine serum albumin each at 1mg/ml concentrations). The flow rate was maintained at 0.5ml/min. with a Pharmacia P1 peristaltic pump. Protein was eluted using the same buffer and fractions (3ml, unless indicated otherwise) were collected. All fractions were monitored for proteinase activity and protein concentrations were continually monitored at 280nm throughout.

2.10.2 Low pressure gel permeation chromatography (under denaturing conditions)

Proteinase samples were applied on to a Pharmacia K16 column containing Sephacryl S 300-HR pre-equilibrated with 20mM Tris-HCl buffer with 0.1% SDS at pH 7.5. Samples,

including standard SDS markers, were pretreated with an equal volume of the same buffer (containing 2-mercaptoethanol at a final concentration of 10% v/v), by boiling for 5-10 minutes and allowing to cool before loading on to the column. Flow rate was set at 0.5ml/min. and protein was monitored continuously at 280nm. Fractions (5ml) were collected and assayed for proteinase activity (Section 2.7).

2.10.3 Fast Protein Liquid Gel Permeation Chromatography (non-denaturing)

Samples of *P. furiosus* cell extracts (200µl) were applied to a Pharmacia Superose 12 HR 10/30 column. The column was pre-equilibrated with 20mM triethanolamine buffer, pH 7.5 before samples were loaded. Proteins were eluted with 20mM triethanolamine buffer-50mM NaCl, pH 7.5 at a flow rate of 1ml/min and 1ml fractions were collected. Protein was monitored throughout at an absorbance of 280nm and fractions were assayed for proteinase activity (Section 2.7).

2.10.4 FPLC Gel permeation (denaturing)

Proteinase samples were treated with 4% (w/v) SDS and 10% (v/v) 2-mercaptoethanol in triethanolamine buffer before application to the Superose 12 column, pre-equilibrated with 20mM triethanolamine-0.1% SDS buffer, pH 7.5.

2.11 Adsorption chromatography

2.11.1 Ion-exchange chromatography (FPLC)

Proteinase samples were applied to an FPLC Mono Q HR 5/5 column (5 x 50mm) pre-equilibrated with 20mM Bis-Tris Propane, pH 7.0. at a flow rate of 1ml/min. The column was washed with the above buffer and proteinase was eluted with a two step gradient of 0-0.35M and 0.35-1M NaCl in 20mM Bis-Tris Propane buffer,pH 7.0. Fractions (1ml) were collected. Protein was monitored at 280nm and all fractions were tested for proteinase activity (section 2.7).

2.11.2 Low Pressure Ion-exchange chromatography

Proteinase samples (10ml) were applied to a column (1.6cm by 30cm) of Q Sepharose (20ml column bed volume) pre-equilibrated with 50mM sodium phosphate buffer, pH 7.5 (unless indicated otherwise). The column was washed with the above buffer before eluting bound protein with a 0-1M NaCl gradient in the same buffer. The flow rate was kept at 2ml/min. Fractions (4ml, unless indicated otherwise) were collected, monitored for protein at 280nm and assayed for proteinase activity (section 2.7.1).

2.11.3 Hydroxylapatite chromatography

Proteinase samples were applied to a hydroxylapatite (Biorad) column (1.6cm by 30cm) equilibrated with 10mM sodium phosphate buffer, pH 7.0. Proteinase activity was eluted with a linear gradient of 0.01M to 2M sodium phosphate (200ml total volume). Fractions (4ml, unless indicated otherwise) were collected and monitored for protein at 280nm and proteinase activity (section 2.7).

2.11.4 Affinity chromatography

Proteinase samples (1-2ml) were applied on to a Pharmacia K16 column (bed volume 10ml) packed with CBZ-Phenylalanine TETA-Sepharose, pre-equilibrated with 0.1M Tris-HCl buffer, pH 7.5. The column was then washed with the above buffer. Protein was eluted with 0.1M acetic acid and the pH of the eluent was adjusted to neutrality with 1M NaOH. Protein was monitored at 280nm and proteinase activity was determined using the azocasein assay (section 2.7.1).

2.12 Hydrophobic Interaction Chromatography

2.12.1 Whatman Hydrogen Bond

A Pharmacia K16 column (dimensions 16cm by 40cm, bed volume 10ml) packed with Whatman Microgranular Modified Cellulose was pre-equilibrated with 3-4 column volumes of 10mM sodium phosphate buffer, pH 7.0, saturated with 2.2M ammonium sulphate (unless otherwise indicated). Cell extracts of *P. furiosus* were also adjusted to the same salt

saturation before being applied to the equilibrated column at a flow rate of 1ml/min. The charged column was consecutively washed with the same salt buffer followed by buffer saturated with 0.8M ammonium sulphate. The proteinase was then eluted with a salt step of 0.8M to 0M salt in 10mM sodium phosphate buffer, pH 7.0 (section 2.2). Fractions (2ml) were collected and assayed for proteinase activity (Section 2.5). Protein was monitored at 280nm.

2.12.2 Phenyl- and Octyl-Sepharose

Hydrophobic interaction chromatography was performed in 1.6cm by 30cm columns containing Phenyl-Sepharose or Octyl-Sepharose (Sigma). The column were pre-equilibrated with 50mM sodium phosphate buffer, pH 7.0 saturated with 50% ammonium sulphate. Cell extracts of *P. furiosus* were also increased to the same salt saturation before being applied to the equilibrated column at a flow rate of 1ml/min. The columns were washed with the above high salt buffer before eluting bound protein with 50mM sodium phosphate buffer, pH 7.0. Fractions (2ml) were collected, monitored for protein and assayed for proteinase activity (section 2.7.1).

2.13 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

Analytical gel electrophoresis under denaturing conditions (Table 2.2) was carried out in 1.5mm thick polyacrylamide gels as described by Laemmli (1970).

2.14 Native (non-denaturing) PAGE

Non-denaturing PAGE of proteinase samples was performed on slab gels of different acrylamide concentrations (up to 10%) as described by Clarke (1964). After electrophoresis (section 2.7.2), gels were immersed in a solution of 1% (w/v) gelatin in 0.1M HEPES buffer, pH 8.0 and gently shaken for 1h. The gels were incubated for 1-3h at 90°C before being fixed with 50% (w/v) trichloroacetic acid. Gels were stained and destained as described in section 2.7.2.

2.15 Electroelution

SDS PAGE of proteinase samples was performed as described in section 2.13. After electrophoresis, activity bands were excised from the denaturing gel. The excised bands were placed in individual dialysis bags and immersed in a horizontal electrophoresis system for submerged gel electrophoresis. The running buffer used for electroelution was the same as used for SDS PAGE. The horizontal unit was operated at 25mA for 6-8h. Following electroelution, the sample was concentrated in an Amicon ultrafiltration unit (10,000Da cut-off membrane). The samples were then analysed using gelatin SDS PAGE (section 2.7.2).

2.16 Ammonium Sulphate Fractionation

Solid ammonium sulphate (BDH, AnalaR) was added to *P. furiosus* cell extract samples to give a final concentration of 22.9g/100ml of solution (40% saturation) and stirred for an hour. The solution was centrifuged at 15,000g for 20 minutes. The volume of the supernatant fluid was measured and increased to 60% saturation by addition of 13.7g of ammonium sulphate per 100ml of solution, stirred for 1h and then centrifuged as above. This procedure was repeated for the 90% saturation step where an additional 24.5g of ammonium sulphate was added per 100ml of solution. At each step, the pellet was suspended in 10mM sodium phosphate buffer, pH 7.0. Both the pellets and samples of supernatants were assayed for proteinase activity (section 2.7.1).

2.17 Effect of Inhibitors

Proteinase samples (100µl) were pre-incubated at room temperature for 1h in the presence of proteinase inhibitors (Table 7.1). Residual proteolytic activity was determined either by the azocasein assay (95°C for 30min) or substrate PAGE.

2.18 Effect of temperature on activity

The influence of temperature on activity was determined by adding 100µl samples of proteinase solution to Eppendorf tubes containing 0.5% azocasein solution pre-incubated at

different temperatures. After a set time, the reaction was terminated with 15% trichloroacetic acid and the tubes were treated as in Section 2.7.1.

2.19 Effect of pH on activity

Proteinase activity was determined with 0.5% (w/v) azocasein at various pH values (5-10) using a buffer mixture consisting 50mM each of MES, Tris and glycine. The pH of buffers was adjusted at 95°C before use.

2.20 Substrate specificity

2.20.1 Protein hydrolysis

Solutions or suspensions of proteins and dye-linked proteins (0.5% w/v) in 0.1M HEPES buffer, pH 8.0 were prepared. Aliquots (100µl) of proteinase solution were added and the reaction mixtures were incubated at 95°C for 1h. The reaction was terminated by the addition of 500µl of 15% (w/v) trichloroacetic acid. The assay tubes were allowed to cool for 5-10 minutes before centrifugation at 12,000rpm for 3 minutes. Absorbances of resulting supernatants were monitored at their specified wavelengths.

2.20.2 Peptide and low molecular weight substrates

Kinetic assays using peptide analogues and low molecular weight substrates at concentrations between 1-10mM were performed as follows. Aliquots (900µl) of substrates, dissolved in 2 parts methoxyethanol and 8 parts 50mM sodium phosphate buffer, pH 7.5 (section 2.2), were preincubated at 85°C before the addition of 100µl of proteinase solution. The progress of the reaction was continuously monitored at specified wavelengths on a Pye Unicam SP8-200 UV/VIS spectrophotometer. One unit of enzyme activity was expressed as µmole of product released per minute.

2.21 Stability-activity studies

2.21.1 Thermostability of Proteinases

Proteinase samples (3-4ml) with and without the addition of 10mM $CaCl_2$, were sealed in Bijoux bottles and immersed in a paraffin oil bath at temperatures between 95°C and 125°C. At appropriate time intervals, tubes were removed and immediately placed on ice. Residual proteinase activity was determined using the azocasein assay (section 2.7.1).

Analysis of kinetics of activity loss

Activity values (U/ml) were converted into residual (percentage of initial value) active enzyme concentrations $[E]_r$. These data were then plotted as $\ln ([E]_o/[E]_r)$ versus time ($[E]_o$ = initial enzyme concentration) and $1/[E]_r$ versus time, as tests for first order and second-order kinetics respectively (Moore, 1962).

Rate constants κ_1 (min^{-1}) and κ_2 ($mol^{-1}.min^{-1}$) were obtained from linear portions of these plots and half-lives were calculated from equations $\tau_1 = \ln 2/\kappa_1$ or $\tau_2 = 1/\kappa_2[E]_o$ (Moore, 1962).

2.21.2 Effect of Chaotropic Agents

The specified reagents (see text) were added to 2-3ml volumes of proteinase samples in sealed Bijoux bottles and immersed in a paraffin oil bath at 95°C and 100°C. At appropriate intervals, samples were removed and immediately placed on ice. Residual proteinase activity was determined using the azocasein assay (section 2.7.1).

2.21.3 Effect of Detergents

The specified detergents (0.1% to 1% w/v) were added to 2-3ml volumes of proteinase solution in sealed Bijoux bottles. The bottles were immersed in a paraffin oil bath at 95°C and 105°C. Samples were removed at specified intervals and placed on ice. Residual activity was determined using the azocasein assay (section 2.7.1).

2.21.4 Effect of Organic Solvents

Water-miscible organic solvents were added to 2-3ml volumes of proteinase solution and sealed in Bijoux bottles. These bottles were immersed in a paraffin oil bath at 90°C. Samples were removed at specified intervals, placed on ice and subsequently assayed for residual proteinase activity (section 2.7.1).

2.21.5 Effect of Ionic Strength

Solutions of NaCl of varying concentrations (0.1M to 2M) in 10mM sodium phosphate buffer, pH 7.5 (section 2.2) were incubated with 2-3ml volumes of proteinase solution at 100°C. At specified intervals, samples were removed, placed on ice and residual proteinase activity was determined using the azocasein assay (section 2.7.1).

RESULTS AND DISCUSSION

CHAPTER 3

GROWTH AND PROTEINASE PRODUCTION FROM *P.FURIOSUS.*

3.1 Aims

The aims of this study were to establish procedures for the routine culturing of *Pyrococcus furiosus*, to investigate the growth characteristics of this hyperthermophile and to determine the control of extracellular proteinase production.

3.2 Static closed cultures.

Static cultures of the hyperthermophile *P. furiosus* were grown anaerobically in a complex medium composed of salts supplemented with 0.5% peptone and 0.1% yeast extract as carbon and energy sources (Stetter *et al.*, 1983). Elemental sulphur (30g/l) was also added. The medium was prepared as described in Section 2.3.1, inoculated with 1% (v/v) *P. furiosus* and incubated overnight at 98°C.

On observation of the cultures after incubation, using phase contrast microscopy, cells appeared as elongated, irregular coccoid-shaped organisms, often occurring as doublets. Cell dimensions were typically 0.5 to 2μm in length. Direct cell counts were used to measure growth with cell densities reaching approximately 1 x 10^8 cells/ml after 17 hours incubation. Cultures were also sampled for extracellular proteinase activity using azocasein as the proteolytic substrate. Proteinase activity was detected in cell-free culture supernatants producing a change of absorbance at 400nm per minute of 0.01.

3.3 Influence of elemental sulphur on growth

It was reported by Fiala and Stetter (1986) that the presence of hydrogen, one of the fermentation products of *P. furiosus* fermentative metabolism, was inhibitory to cell growth. This problem was alleviated by the addition of elemental sulphur to the medium, promoting the biotic conversion of hydrogen to hydrogen sulphide. However, the organism was also reported to grow in the absence of sulphur.

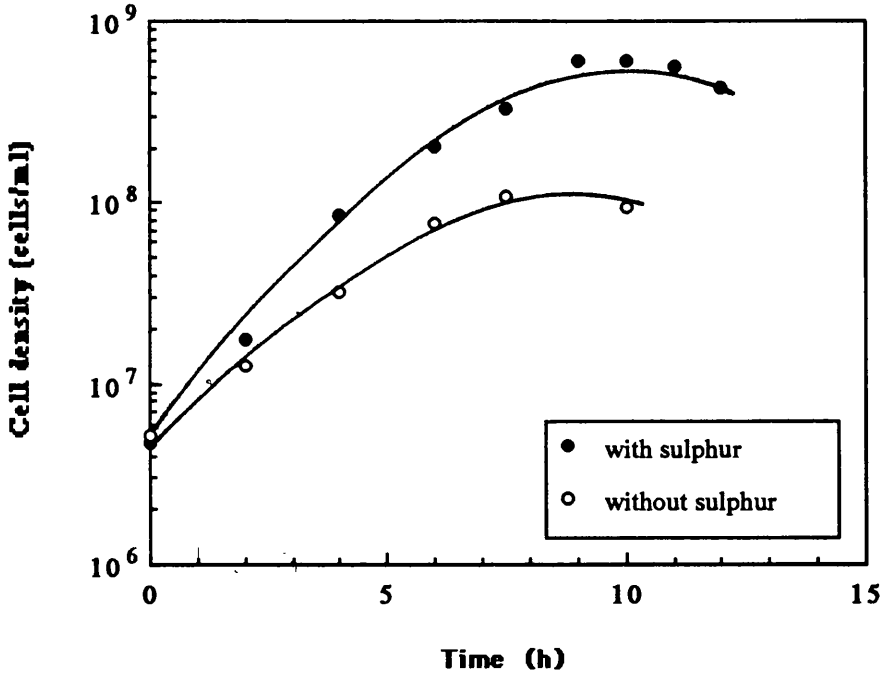
Comparative studies on the growth of *P. furiosus* in the presence and absence of elemental sulphur were therefore performed. All fermentations were carried out in a 3 litre glass bioreactor linked to a microprocessor. The medium used for growth was essentially the same as described for static cultures (Section 2.3.1). However, for sulphur-free medium, elemental sulphur was omitted and a solution of 0.5%(v/v) titanium chloride was used to replace sodium sulphide as the reducing agent. A continuous gas stream of 100% oxygen-free nitrogen was also used for the sulphur-free fermentation.

Figure 3.1 illustrates the growth profiles of *P. furiosus* in the presence and absence of elemental sulphur at 95°C. Growth was observed in both cases, with optimal cell growth being achieved in under 10 hours instead of 17-18 hours as seen with static closed cultures. Cell densities in medium containing sulphur were highest with maximal cell densities reaching 6 x 10^8 cells/ml as compared to 1 x 10^8 cells/ml in the absence of sulphur. The inhibitory effect of hydrogen was observed under static conditions in the absence of sulphur, where no growth was detected in closed culture bottles. The growth in the fermenter in this medium was possibly due to the hydrogen gas being continually flushed out of the reactor by a stream of nitrogen gas, thereby alleviating inhibition.

The physiology of growth in the presence and absence of sulphur has been studied in some detail (Malik *et al.*, 1988). The presence of sulphur is thought to induce a sulphur reductase which reduces elemental sulphur to hydrogen sulphide (Adams, 1990). Although this enzyme has not been isolated, studies from SDS-PAGE of cell extracts of *P. furiosus* have shown that at least 5 more bands appeared when cells were grown in the presence of sulphur (Kelly and Deming, 1988). It is possible that one of these bands represented a sulphur reductase.

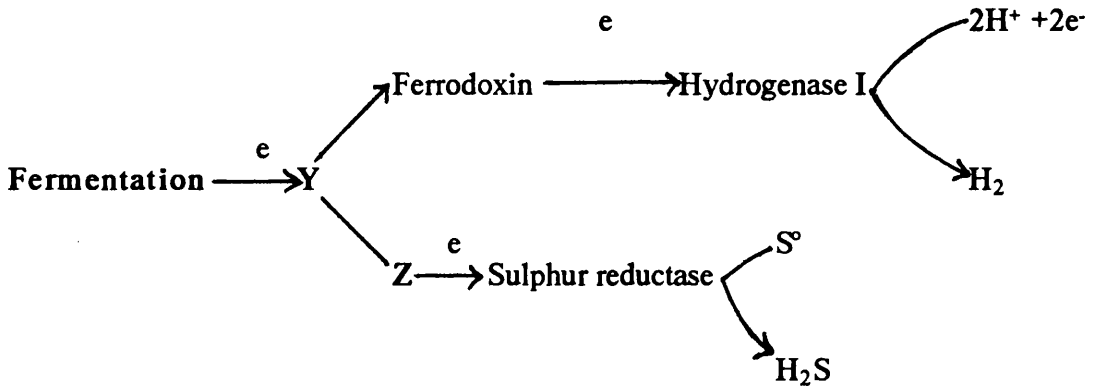
In the absence of sulphur, hydrogen must be channelled via another pathway to allow reoxidation of reduced electron carriers, such as NADH, produced during fermentation. Evidence that an alternative pathway exists for the channelling of the reductant was shown

Figure 3.1 Growth of *P. furiosus* in the presence and absence of elemental sulphur at 95°C.



by Bryant and Adams (1989) who isolated a H_2-evolving hydrogenase from *Pyrococcus*. The transfer of electrons was thought to be carried via a unique thermostable ferrodoxin (Aono *et al.*, 1989) which serves as the physiological electron donor to the hydrogenase producing molecular hydrogen. Inhibition of cell growth would thus depend on inhibition of the H_2-evolving hydrogenase by hydrogen. Growth in the absence of sulphur is resumed if the hydrogen is removed from the system by continual flushing with an inert gas. Fig.3.2 summarises the pathways for the relief by sulphur of the inhibition of growth.

Figure 3.2 Pathways for the relief by elemental sulphur of the inhibition of growth by hydrogen (reproduced from Adams, 1990).



where Y and Z are hypothetical electron carriers.

In addition to measuring cell density during growth, extracellular proteinase production was also monitored. Figure 3.3 shows proteinase levels from *P. furiosus* grown in a sulphur-free medium at 95°C. Proteinase levels were found to increase throughout growth, reaching peak activity titres at around 8-9 hours after inoculation. On entering stationary phase where rapid cell lysis occurred, extracellular proteinase levels decreased. Activity appeared to be growth associated, as specific proteinase production (expressed as units of activity per gram of protein) increased during exponential phase (Fig. 3.3). When *Pyrococcus* was cultured in a 25 L stainless steel reactor (Figure 3.4), activity appeared not to be growth dependent, as specific proteinase production decreased throughout the growth phase. This could be due to residual enzyme being carried over when active culture

Figure 3.3 Growth of *P. furiosus* and extracellular proteinase production in the absence of elemental sulphur at 95°C.

Graphs represent (a) cell density, cells/ml; (b) extracellular proteinase activity, U/ml x 10^{-3}, and (c) specific proteinase production, U/g protein.

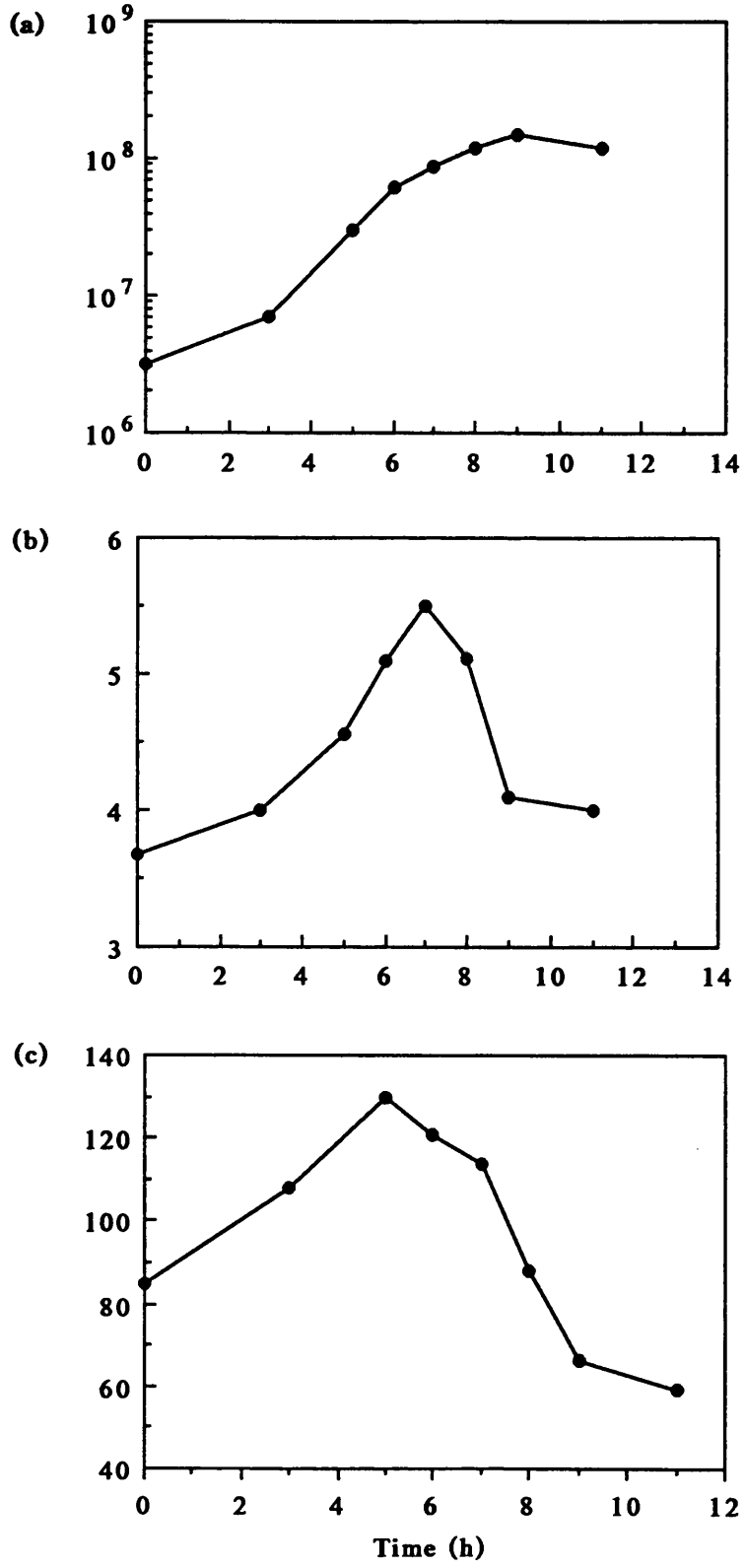
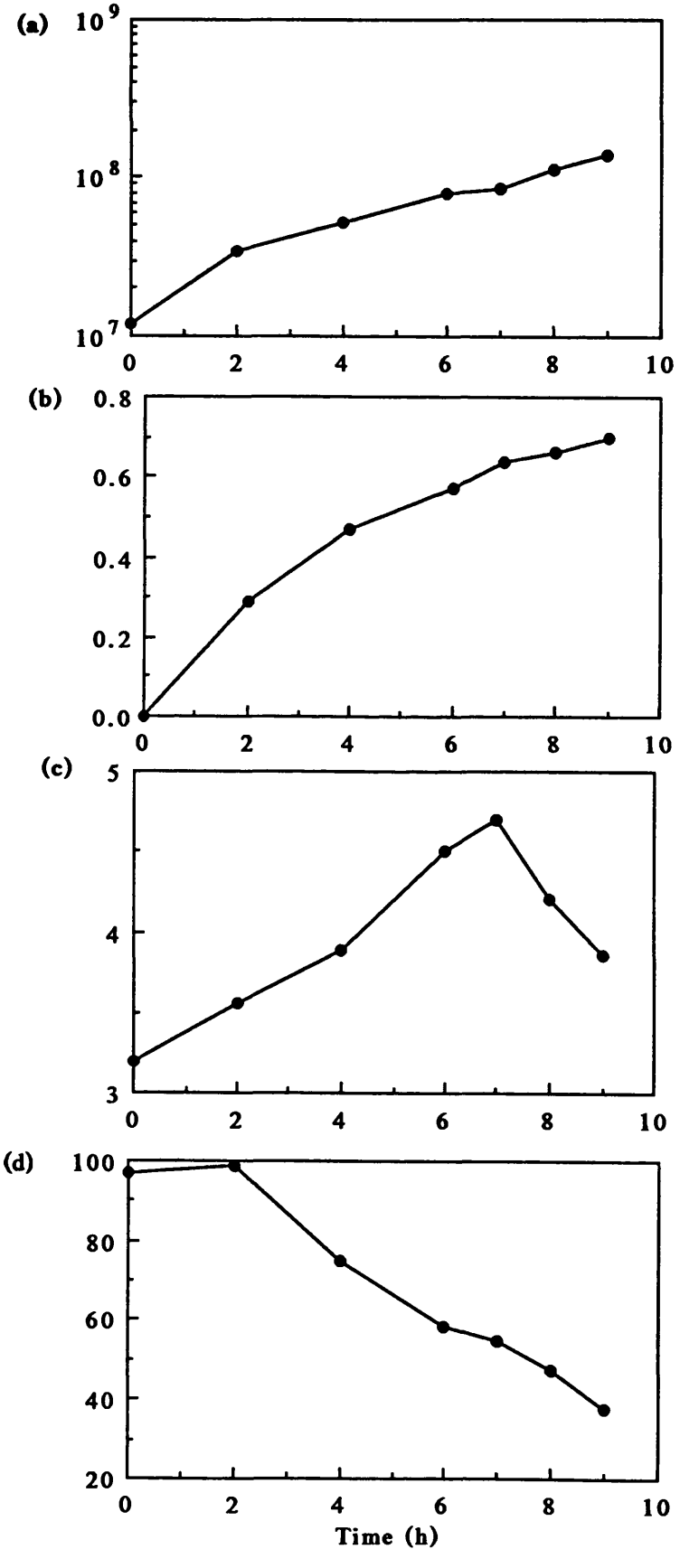


Figure 3.4 Growth of *P. furiosus* and extracellular proteinase production in a 25 litre stainless steel fermenter.

Graphs represent (a) direct cell counts, cells/ml; (b) release of CO_2, (%); (c) extracellular proteinase activity, U/ml x 10^{-3}; (d) specific proteinase production, U/g protein.



inoculum was transferred into a large volume of medium (2L to 20L working volume). As a result, the lag phase was abolished, and an initially high level of extracellular proteinase was produced compared to protein. This corresponded to a high initial value for specific proteinase production (calculated from the ratios of proteinase to protein). In addition, the initial cell density for the 25 litre fermenter data was calculated to be 10-fold higher to that measured for the 3 litre fermenter, supporting the high initial value obtained for specific proteinase production at the 25 litre scale.

Since it has been demonstrated that *Pyrococcus* can grow in the absence of sulphur, it was decided to perform further studies on growth and proteinase production in sulphur-free medium. The reasons for this were as followed. Firstly, the production of hydrogen sulphide, normally associated with sulphur metabolism, would be reduced significantly, as the gas is toxic at high concentrations, and secondly, cell harvesting would be simplified in that the separation of sulphur particles from cells can be avoided.

3.4 Influence of temperature on growth and extracellular proteinase production.

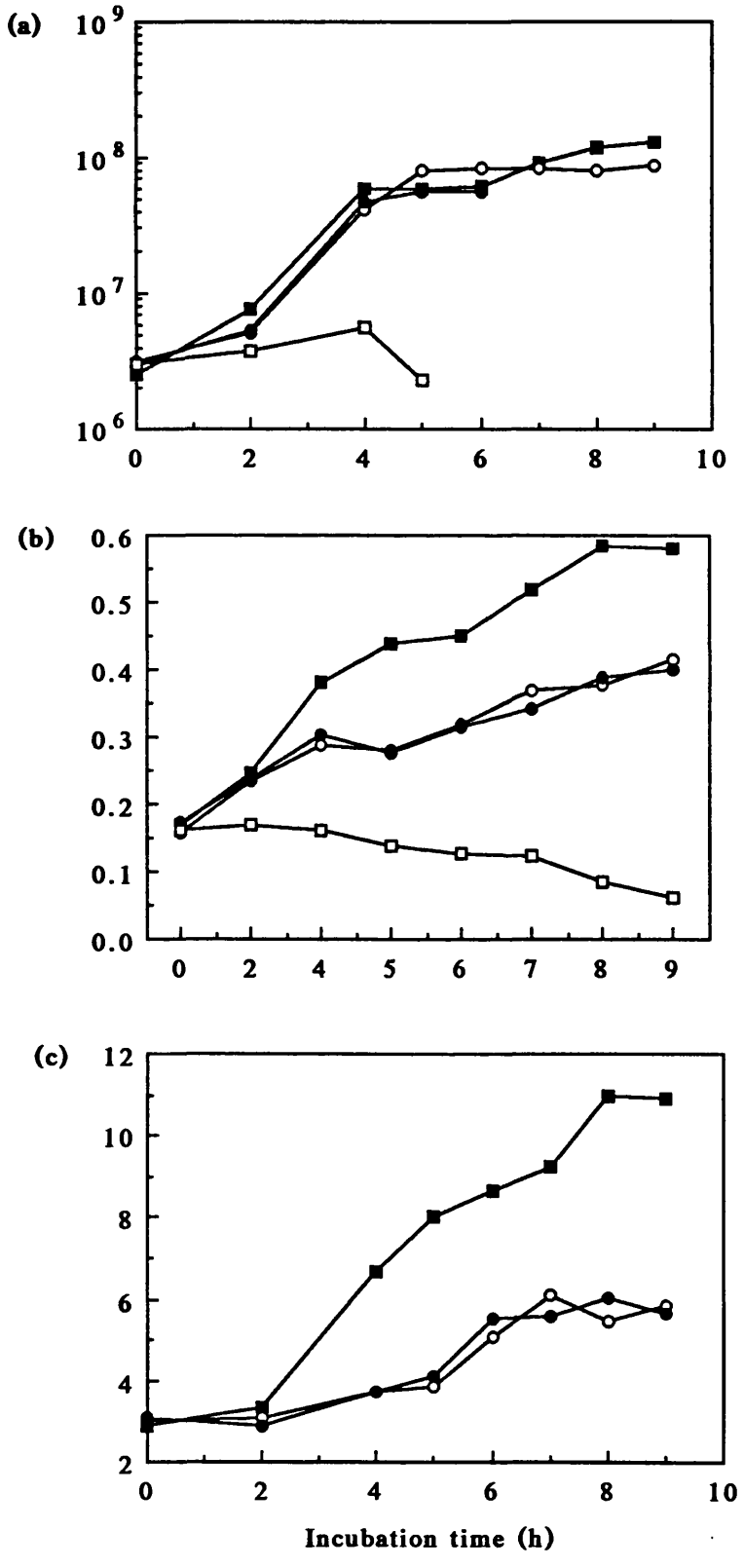
Studies on the effect of temperature on the growth of *Pyrococcus* and extracellular proteinase production were carried out to determine optimal growth temperature and proteinase production.

A 3 litre batch culture of *Pyrococcus* was grown in the absence of sulphur and used to inoculate five 8L fermenters set at temperatures of 95°C, 98°C, 100°C, 103°C and 105°C respectively. Aliquots of culture were sampled at periodic intervals to monitor growth and proteinase production.

Figure 3.5 shows the effect of temperature on *Pyrococcus* with respect to growth and proteinase production. The increase in temperature appeared to have a marked effect on cell proliferation. At 105°C, rapid lysis of cell material occurred and no growth was detected. The optimum growth temperature was calculated to be 100°C which corresponded to the shortest doubling time of 36 minutes (Table 3.1). The calculated optimum temperature

Figure 3.5 Effect of temperature on growth and proteinase production

(a) cell density, cells/ml; (b) protein content, mg/ml; (c) extracellular proteinase activity, U/ml x 10^{-3}.Temperature is represented by symbols (■) 95°C, (●) 98°C, (○) 100°C, and (□) 103°C.



also corresponded to that measured by Fiala and Stetter (1986). Specific growth rates were also calculated, the highest occurring at 100°C with a value of 1.15h^{-1} (Table 3.1). Cell density and proteinase levels were higher at 95°C with values of 1.3 x 10^8 cells/ml and 0.011 U/ml respectively.

Table 3.1. Doubling times and specific growth rates of *P. furiosus* at different growth temperatures.

Temperature (°C)	Doubling Time (min)	Specific Growth Rate (h^{-1})
95	75	0.55
98	42	0.99
100	36	1.15
103	218	0.19

3.5 Effect of different substrates on growth of *Pyrococcus* and proteinase production.

It has been shown that extracellular proteolytic enzymes from a variety of micro-organisms can be induced or repressed by the addition of suitable nutrients to the growth medium. For instance, the amino acids isoleucine and proline have a repressive effect on proteinase production in some *Bacillus* spp (Glenn*et al.*, 1973). This repressive effect was found to occur at the level of transcription where end-product repression appears to take place.

Some peptides and proteins have been shown to induce proteinase synthesis. Shinmyo *et al.* (1968) demonstrated that with *Aspergillus terricola*, the addition of either peptone, casein, various amino acids (particularly glutamate) or a nitrogen-free structural analogue of glutamate, all stimulated proteinase production.

The use of carbohydrates, such as starch or starch hydrolysates, has often been shown to repress extracellular proteinase production by catabolite repression mechanisms. As a carbon source for some bacteria, such as *Thermus* species, some carbohydrates such as

starch have also been shown to stimulate high yields of extracellular proteinases as well as improving the growth of the micro-organism (Lehmann *et al.*, 1977; Cowan, 1980).

The experiments below attempted to relate the production of extracellular proteinases from *Pyrococcus* to the nutrient state of the growth medium.

3.5.1 Effect of different peptones and hydrolysates

The effects of different peptones and hydrolysates on cell yield and proteinase production were determined. Cultures of *Pyrococcus* were grown in complex culture media containing 0.1% yeast extract and 0.5% peptone or protein hydrolysate (specified). The results showing the specific production of extracellular proteinase at maximal growth of *Pyrococcus* in relation to substrate are presented in Table 3.2.

Table 3.2. Effect of different peptones and their hydrolysates on growth of *Pyrococcus* and specific proteinase yield.

Substrate	Cell density at maximal growth (cells/ml x 10^7)	Specific Proteinase Production (U/mg cell protein x 10^{-4})
Peptone	8.1	1.6
Casitone	7.1	1.2
Tryptose	6.9	1.6
Proteose Peptone No.3	5.9	1.5
Soya Peptone	5.5	1.2
Tryptone	4.9	2.1
Neopeptone	4.8	2.9
Proteose Peptone	4.6	3.0
Casamino acids (T)	3.6	1.6
Casein	3.5	0.4
Trypticase Peptone	2.3	4.5
Casamino acids	1.9	4.6
Casein Enzyme Hydrolysate	1.5	2.4

The peptones appeared to be the better substrates for growth of *Pyrococcus*, with bacto-peptone being the best substrate for cell yield. Cultures grown on hydrolysates produced lower cell yields but higher proteinase levels. These results suggested that certain peptides and amino acids may be responsible for stimulating proteinase production but were not essential for increased cell growth. Growth on casein gave the lowest level of specific proteinase production of all the substrates tested. From this result, it can be suggested that casein may show a repressive effect with respect to proteinase production. It is also likely that due to the low solubility of casein, the organism cannot utilise this substrate efficiently and uses other carbon/energy source for growth, such as yeast extract (a complex substrate, which probably contains the essential nutrients for growth).

3.5.2 Effect of amino acids.

It has been well established that extracellular proteinases from a variety of micro-organisms can be induced or repressed by certain amino acids (Ward, 1983).

In order to study the control of proteinase production from *Pyrococcus*, it was necessary to reduce the nitrogen source (i.e peptone) to a level able to sustain growth. Further experiments were carried out to determine the influence of amino acids on proteinase production and growth.

Cultures of *Pyrococcus* were grown in media with and without peptone and containing basal salts, 0.1% yeast extract, and 0.2% amino acid pools (as specified). The results are presented in Table 3.3.

Specific proteinase production appeared to be affected by the presence of amino acid pools in the absence of peptone. In the presence of the pyruvate pool (alanine, valine and leucine), specific yield levels of 0.03 x 10^{-4} U/mg were obtained compared to approximately 0.7 to 2.5 x 10^{-3} U/mg for the other pools. However, on the addition of a very low concentration of peptone (0.005%), activity increased 57-fold, suggesting that the amino acids of the pyruvate family are responsible for repressing extracellular proteinase production. The aromatic amino acids (tyrosine, tryptophan and phenylalanine) gave the

Table 3.3. Effect of amino acids on cell growth and specific proteinase production in the presence and absence of peptone.

Amino acid[a] pool	0% peptone		0.005% peptone	
	Cell density (cells/ml x 10^7)	S.P.P[b] (U/mg x 10^{-4})	Cell density (cells/ml x 10^7)	S.P.P. (U/mg x 10^{-4})
Glutamate	2.7	0.7	4.1	0.51
Aspartate	1.2	1.16	2.1	0.42
Aromatic	3.4	0.85	5.7	0.64
Serine	0.8	2.5	0.8	2.4
Pyruvate	0.9	0.03	1.2	1.7

[a] Final concentration, 0.2% (w/v).
[b] SPP, specific proteinase production expressed as activity per milligram of cell protein.

highest level of growth with and without peptone in the culture medium while the serine amino acids produced the highest specific proteinase yield of 2.5 x 10^{-4} U/mg protein. In the absence of peptone and amino acids, weak growth took place on yeast extract alone (0.1%). This suggested that this carbon substrate was required for growth. This was further supported by the observation that *Pyrococcus* could not grow without yeast extract even if this was substituted by a cocktail of amino acids (all amino acids to give a final concentration of 0.2%) and vitamins.

3.5.3 Maltose as an alternative carbon source.

It has been demonstrated that *Pyrococcus* can grow on carbon sources such as maltose or starch in the presence of yeast extract and ammonium chloride at a lower temperature of about 85-88°C (Fiala and Stetter, 1986; Bryant and Adams, 1989). No growth, however, was reported on many other carbohydrates including glucose. Experiments were carried out to determine the influence of maltose on proteinase production.

Cultures of *Pyrococcus* were grown and subcultured in media containing 0.1% yeast extract, ammonium chloride (1.25g/l) and 0.5% maltose. (For detailed composition of medium, see Table 3.4). All cultures were incubated at 88°C instead of 98°C. After successful subcultures, cell density reached 8 x 10^7 cells/ml with a proteinase titre of 2.64 x 10^{-3} U/ml. However, on observation of the cultures under phase contrast microscopy,

Table 3.4. Composition of growth medium for *Pyrococcus* containing maltose as the carbon source (from Bryant and Adams, 1989).

Substance	*grams per litre distilled water*
Maltose	5.0
NH_4Cl	1.25
Elemental sulphur	5.0
Na_2S	0.5
Basal salts mixture	(see Table 2.1, section 2.3.1.)
Vitamin mixture (see below)	10ml
$FeCl_2$	25μM
Na_2WO_4	10μM
Yeast extract	1.0
Vitamin mixture	*mg per litre distilled water*
Biotin	2
Folic acid	2
Pyridoxine hydrochloride	10
Riboflavin	5
Nicotinic acid	5
D-L-calcium pantothenate	5
Vitamin B_{12}	0.1
p-aminobenzoic acid	5
Lipoic acid	5
Thiamine hydrochloride	5

cells appeared to be much smaller in size (max. 0.5μm in diameter) compared to cultures grown on peptone and yeast extract.

3.6 Small scale batch fermentations of *Pyrococcus*

Small scale batch fermentations (8L) of *Pyrococcus* were performed in basal salts media containing the following substrates:

(i) 0.5% peptone and 0.1% yeast extract

(ii) 0.5% maltose and 0.1% yeast extract

(iii) 0.5% peptone, 0.5% maltose and 0.1% yeast extract

(iv) 0.03% yeast extract and 4% maltose feed (100ml/h)

(v) 0.03% yeast extract and 2% maltose feed (100ml/h)

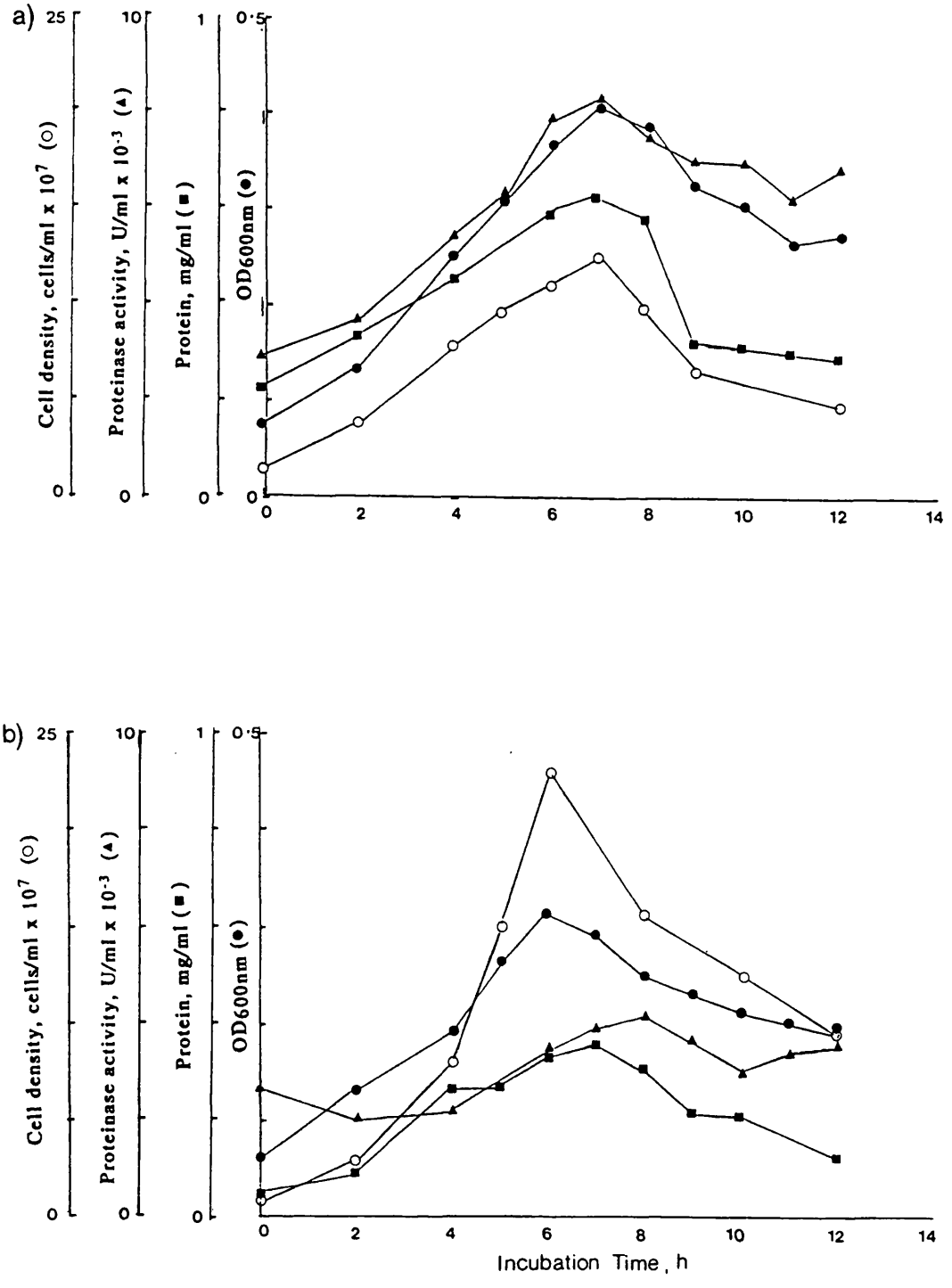
Maltose solutions of different concentrations were continually added during batch fermentation to establish whether growth could be sustained over prolonged periods of time at 95°C without caramelisation occurring.

Fig.3.6 illustrates the growth and proteinase profiles of *Pyrococcus* from the different batch fermentations. Table 3.5 lists the doubling times for each reactor. From these profiles, a comparison of the major carbon substrates, peptone and maltose was made on growth and extracellualr proteinase production (Table 3.6). It can be seen that the protein content of cultures grown on maltose was 44% lower (0.051mg/ml) than cells grown on peptone, despite having a much higher cell density than peptone-grown cultures (2×10^8 cells/ml and 1.28×10^8 cells/ml respectively). Proteinase activity was highest with peptone-grown cultures with a specific proteinase yield of 91.4 U/g protein.

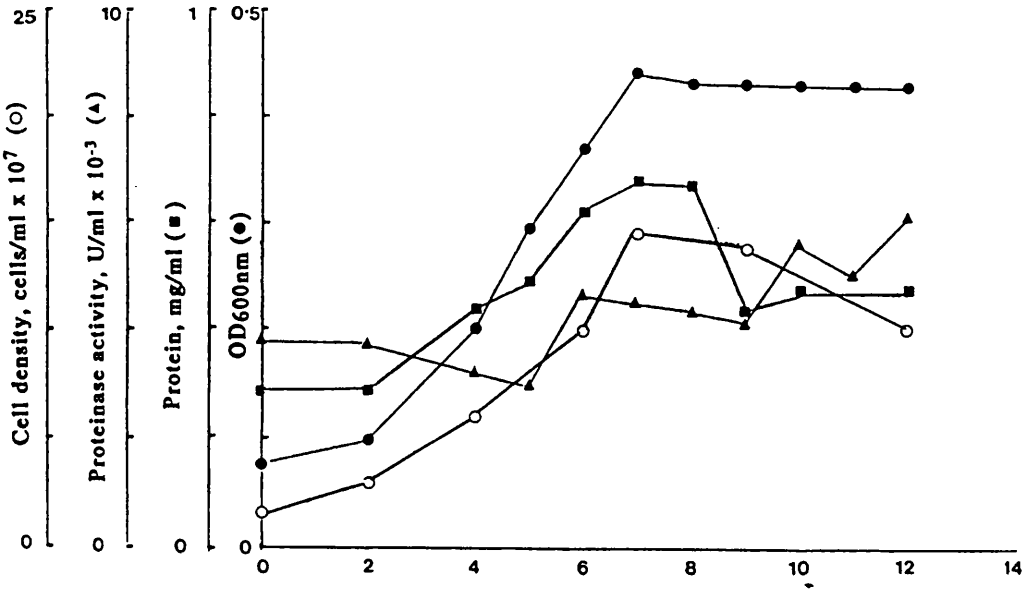
When cultures were grown on a peptone/maltose/yeast extract medium, an extended growth curve was observed (Fig.3.6b). Cell doublets, characteristic of log phase growth, were still observed after 8 hours growth. In comparison, cultures grown on a peptone-yeast extract medium were found to have entered stationary phase after this period. Growth on peptone/maltose/yeast extract medium corresponded to a higher cell density and protein

Figure 3.6 Influence of different carbon and energy sources on the growth of *P. furiosus* and extracellular proteinase production.

Growth and proteinase production was monitored in media containing (a) 0.5% peptone and 0.1% yeast extract; (b) 0.5% maltose and 0.1% yeast extract; (c) 0.5% peptone, 0.5% maltose and 0.1% yeast extract; (d) 0.03% yeast extract with 4% maltose feed; and (e) 0.03% yeast extract and 2% maltose feed. Symbols represent OD_{600nm} (●); cell density, cells/ml x 10^7 (○); extracellular proteinase activity, U/ml x 10^{-3} (▲); and protein, mg/ml (■).



c)


Cell density, cells/ml x 10⁷ (○)
Proteinase activity, U/ml x 10⁻³ (▲)
Protein, mg/ml (■)
OD600nm (●)
25
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2
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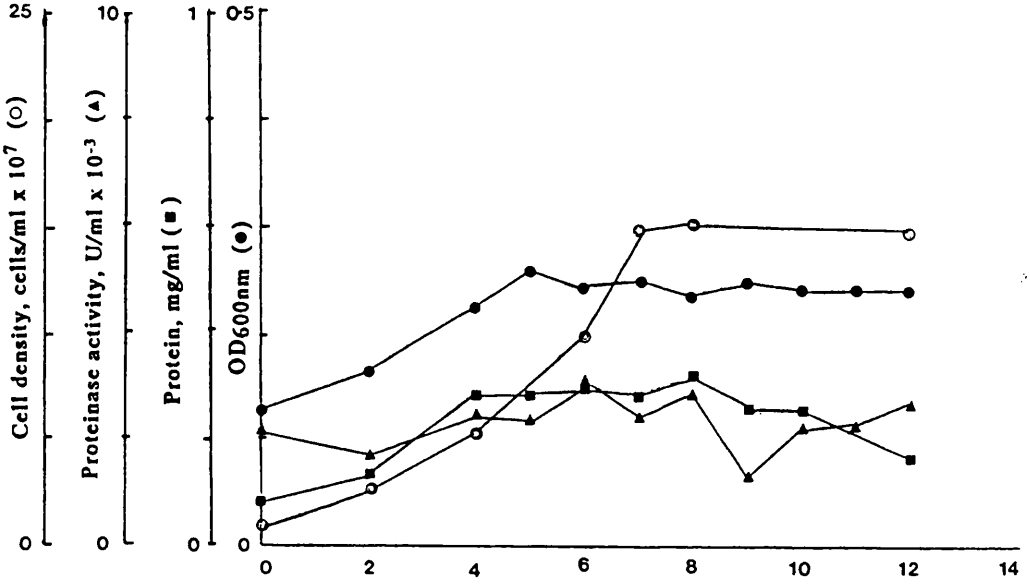
10
12
14

d)


Cell density, cells/ml x 10⁷ (○)
Proteinase activity, U/ml x 10⁻³ (▲)
Protein, mg/ml (■)
OD600nm (●)
25
10
1
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14

e)

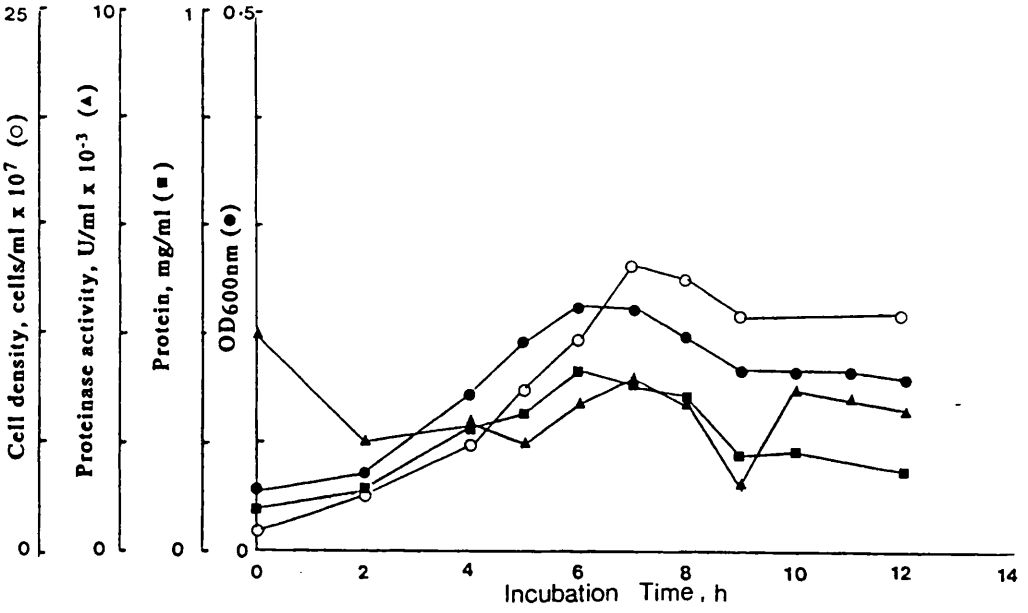

Cell density, cells/ml x 10⁷ (○)
Proteinase activity, U/ml x 10⁻³ (▲)
Protein, mg/ml (■)
OD600nm (●)
25
10
1
0·5
0
0
0
0
0
2
4
6
8
10
12
14
Incubation Time, h

Table 3.5 Doubling times of *P. furiosus* in different growth media at 95°C.

Substrate	Doubling time at 95°C (min)
0.5% peptone + 0.1% yeast extract	75
0.5% peptone + 0.1% yeast extract + 0.5% maltose	126
0.5% maltose + 0.1% yeast extract	78
4% maltose feed + 0.03% yeast extract	114
2% maltose feed + 0.03% yeast extract	114

Table 3.6. Comparison of two different substrates on growth and extracellular proteinase production of *P. furiosus* after 6-7 hours growth at 95°C.

Substrate	Cell density (cells/ml)	Protein (mg/ml)	Proteinase Activity (U/ml x 10^{-3})	Specific Proteinase Production (U/g protein)
Peptone	1.28 x 10^8	0.090	8.23	91.4
Maltose	2.00 x 10^8	0.051	3.90	76.5
Peptone and Maltose	1.45 x 10^8	0.098	4.46	45.5

level compared to growth on peptone and yeast extract, but specific proteinase production was lower (45.5U/g). The extended growth phase suggests that *Pyrococcus* possibly employs a mechanism where one of the major carbon sources is utilised before the organism switches to other remaining carbon source for cell growth.

In both maltose-fed batch fermenters, cell densities reached approximately 1.4 x 10^8 cells/ml. However, total protein levels were low with equally low proteinase activity. The doubling time was lower than for cultures grown in a medium containing a fixed amount of maltose at the start of the fermentation. At 95°C, the problem of caramelisation was evident. Caramelisation was observed to occur later in the growth cycle for *Pyrococcus* in the maltose-yeast extract reactor but did not not occur in maltose-fed reactors.

3.7 Continuous Culture of *Pyrococcus*

The use of chemostat cultures is important for optimising growth parameters and understanding the metabolic behaviour of cultures. Continuous culture of micro-organisms is known to be particularly useful for the production of enzymes which suffer from catabolite repression.

Very few reports on continuous culture of extreme thermophilic organisms have been published. To date, the only extremely thermophilic sulphur-metabolising archaeobacteria grown in chemostat cultures were *Sulfolobus* BC (Norris *et al.,* 1988; Buckingham *et al.,* 1989) and *Pyrococcus furiosus* (Brown and Kelly, 1989). Norris and coworkers (1988) found that *Sulfolobus* cultures oxidising tetrathionate were not only tetrathionate limited but molybdenum limited. The addition of molybdenum (1.3μM) to cultures oxidising 15mM tetrathionate at a dilution rate of 0.029h-1 increased the steady state biomass from absorbance (660nm) 0.160 to 0.315 giving a corresponding increase in yield from 5.93g to 11.52g dry weight/mol tetrathionate oxidised. Brown and Kelly (1989) developed a continuous culture system for *P. furiosus* using a sulphur-free medium containing peptone and yeast extract. Steady state was reached at a dilution rate of 0.1h^{-1} at 98°C with a cell

density of 1.6 x 10^8 cells/ml. This was maintained up to a dilution rate of 0.8h^{-1} before gradually declining as dilution rate was increased further.

In order to investigate growth parameters and study the control of proteinase production from *Pyrococcus*, a chemostat system was developed using a sulphur-free medium composed of peptone and yeast extract. Experiments were carried out in a 1 litre chemostat (working volume of about 800ml) and the system was set up as described in Chapter 2, section 2.3.2b. A dilution rate of 0.3h-1 was required to reach steady state conditions. This corresponded to a cell density value of 3 x 10^8 cells/ml. Growth was sustained for 2 weeks before technical problems (such as leaking pipes due to prolonged heating at high temperatures or depletion of the inert gas) terminated the fermentation. The dilution rate and cell density values obtained compared well with those reported by Brown and Kelly (1989).

Continuous culture of *Pyrococcus* was also attempted using a maltose-yeast extract medium at a lower temperature of 85°C. Results from batch culture studies of *Pyrococcus* in a sulphur-free maltose-yeast extract medium showed that a low level of proteinase was produced even though the cell density was high (2 x 10^8 cells/ml, Table 3.4). Therefore, a 1L chemostat was set up to study growth and proteinase production using the above medium. Using 0.5% maltose as the carbon source, growth to stationary phase was achieved after 17 hours (compared to 10h in batch reactors). The reduced growth rate was attributed to the operating temperature of the chemostat being below the optimum growth temperature of the organism. However, growth was not maintained due to severe caramelization of the medium after 1-2 days.

Raven *et al.*, (1991) have recently improved and optimised growth of *Pyrococcus* in continuous culture at 90°C by altering the gas flow rates to the chemostat. They reported that in batch culture, cell density increased in direct proportion to gas flow up to 0.05v/v/min (1L of nitrogen passed through a sintered glass bulb/min in a 20L culture). At this flow rate, maximum cell densities of approximately 10^9 cells/ml were obtained with

sulphur and 6 x10^8 cells/ml without sulphur. The effect of sparging inert gas was also carried out using an all glass gas-lift chemostat (Raven *et al.*, 1991). Values of 3 x 10^9 cells/ml were obtained in the absence of sulphur when argon gas flow reached 0.3v/v/min. However, it was suggested that greater gas flow rates reduced cell density, possibly as a result of foaming. The use of nitrogen gas produced similar cell densities as with argon but at a flow rate of 0.5v/v/min. Raven *et al.* (1991) also reported that *Pyrococcus* can be cultured continuously for up to 1000h producing approximately 1.56g wet weight of cells/h/litre culture volume in a peptone/maltose/yeast extract medium.

RESULTS AND DISCUSSION

CHAPTER 4

PROTEINASE HETEROGENEITY IN *PYROCOCCUS FURIOSUS*

4.1 Introduction

In the course of our investigations on the production of extracellular proteinases from *P. furiosus*, a number of reports were published by other groups also investigating proteolytic activity from this hyperthermophile. It was reported that proteolytic activity was detected in both intracellular and extracellular fractions, activity being detected up to 120°C using azocasein as the proteolytic substrate (Skaja *et al.*, 1990). Blumentals *et al.* (1990) and Eggen *et al.* (1990) both demonstrated the presence of more than one intracellular proteinase using a gel-detection method. However, Eggen *et al.* (1990) could not detect any proteinases in the culture medium.

In light of this evidence, it was necessary to determine whether extracellular proteinase production was due to enzyme secretion into the culture medium or whether it was derived from intracellular contents released into the culture medium, as a result of lysis occurring at the end of the exponential phase.

4.2 Enzyme localisation

Preliminary studies on intracellular and extracellular proteinase activity from static cultures of *Pyrococcus* were carried out using azocasein as the proteolytic substrate. Aliquots of culture (1ml) were removed throughout growth and treated as followed. Cell pellets were removed after centrifugation for 5 minutes at high speed in an MSE microcentrifuge. The pellets were washed and resuspended in 10mM sodium phosphate buffer, pH 7.5, prior to sonication and recentrifugation to obtain cell-free extracts (Section 2.6.1). Cell-free culture supernatants were treated as described in Section 2.6.2. Both cell extracts and culture supernatants were assayed for protein (Section 2.8) and proteinase activity (Section 2.7.1).

Figure 4.1 illustrates the growth and proteinase profiles from *Pyrococcus*. Proteinase activity was detected in both cell extracts and culture supernatants, reaching a peak at around 17-18 hours after inoculation (Fig. 4.1*a* and *b*). Specific proteinase production was also calculated and was observed to be 'roughly' linear throughout growth (Fig.4.1*c*) suggesting that intracellular and extracellular proteinase production was not growth-dependent. However, the disadvantage of the azocasein assay is that it does not provide any information on enzyme multiplicity since the assay measures only the total titre of proteinase activity in the samples.

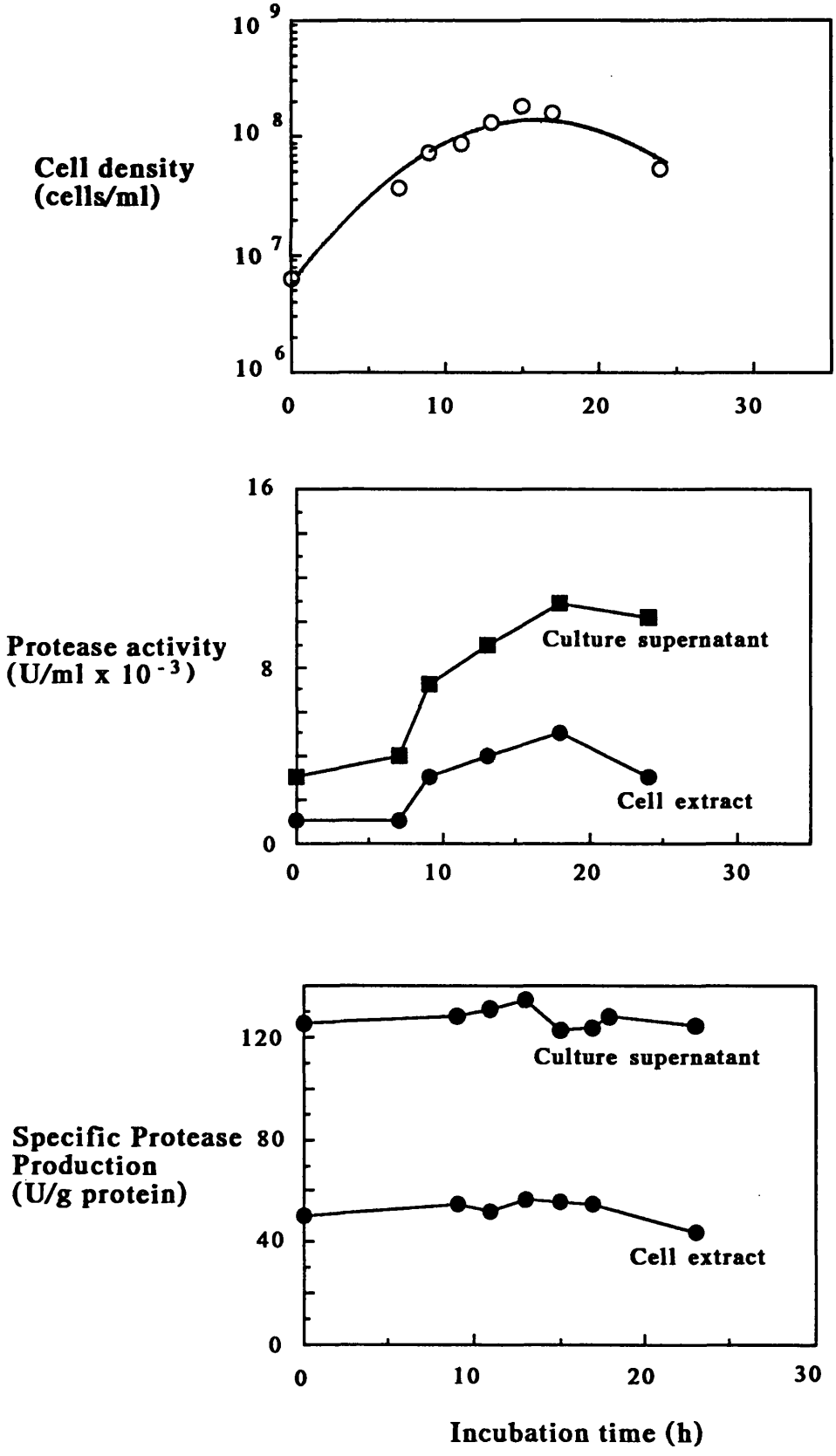
4.3 Proteinase heterogeneity using substrate PAGE

A useful alternative assay for the qualitative analysis of individual proteinases in complex mixtures is the substrate PAGE technique (Heussen and Dowdle, 1980). Substrate PAGE is a gel detection system which relies on the unfolding and refolding of proteins *in situ*. Refolding depends on the efficient removal of bound sodium dodecyl sulphate (SDS) by the non-ionic detergent Triton X-100 to allow the protein to regain its native conformation and hence its activity (Heussen and Dowdle, 1980; Horie *et al.*, 1984).

Substrate PAGE involves either the incorporation of a protein substrate within the acrylamide gel (Heusson and Dowdle, 1980; Kelleher and Juliano, 1983) or contact prints (zymograms) where proteinases are allowed to diffuse onto substrate-coated supports (Granelli-Piperno and Reich, 1978). However, some loss of resolution in the detection of proteinase bands can result using the latter process.

The sensitivity and resolution of substrate PAGE is much greater when the substrate is covalently linked with the acrylamide, since the protein-acrylamide conjugate will be retained during electrophoresis of enzyme samples. As with related substrate PAGE methods, proteolytic action forms clear zones of hydrolysis on subsequent staining of the gel. This technique has been used successfully for the determination of the number and the apparent molecular weights of both thermophilic and mesophilic proteinases from a large number of crude biological samples (Lacks and Springhorn, 1980; Horie *et al.*, 1984;

Figure 4.1 Growth and proteinase production from *P. furiosus*.



Kelleher and Juliano, 1984; Eggen *et al.*, 1990; Klingeberg *et al.*, 1991). In addition, a semi-quantitative determination of activity in individual bands using laser densitometry can be achieved with this method, allowing the measurement of the relative amounts of individual proteins in a particular biological sample.

Using substrate SDS PAGE containing 0.01% gelatin, studies were carried out on cell extracts and culture supernatant samples obtained during growth of *Pyrococcus* (Fig. 4.1). The samples were prepared as described in Section 2.6. Electrophoresis of samples was then performed in slab gels prior to activity staining (Section 2.7.2). The results of activity gels are shown in Figure 4.2.

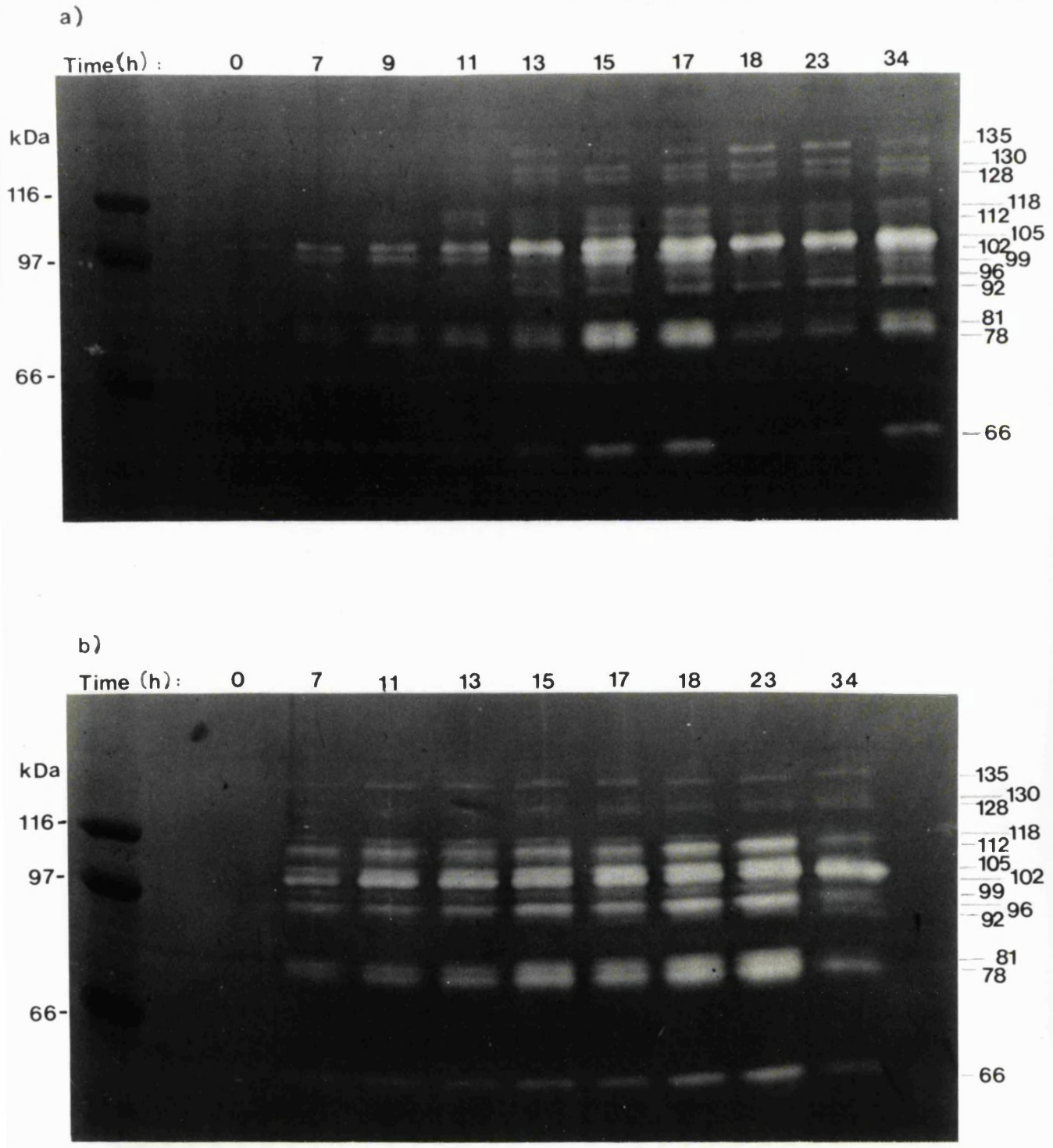
Substrate PAGE analysis of cell extracts and culture supernatants revealed a multiple band pattern of up to 13 distinct proteolytically active "monomers". These were observed as clear zones of enzyme hydrolysis against the blue background of undegraded stained gelatin. Levels of these proteinases were seen to increase until late exponential phase in a manner consistent with the analysis of total proteinases titres as seen in Fig. 4.1*b*. Using standard marker proteins as an indication of molecular weight, the apparent molecular masses of these hydrolases were found to occur between 66kDa and 135kDa (Fig. 4.2).

The observation of multiple proteinase bands from *P. furiosus* confirmed reports of the presence of more than one proteinase from this hyperthermophile (Blumentals *et al.*, 1990; Eggen *et al.*, 1990). However, while Blumentals *et al.* (1990) and Eggen *et al.* (1990) both reported a multiple band pattern using the substrate gel method, neither study indicated the existence of more than 5 activity bands. The observation in this study of a minimum of 13 activity bands is clearly a significant point of disagreement with these data.

On closer inspection of the data produced by Blumentals *et al.* (1990) and Eggen *et al.* (1990), it was discovered that the differences in band patterning were possibly due to the sensitivity and resolution of the gel detection method. Blumentals *et al.* (1990) reported extensive streaking in gels resulting from gelatinolysis during electrophoresis. This was

Figure 4.2 Analysis of cell extracts (a), and culture supernatants (b), obtained during growth of *P. furiosus* using gelatin SDS PAGE.

Numbers along the top of gels indicate incubation time of culture (in hours). Numbers on RHS of photograph indicate proteinase band numbers in ascending molecular mass (kDa), whereas numbers on the LHS denote molecular masses of standard markers (66kDa, bovine serum albumin; 97kDa, phosphorylase b; 116kDa, β-galactosidase).



possibly due to incomplete unfolding of the proteinases where functional enzymes might interact with the substrate during electrophoresis. However, using an SDS-PAGE/gelatin overlay technique, Blumentals *et al.* (1990) were able to distinguish 5 discrete activity bands.

Eggen *et al.* (1990) also reported the presence of 5 activity bands but this was observed using gelatin-containing SDS PAGE. Unlike Blumentals *et al.* (1990), no evidence of streaking was obtained.

Further investigation of the methods used by all groups revealed differences in the details of operation. It was discovered that the substrate concentration used by Blumentals *et al.* (1990), Eggen *et al.* (1990) and in this study differed by as much as 50-fold (0.5%, 0.1% and 0.01% respectively). In using a low concentration of gelatin, minor proteinases would be revealed during incubation which would otherwise not be obvious if higher concentrations were used. For instance, a high substrate concentration may cause inhibition of the minor proteinase bands. It is also likely that substrate hydrolysis by the minor proteinase bands may have occurred within the gel, but the rate of hydrolysis may be too low to observe a distinct zone of proteolytic action (due to gel thickness and a high concentration of undigested protein).

4.4 Possible origins of proteinase multiplicity in substrate gels

To establish whether the apparent band multiplicity observed in substrate gels was due to artefacts of the gel system or due to the existence of discrete, distinct polypeptides, studies were performed using both cell extracts and culture supernatants.

4.4.1 Generation of heterogeneous active processing products

It has been well established that heterogeneous active proteinase fragments can be generated by proteolytic or autolytic degradative processes under conditions of environmental stress (Fontana, 1988). The thermostable, neutral metalloendopeptidase thermolysin, isolated from *B. thermoproteolyticus* (Endo, 1962), is known to undergo both proteolytic and

autolytic degradation to produce functional cleavage products. Limited proteolysis of thermolysin occurred when samples of the proteinase were incubated at pH 9-10 in the presence of 10mM $CaCl_2$ for 2 days at room temperature with subtilisin, producing an active derivative, albeit with lower intrinsic catalytic activity (Vita *et al.*, 1985). Thermolysin was subject to autolytic cleavage when the enzyme (1mg/ml) was incubated in 50mM Tris-HCl buffer, pH 9 containing 10mM $CaCl_2$ at 55°C for 48h. As a result, four active lower molecular weight protein fragments were produced (Fassina *et al.*, 1986).

Experiments were designed to investigate the possibility that the multiplicity of proteolytic bands observed in substrate gels were the products of autolytic or proteolytic degradative processes.

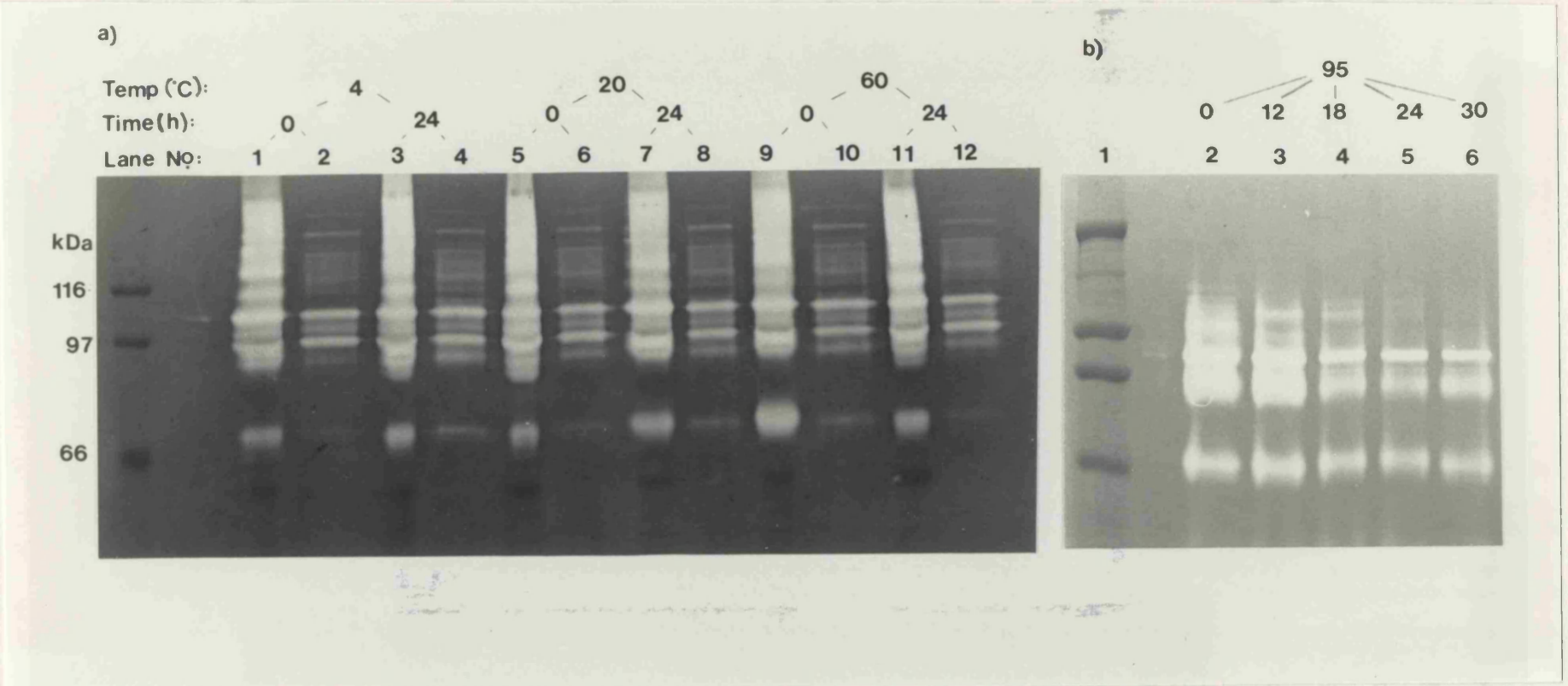
Samples (1ml aliquots) of *P. furiosus* cell extracts and culture supernatants (prepared as described in Section 2.6) were preincubated at different temperatures (4°C, 20°C, 60°C and 95°C) for up to 24-30 hours before analysis by gelatin SDS PAGE.

As shown in Fig. 4.3, a consistent multiple band pattern was observed when samples were preincubated at the specified temperatures. Moreover, there was no marked difference in the intensity of the activity bands over the range of incubation times used. If autolysis was a significant factor, a gradual decrease in the intensity of higher molecular mass bands, possibly corresponding to an increase in the intensity of the lower molecular mass bands, might be observed. New low molecular weight active bands might also be observed. However, the results obtained in this study indicate that the activity bands shown in Fig. 4.3 are unlikely to be degradation products of autolytic processing, and it is reasonably concluded that autolytic degradation is not a significant factor under the conditions used.

Evidence obtained by Blumentals *et al.* (1990) and Eggen *et al.* (1990) appears to contradict these results. Immunoblot analysis of the SDS-resistant proteinase 66kDa from *P. furiosus* reported by Blumentals *et al.* (1990) showed a decrease in molecular mass to an immunologically related, inactive proteolytic fragment of 49kDa. Lower molecular mass bands were recognised during a time course of up to 24 hours at 98°C but none were

Figure 4.3 Effect of temperature and time on *P. furiosus* cell extracts and culture supernatants.

Photograph (a) illustrates activity gel of cell extracts (odd number lanes) and culture supernatants (even number lanes) incubated at 4°C, 20°C, and 60°C for up to 24h. Photograph (b) is an activity gel showing a time course for *P. furiosus* cell extract up to 30h at 95°C. Molecular masses of standard markers are indicated on the LHS of the gels.



reported to exhibit proteolytic activity. It was suggested that these fragments were degradation products of larger proteins (not necessarily proteolytic). Furthermore, the three other proteolytically active bands (116kDa, 125kDa and 140kDa) isolated from *P. furiosus* cell extracts were not immunologically related to the two SDS-resistant proteinases, S66 and S102, suggesting that these bands were not active degradative products of larger protein species.

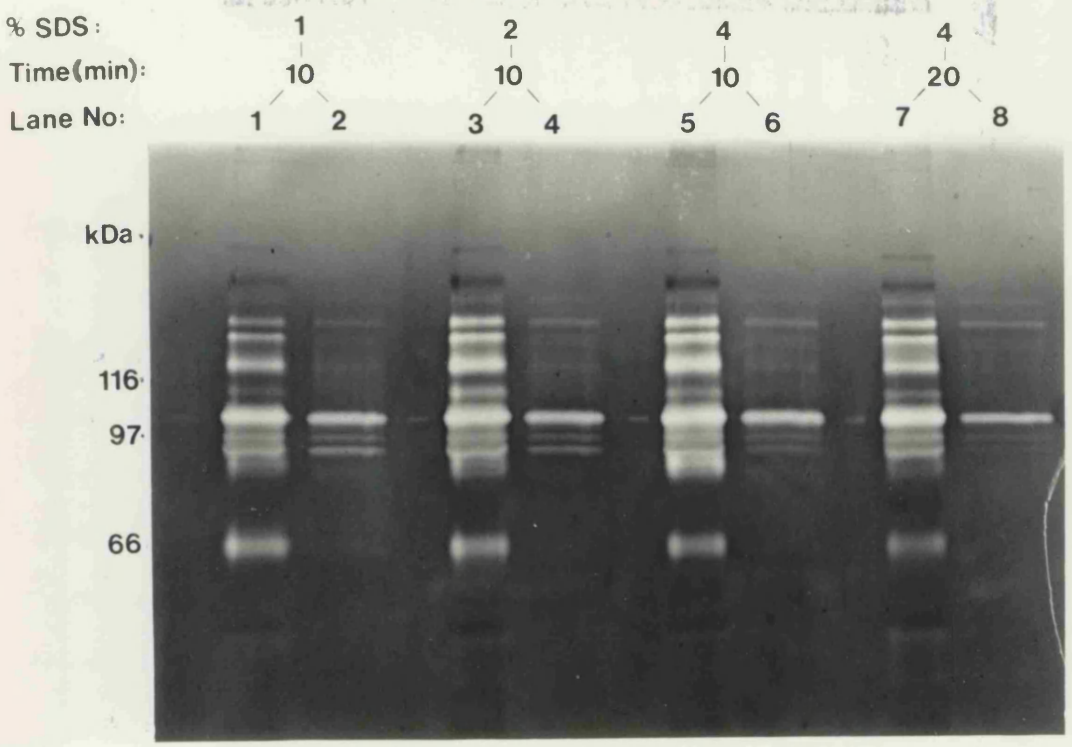
In experiments reported by Eggen *et al.* (1990), preincubation of cell extracts at 95°C up to 28 hours in the absence of substrate followed by activity staining using gelatin PAGE, showed a concomitant increase in the intensity of the lower molecular mass band at 65kDa. This result suggested that autolytic processing of higher molecular mass proteinases was significant. However, results obtained in this investigation did not show an increase in intensity of the 66kDa band. In fact, a slight decrease in the intensity of the activity bands was observed after 30 hours at 95°C (Fig. 4.3), an observation which does not support the view that these bands are active processing products of autolytic degradation (Eggen *et al.*, 1990).

4.4.2 Artefacts resulting from incomplete unfolding.

It has been reported that apparent enzyme heterogeneity can occur in SDS-PAGE, resulting from the incomplete unfolding of proteins by SDS (Heusson and Dowdle, 1980). This has been shown to be a major cause of artefacts in substrate gels (Horie *et al.*, 1984; Kelleher and Juliano, 1984). It is known that even in the presence of SDS, partially unfolded proteins can retain activity and interact with the substrate during electrophoresis thereby producing multiple bands and "streaking" across gels. Proteins which are resistant to SDS will remain active and thus interact with substrate during electrophoresis. This "binding mode" electrophoresis (Brown *et al.*, 1982) has been observed by Blumentals *et al.* (1990) with the two thermostable SDS-resistant proteinases isolated from *P. furiosus.*

Figure 4.4 Effect of SDS on proteinase multiplicity from *P. furiosus*.

Lanes numbers indicate cell extracts (odd numbers) and culture supernatants (even numbers) incubated for up to 20 minutes in SDS (1 to 4% w/v). Numbers on the LHS indicate molecular masses of standard markers.



Experiments were carried out to investigate whether the multiplicity of activity bands in substrate gels were due to artefacts resulting from incomplete unfolding of proteinases and interactions with the substrate during electrophoresis.

Samples (100µl aliquots) of cell extracts and culture supernatants (prepared as described in Section 2.6) were incubated for 10 to 20 minutes at 100°C in an equal volume of sample buffer containing different concentrations of SDS (1%, 2%, and 4% final concentration respectively). In addition, samples were autoclaved for 20 minutes in the presence and absence of 1% SDS.

Figure 4.4 illustrates the results of gelatin-containing SDS PAGE analysis of cell extracts and culture supernatants subjected to a variety of SDS treatment conditions (above). The reproducible band patterns, together with the absence of "streaking" suggests that none of the proteinases were particularly SDS-resistant. There is thus no evidence of incomplete unfolding as a source of multiple band patterns since the absence of streaking and the consistently reproducible band pattern was observed under all conditions. In addition, the absence of the multiple band pattern after samples were autoclaved confirm these bands to be true biological entities and not artefactual. The implication of the above results is that the proteinases are fully and reversibly denatured under the conditions used and that they apparently renature with relative ease when SDS is removed during the Triton-X100 step. These observations, however, do not support those of Blumentals *et al.* (1990) who reported extensive streaking in gelatin-containg SDS PAGE on applying cell extracts pretreated with 1% SDS. Further studies by Blumentals and coworkers (1990) demonstrated that two of the proteinases exhibited resistance to SDS since they were not denatured when boiled with this reagent.

4.4.3 Thiol interchange mechanisms

Among the many processes which occur during protein folding, the formation of disulphide bonds by thiol-disulphide interchange reactions (Eldjarn and Pihl, 1957) can be a major contributer to the stability of the protein structure. Occasionally, the formation of

incorrect disulphide bridges and reshuffling processes can occur during protein refolding, leading to intermediates which may or may not have enzymic activity. This process can also lead to intermolecular interactions with other reduced, unfolded proteins particularly in crude preparations, possibly resulting in the formation of heterogeneous artefacts with enzymic activity.

Evidence has shown that the formation of incorrect disulphide bonding can occur in early stages of the refolding process of reduced and denatured proteins (Ghelis and Yon, 1982). The first well documented studies of the formation of the tertiary structure of proteins from the linear polypeptide chain were performed using bovine pancreatic ribonuclease (Anfinsen and Haber, 1961). Reformation of the tertiary structure was achieved from a protein unfolded in 8M urea and reduced by β-mercaptoethanol with a yield approaching 100%. In earlier studies, reduction was performed with thioglycolate which produced a series of thiol artefacts but was prevented when β-mercaptoethanol was used (White, 1961). Later studies also showed that the reoxidation of ribonuclease in 8M urea or 4M guanidinium chloride gave a product which contained a great number of species with incorrectly formed S-S bonds (Haber and Anfinsen, 1962). The correct native structure was allowed to reform on removal of urea and exposure to a small amount of reducing reagent to allow disulphide interchange.

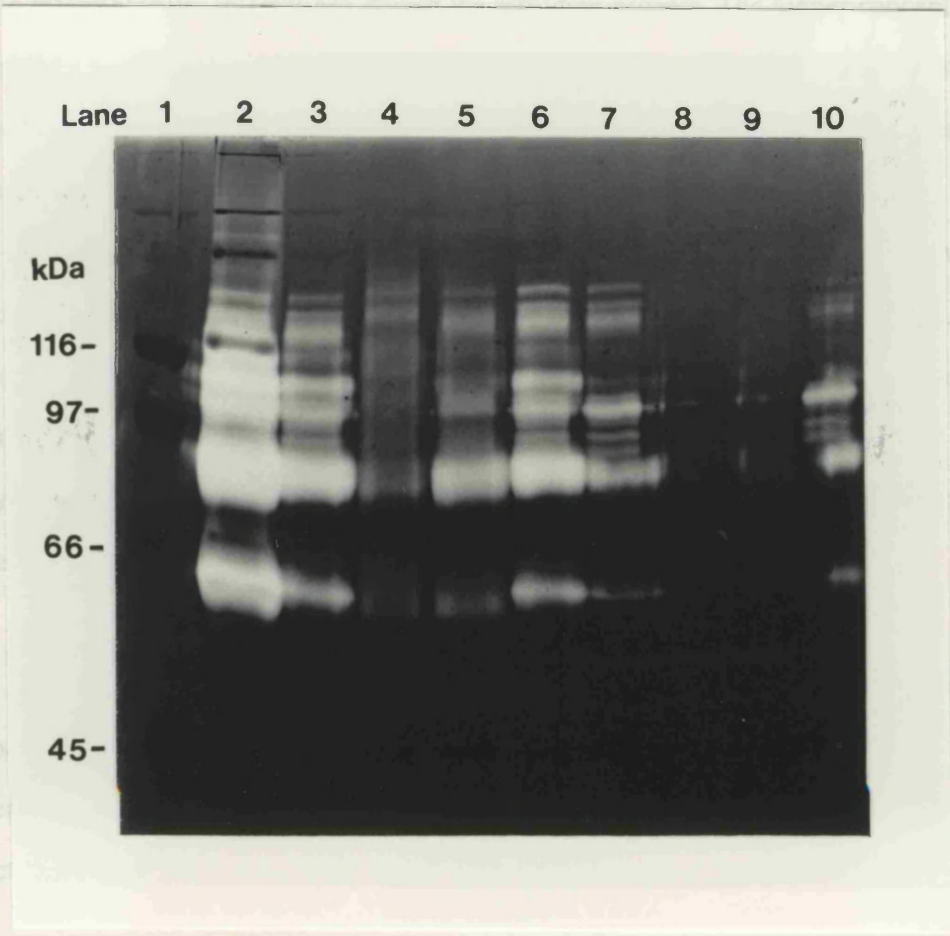
The following experiments were devised in order to determine whether the apparent enzyme heterogeneity of proteinases observed in substrate gels resulted from cross-linked formation (by disulphide bonding) of unfolded polypeptides during electrophoresis in substrate gels or by covalent interactions with other proteins present in *P. furiosus* cell extracts.

The first of these experiments involved the unmasking of buried, free thiol groups within the protein structure. Samples of crude cell extract (1ml) were incubated with 6M guanidinium chloride and 10mM DTT in 0.1M sodium phosphate buffer, pH 7.3 with 1mM EDTA, before the addition of 50µl of the thiol reagent, iodoacetamide at

Figure 4.5 Effect of the thiol reagent iodoacetamide on proteinase multiplicity from *P. furiosus*.

Photograph shows the following:

Lane	1	Molecular masses of standard markers
	2	Cell extract, undiluted
	3	Cell extract, 1 in 10 dilution
	4	Cell extract (1 in 10 dilution), treated with 0.1M iodoacetamide
	5	" " " " 0.05M "
	6	" " " " 0.01M "
	7	Cell extract, 1 in 20 dilution
	8	Cell extract (1 in 20 dilution), treated with 6M guanidinium chloride and 0.1M iodoacetamide
	9	As in (8), but treated with 0.05M iodoacetamide
	10	As in (8), but treated with 0.01M iodoacetamide.



concentrations of 0-0.1M. The samples were incubated at room temperature for 30 minutes before removing excess thiol reagent by gel filtration using Sephadex G25. The treated samples were then analysed using gelatin SDS PAGE (section 2.7.2).

The second experiment was designed to block free thiols on the exposed surfaces of the protein structure in order to prevent the formation of cross-links with other proteins *in vitro*. Samples of cell extract (100µl) were incubated for 30 minutes at room temperature with 10µl of iodoacetamide to give a final concentration of 0.1M, 0.05M and 0.01M respectively. Excess thiol reagent was removed as described above before analysis using gelatin SDS-PAGE. The results of the above two experiments are illustrated in Figure 4.5.

In both experiments, a reproducible pattern of proteinase activity, qualitatively identical to control patterns, was observed. It was concluded that the apparent heterogeneity seen in substrate gels was probably not due to the formation of cross-links of intermediate species nor to intermolecular interactions during the refolding process. The higher concentrations of the thiol reagent iodoacetamide affected activity in diluted cell extracts (intensity of bands decreased compared to the control lane) but did not hinder the reformation of the multiple band pattern observed at lower concentrations or in the absence of this reagent (Fig. 4.5). It has been shown that high concentrations of iodoacetate can interfere with activity by reacting with side groups of a number of amino acids (Means and Feeney, 1971). These results suggest, but are not definitive evidence, that the proteinases may not contain disulphide bonds or free thiol groups since reformation of the individual active polypeptides was observed to occur in a manner very similar to that in the control experiments.

4.4.4 Artefacts resulting from heterogeneous associations with substrate

Apparent banding multiplicity in substrate gels might arise from heterogeneous non-specific interactions between the denatured protein and gel-encapsulated substrate during electrophoresis. This possibility could be easily tested by comparing, under similar

electrophoresis conditions, the activity band pattern generated in gels containing different proteinaceous substrates.

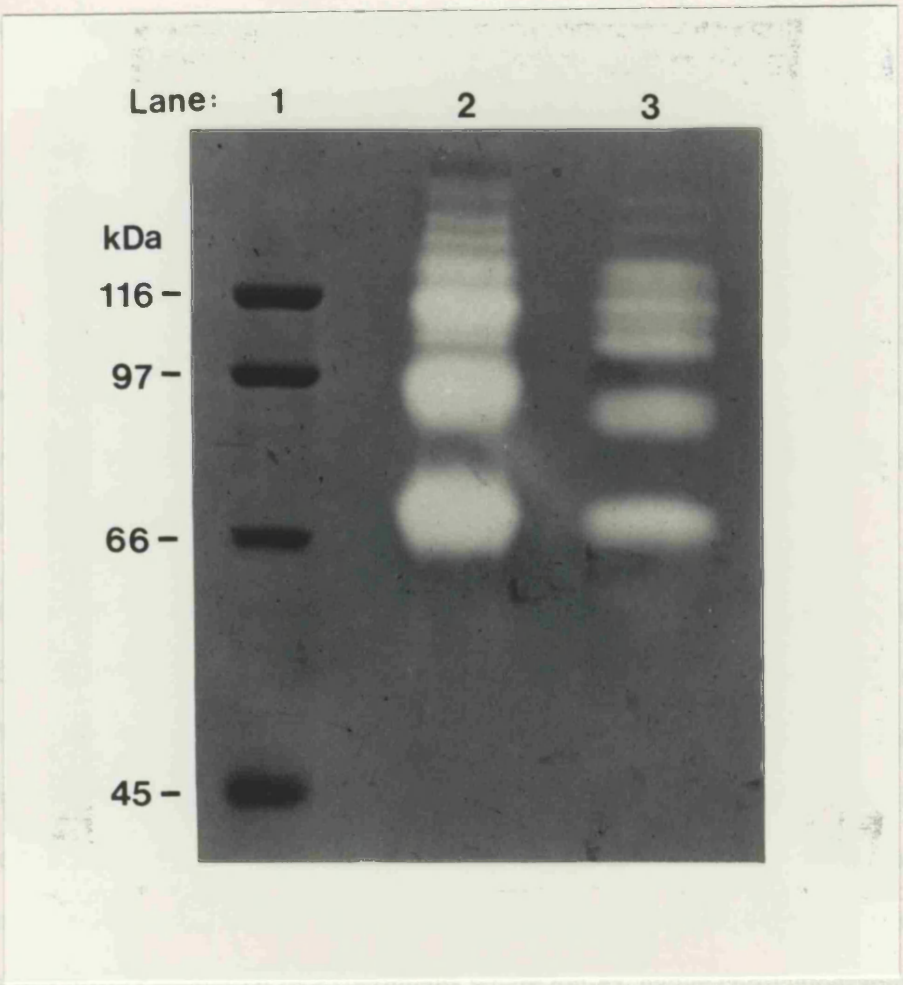
Samples of cell extract and culture supernatant (100µl aliquots) were prepared as described in Section 2.6. SDS PAGE gels were prepared with casein at a final concentration of 0.01% (w/v) as an alternative substrate to gelatin. After electrophoresis, the gels were treated as previously described. The results are illustrated in Figure 4.6

It was evident that the apparent enzyme multiplicity observed in the substrate gels was not an artefact of non-specific binding interactions between denatured proteinase and gelatin as casein-containing gels produced multiple band patterns similar to those observed with gelatin-containing gels. The incubation time for the hydrolysis of casein was however increased (up to 14 hours) compared to gelatin gels suggesting that the *Pyrococcus* proteinases hydrolyse casein at a much slower rate. As a consequence of the increased incubation period, the resolution of activity bands was not as high as that seen with gelatin-containing gels.

All the observations discussed above contribute to the conclusion that the multiplicity of activity bands produced in substrate gels is not artefactual, but that the bands are products of distinct active polypeptides. Neither the genetic origins of these polypeptides (whether genetically related or unrelated), nor the *in vivo* state of these activities, is clear from these results, however. Studies published to date have shown that some of the proteinases from *P. furiosus* are not genetically related and correspond to different enzymes (Blumentals *et al.*, 1990). In contrast to these findings, Eggen *et al.* (1990) contend that the multiple proteinase bands have evolved from a high molecular weight active protein which is processed to give a final proteolytically active 66kDa band. Nevertheless, the data obtained in this study support the conclusions of Blumentals *et al.* (1990) that the proteinase heterogeneity observed in substrate gels is the result of discrete polypeptides.

Figure 4.6 Casein-containing SDS PAGE of *P. furiosus* cell extracts and culture supernatants.

After renaturing, the casein gel was incubated for 14h at 90°C, before staining and destaining. Lane 1, standard molecular mass markers; lane 2, cell extract, and lane 3, culture supernatant.



RESULTS AND DISCUSSION

CHAPTER 5

SEPARATION OF *P.FURIOSUS* PROTEINASES

5.1 Introduction

In order to investigate the *in vivo* state of the *Pyrococcus* proteinases and to facilitate studies of the individual activities, attempts were made to separate individual proteinases corresponding to single bands observed on SDS-substrate PAGE gels.

5.2 Ion exchange chromatography.

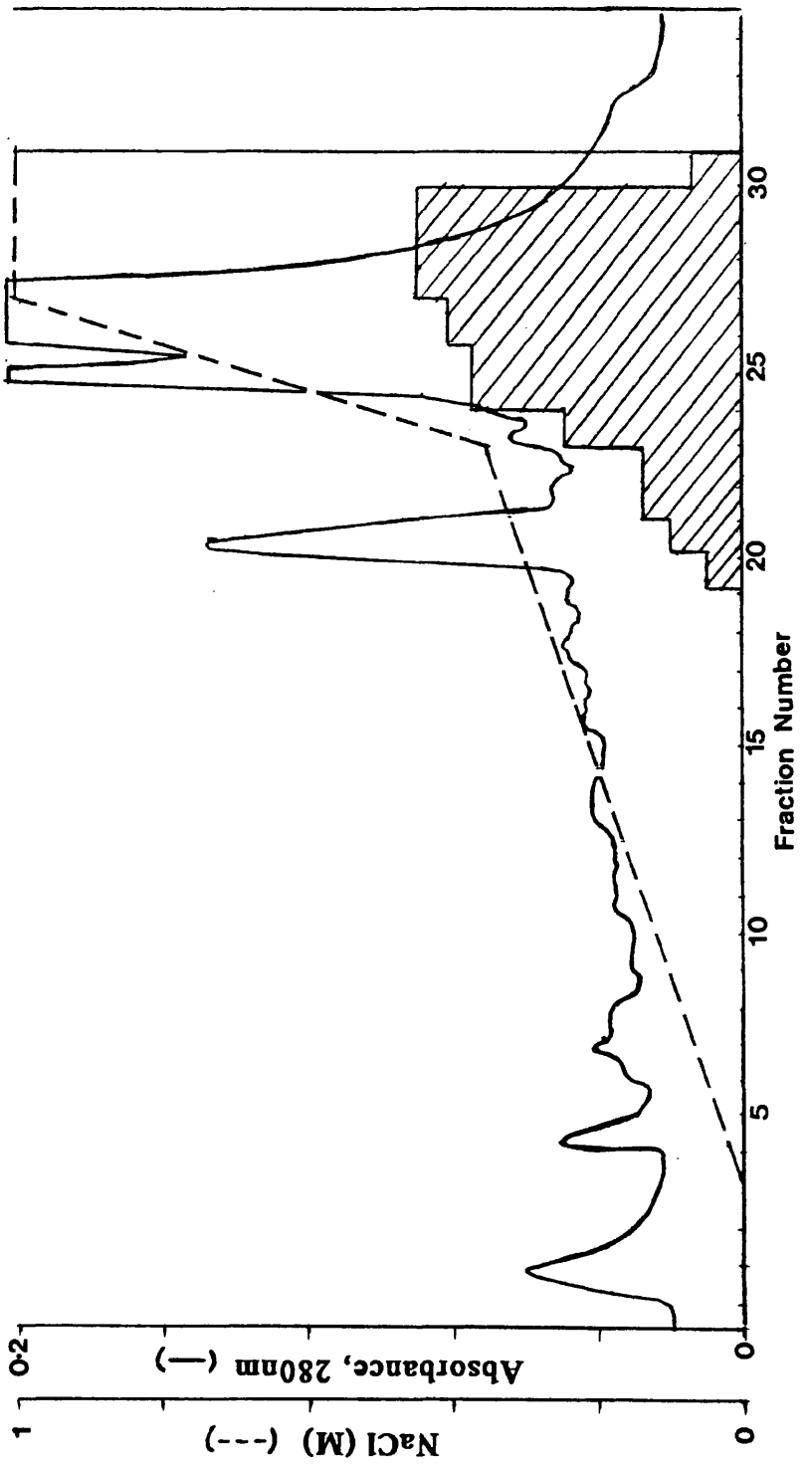
Samples of *P. furiosus* cell extract (1ml) were applied to a Mono Q HR 5/5 column pre-equilibrated with 20mM Bis Tris Propane, pH 7.0 and eluted with a 0-1M NaCl gradient in the same buffer (section 2.11.1). Eluted material was monitored for protein at 280nm and collected in 1ml fractions. These were subsequently assayed for proteinase activity using gelatin SDS PAGE. An elution profile is reproduced in Fig. 5.1 together with an analysis of active proteinase fractions by gelatin SDS PAGE.

Separation of the individual polypeptides by ion exchange was not successful under the conditions used. Activity was observed in fractions 19-30, corresponding to an NaCl gradient concentration of 0.3M to 1M NaCl. However, each of the fractions showed the same multi-component activity pattern after substrate SDS PAGE as the unpurified material. This suggests that the proteinase activities were chromatographing as a heterogeneous complex since no separation of the individual polypeptides was achieved. This was further supported by experiments where ion-exchange chromatography on Mono Q was attempted under different pH conditions (3 to 8). However, although binding was observed from pH 7 onwards, separation of the proteinases was not achieved. It was concluded that the proteinase "complex" exhibited an isoelectric point of approximately 6.5.

Figure 5.1 Elution profile (a), and activity gel analysis (b) of fractions from Mono Q chromatography

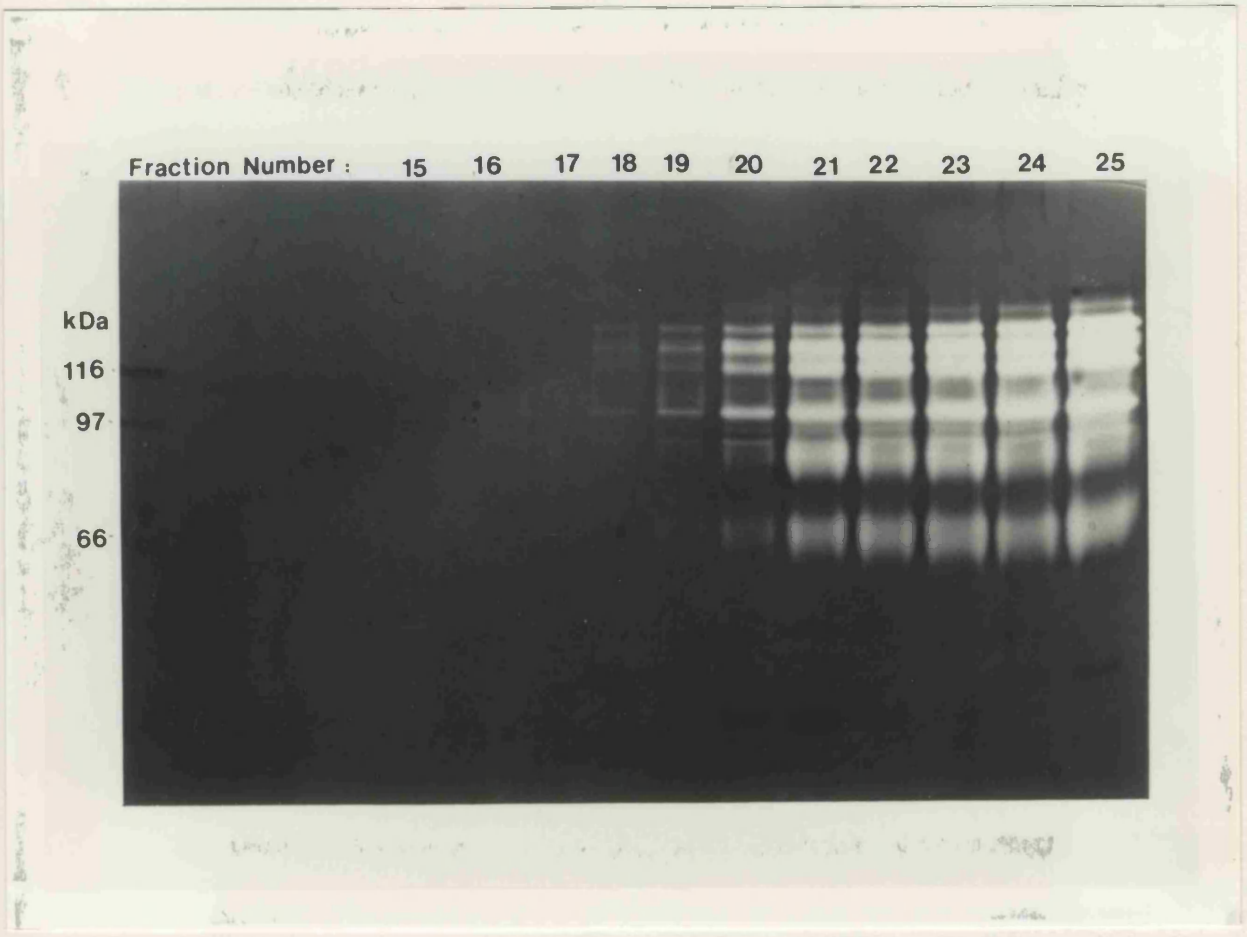
(a) Elution profile

Shaded area represents level of proteinase as adjudged by the intensity of hydrolysis in activity gel (see b)



(b) Gelatin-SDS-PAGE of fractions from Mono Q chromatography

Numbers along the top of photograph indicate fraction number during chromatography. Standard molecular mass markers are denoted on the LHS of the photograph.



5.3 Gel Permeation Chromatography

Separation of the individual proteinase activities on the basis of molecular weight was attempted using gel permeation chromatography under non denaturing (native state) conditions. Since proteinase separation was observed in the presence of SDS, denaturing conditions were also applied in an attempt to exploit the proteinases denaturation-renaturation properties to achieve separation.

5.3.1 Non-denaturing gel permeation chromatography

Superose 12 gel permeation chromatography (fractionation range 10^3 to 10^6 Da) under high pressure conditions was used to separate proteinases on the basis of their native molecular weights (section 2.10.3). Samples of cell extract and culture supernatants (200µl) were applied to the column pre-equilibrated in 20mM triethanolamine buffer, pH 7.5. Elution was carried out in the same buffer containing 50mM NaCl (section 2.10.3). Fractions (1ml) were monitored for protein and assayed for proteinase activity using gelatin SDS PAGE.

An analysis of active fractions using gelatin SDS PAGE is shown in Fig. 5.2. Separation of the individual proteinases was not apparently achieved since the same pattern of active proteinases was observed in all fractions. The apparent molecular weight of the "complex" corresponded to that of the molecular weight standard Blue Dextran (approximately 2 x 10^6) which was used to indicate the void volume (V_o= 9ml). This result suggested that the combined molecular mass of these proteinases is approximately 10^6 Da or more (since the exclusion limit of the gel matrix is 1 x 10^6), and that no separation of individual proteinase components was achieved.

Attempts to separate the proteinases using an alternative chromatography medium Sephacryl S300 HR (fractionation range 10^3 to 10^6 Da) was also performed (section 2.10.1). In this case, separation was performed in a 1 metre Pharmacia K16 column using the same buffer conditions described for high pressure gel permeation chromatography.

Figure 5.2 Gelatin-containing SDS PAGE of fractions from FPLC Superose 12 gel permeation of *P.furiosus* cell extract.

Approximately 1.6mg/ml of protein was loaded to Superose 12. Fraction number is indicated along the top of the photograph; standard molecular weight markers are denoted on the left.

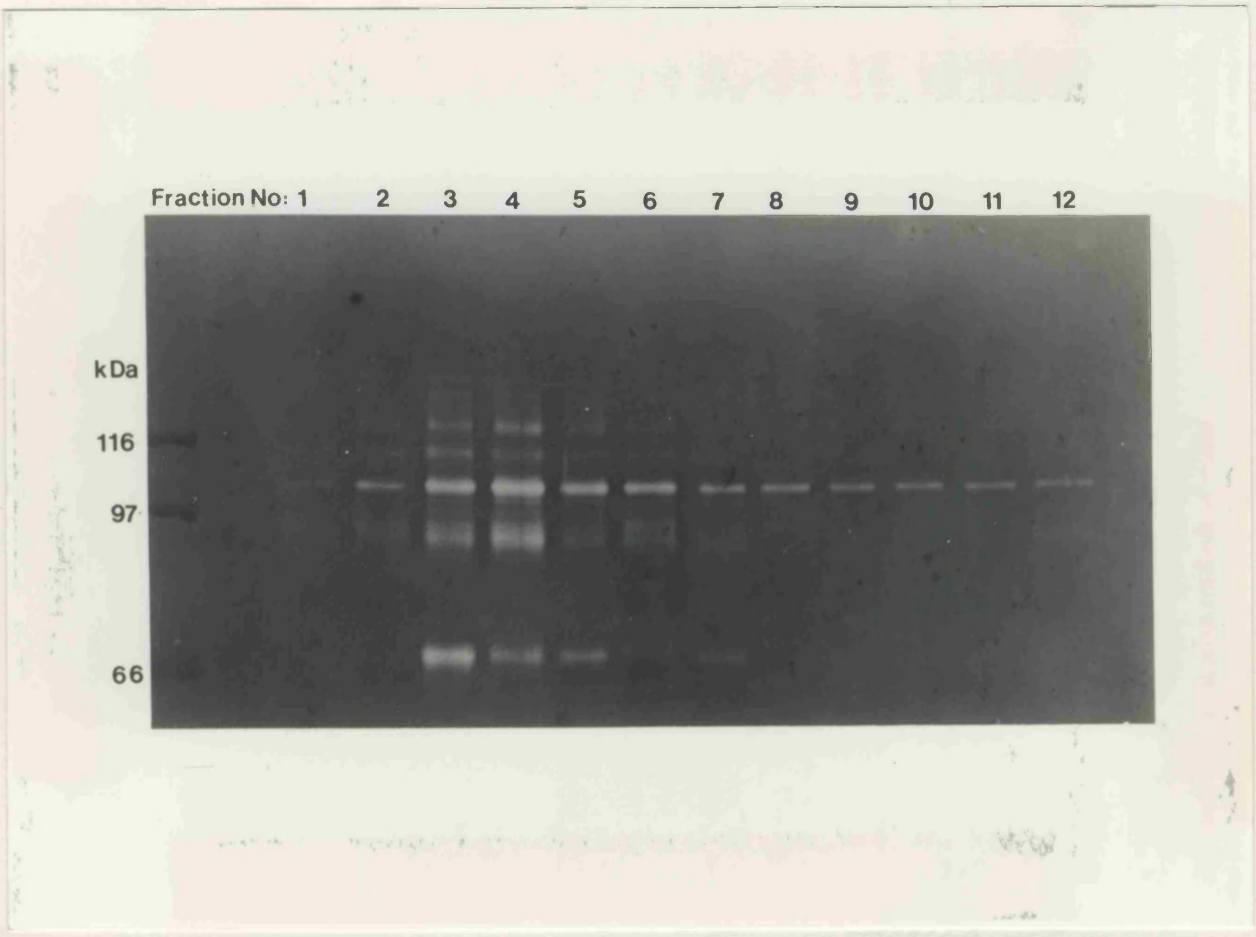
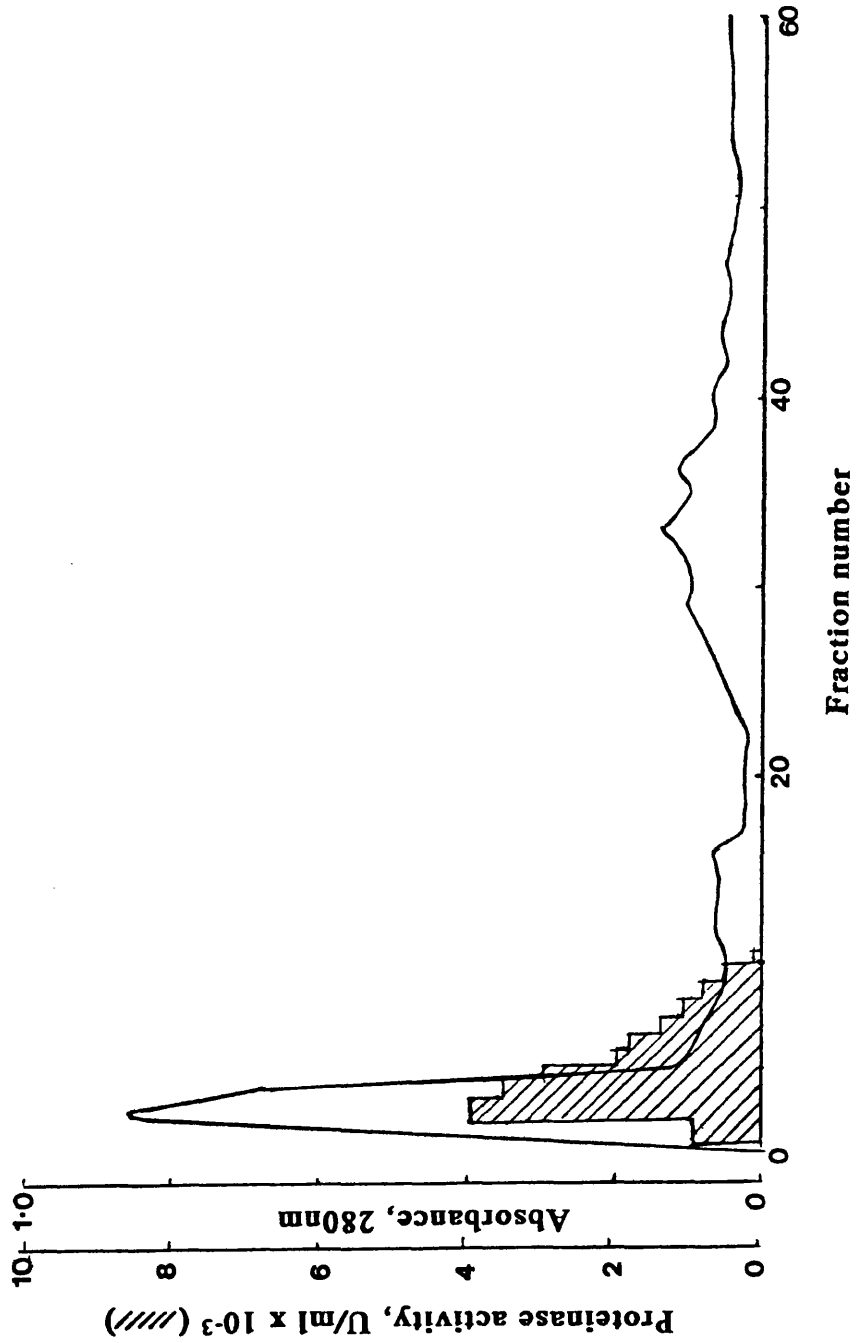


Figure 5.3 Elution profile of chromatographic separation of *P.furiosus* cell extract using Sephacryl S300

(fractions 1 to 60 were collected from void volume i.e. Blue Dextran)



The elution profile from the chromatographic separation is reproduced in Fig. 5.3

Analysis of the fractions using the azocasein assay demonstrated the presence of a major activity peak eluting in the void volume (Vo = 78ml). All active fractions produced a similar multiple band pattern as observed in Fig. 5.2, suggesting that no separation of the individual proteinases had been obtained. The observations of both high and low pressure gel permeation chromatography of *P. furiosus* proteinases strongly supports the view that proteinase components exist in the form of a high molecular weight complex. The mechanism of formation of such a complex is not clear but both an *in vitro* aggregation phenomenon or the existence of an "*in vivo*" multicatalytic proteinase complex are possible. Such multi-component complexes have been recently described (Rivett, 1989; Dahlmann *et al.*, 1989).

5.3.2 Gel permeation chromatography under denaturing conditions.

Samples of cell extract and culture supernatants (100µl) were denatured in an equal volume of protein solubilisation solution (section 2.2) before application on to a Pharmacia Superose 12 column pre-equilibrated in 20mM Tris-HCl buffer, pH 7.5 containing 0.1% SDS (section 2.10.4). Elution of the proteinases was carried out using the same buffer and fractions were monitored for protein and assayed for proteinase activity using gelatin SDS PAGE. Standard molecular weight markers were used for the calibration of the column.

Fig. 5.4 illustrates the elution profile obtained after chromatography of *P. furiosus* proteinases on Superose 12 in the presence of 0.1% SDS. Analysis of the active fractions showed a multiple band pattern in each fraction as observed previously. This suggests that little or no separation of the proteinase "monomers" had occurred. However, the standard SDS molecular weight markers bovine serum albumin (66kDa monomer) and β-galactosidase (116kDa monomer) were separated by a few fractions only (Fig. 5.4). Thus the possible resolution of the multiple proteolytically active polypeptides, with

Figure 5.4 Elution profile of *P. furiosus* cell extract using Superose 12 under denaturing conditions.

Bar represents location of proteinases during chromatography. Abbreviations represent BD, Blue Dextran; 116kDa, β-galactosidase marker protein; 66kDa, bovine serum albumin marker protein.

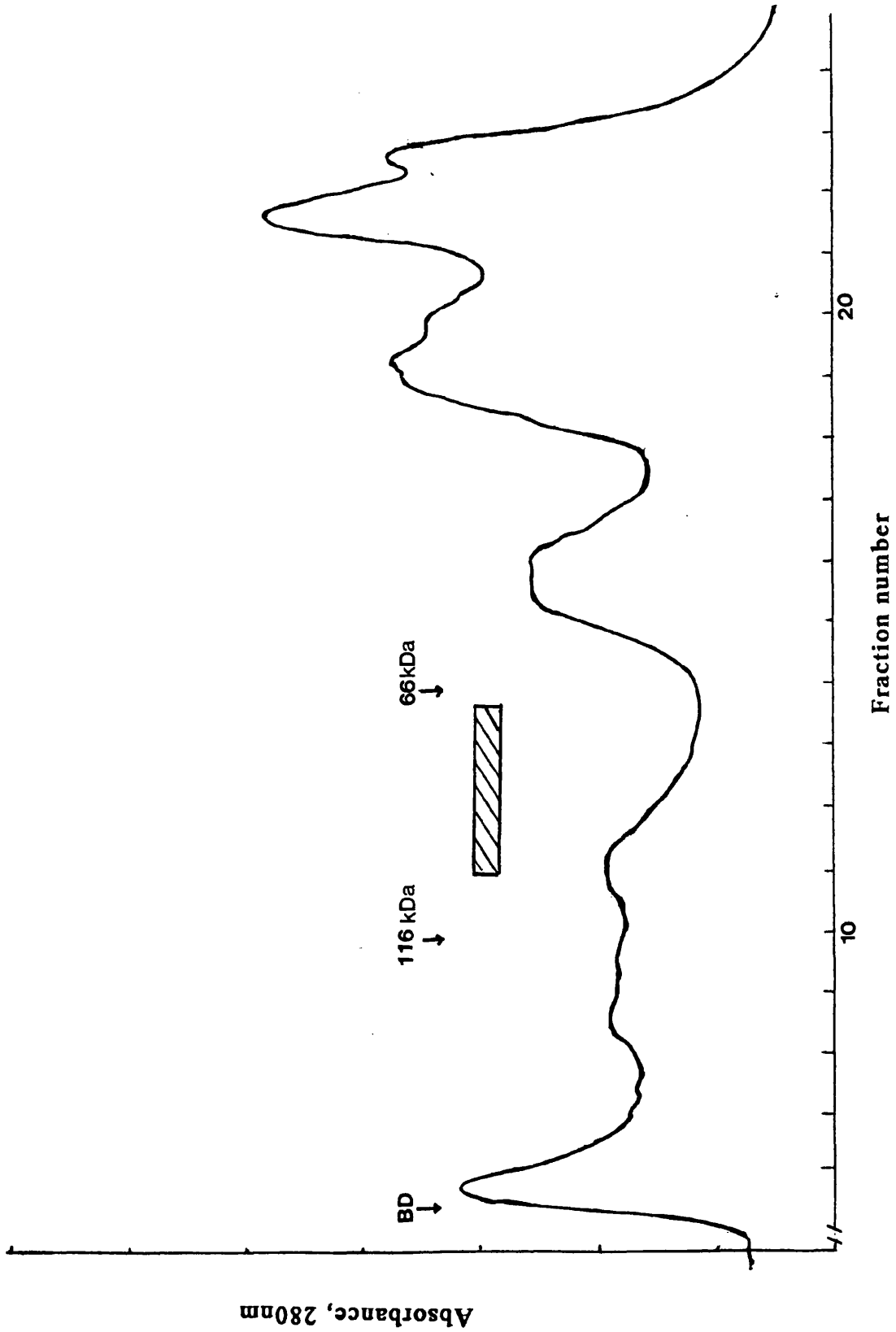
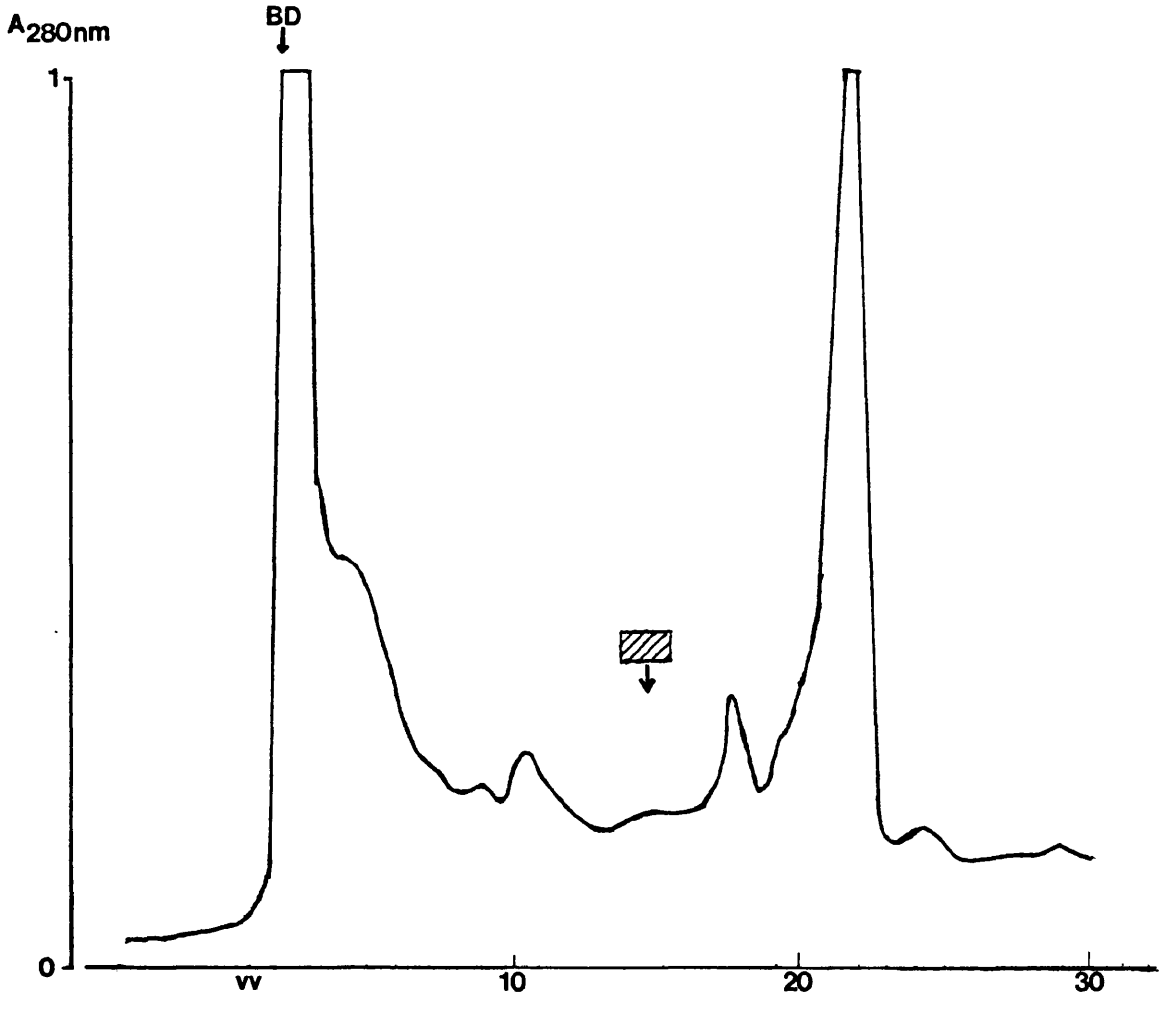


Figure 5.5 Elution profile of *P. furiosus* cell extract (a), and activity gel analysis (b) of fractions obtained from gel permeation chromatography using Sephacryl S300 under denaturing conditions.

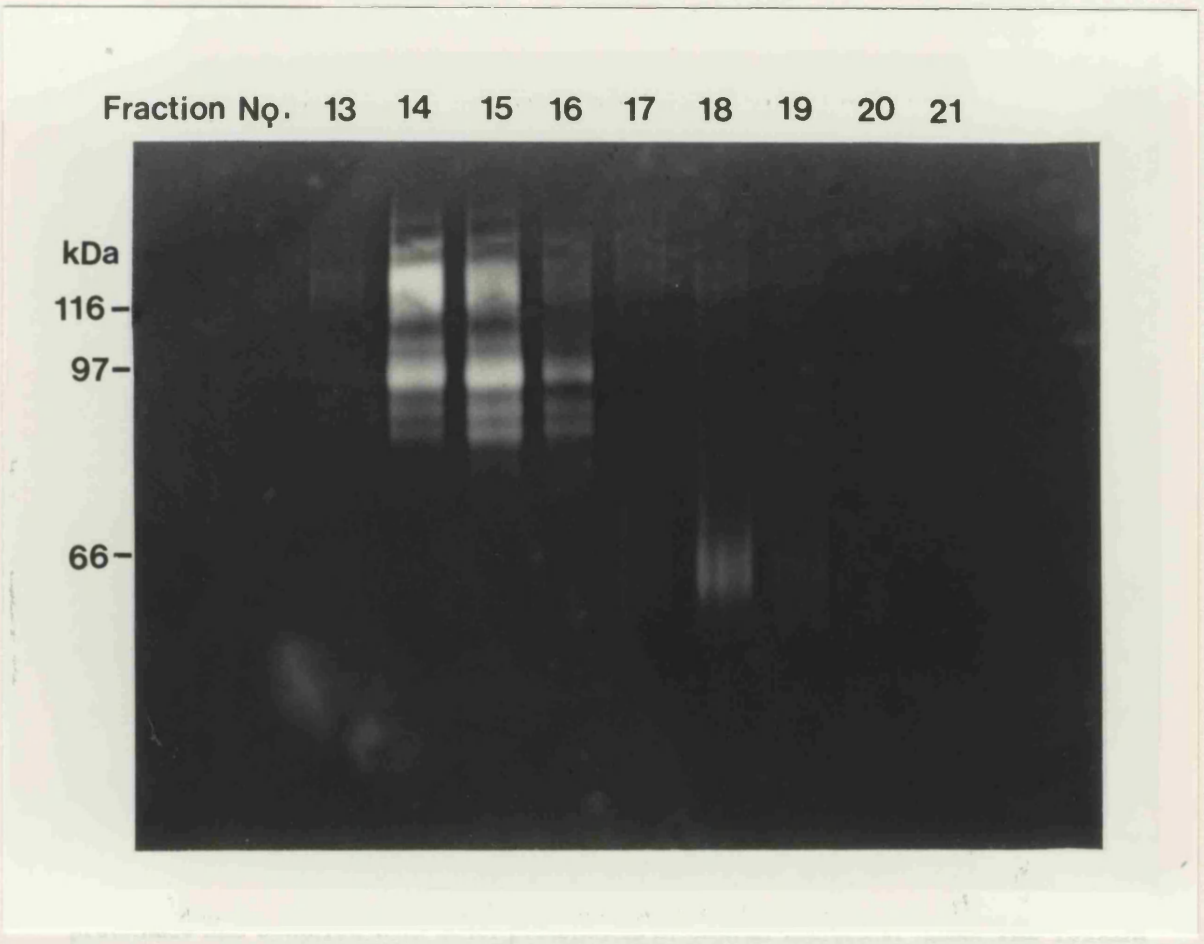
(a) Elution profile (reproduced from original trace)

Approximately 1.3mg/ml of protein was loaded to the column. Bar indicates position of proteinase activity (BD = Blue Dextran; vv = void volume).



(b) Activity gel

Fraction number of column chromatography is indicated along the top of photograph. Standard molecular weight markers are shown on the left.



molecular weights ranging from 66kDa to 135kDa is limited. In order to expand the separation range, low pressure gel permeation chromatography using Sephacryl S300 under denaturing conditions (fractionation range 10^3 to 4 x 10^5) was attempted (section 2.10.2).

The elution profile and gel analysis of the active fractions are shown in Fig. 5.5. Limited separation of the polypeptides was achieved with apparent dissociation of the 66kDa proteinase from the other high molecular weight active polypeptides (fractions 17-19). Due to the dilution factor involved during chromatography in Sephacryl S300, the substrate gel was incubated for approximately 4-5 hours before a result was obtained. Further attempts to isolate another proteinase using gel permeation under denaturing conditions were not performed due to the narrow molecular weight range of the proteinases to be separated.

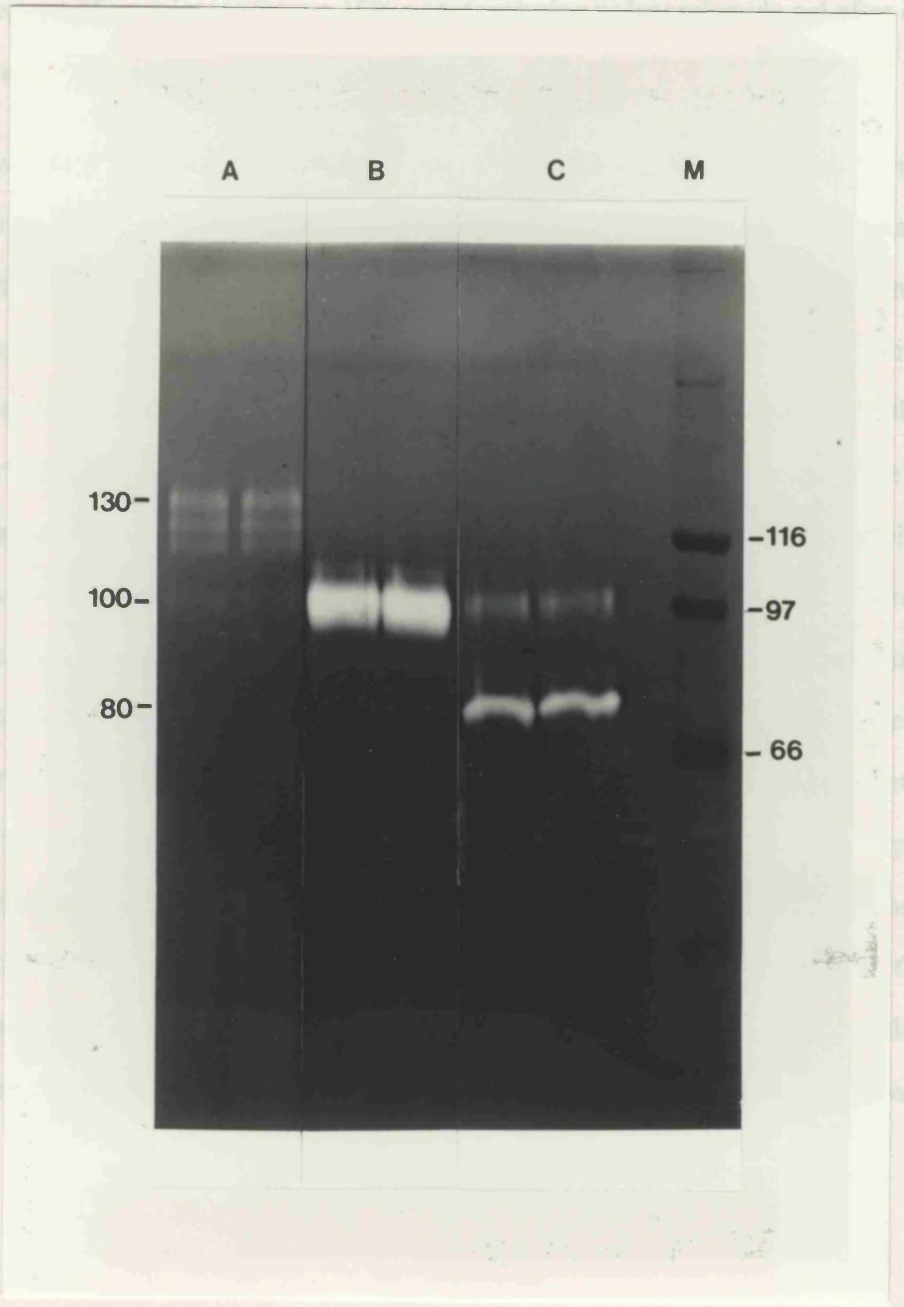
5.4 Electroelution of proteinases

Electroelution of the proteolytic bands from substrate SDS polyacrylamide gels was attempted as described in Section 2.15. Three randomly chosen activity bands (molecular masses approximately 80kDa, 102kDa and 130kDa) were removed from the polyacrylamide gel by excision, electroeluted and then analysed using gelatin SDS PAGE (Fig 5.6).

From the results obtained (Fig. 5.6), it appears that some contamination of the 130kDa proteinase had occurred with other proteinases of similar molecular mass. The 102kDa proteinase produced an intense resolved band, possibly including other similar molecular weight proteinases, such as the 99kDa and the 105kDa proteinases (Fig. 5.6, lane 2). These results suggest that the proteinases can be physically separated from each other under 'extreme' conditions. However, a surprising result was obtained after electroelution of the 80kDa proteinase. A higher molecular weight proteinase was observed at around 100kDa. The reason for the appearance of this band is not clear. It is possible (although unlikely) that the high molecular weight band may represent an aggregate of the 80kDa proteinase

Figure 5.6 Activity gel analysis of three proteolytic bands 80kDa, 102kDa and 130kDa after electroelution.

Lane A represents 130kDa proteinase; lane B, 102kDa proteinase; lane C, 80kDa proteinase. M denotes standard molecular weight markers.



with a non-proteolytic protein fragment.

5.5 Native (non-denaturing) gel electrophoresis

Non denatured samples of cell extract and culture supernatant were applied to a series of native polyacrylamide gels of different acrylamide concentrations as described in Section 2.14. After electrophoresis, the gels were tested for proteinase activity (Section 2.7.2.). A zymogram of proteinases from a 10% non-denaturing polyacrylamide gel after diffusing substrate into the gel is reproduced in Fig. 5.7.

Native PAGE indicated the presence of more than 7 proteinases in cell extracts and culture supernatants. Analysis of gels containing different acrylamide concentrations (5-10% acrylamide) demonstrated similar band patterning with proteinase mobility increasing throughout the gel with decreasing acrylamide concentration. The apparent molecular masses of these proteinases were calculated to be in the range of 95kDa to 280kDa. The observation of streaking in the lane containing the cell extract sample suggests the presence of high molecular mass proteinases. However, this streaking effect was not resolved even when the acrylamide concentration was reduced to 5%. This suggests that heterogeneous association of proteinases or other proteins may have occurred. The implication that *P. furiosus* proteinases exist as high molecular weight aggregates is supported by the observation of the series of high molecular weight bands, where some components such as the 95kDa native proteinase, appear to be more readily dissociated from the "complex" than others. Since it has been previously demonstrated that the smallest active monomer in substrate SDS PAGE has a relative molecular mass of approximately 66kDa, the above results suggest that interactions with other proteinases are frequent. However, there is no evidence to suggest that this form of aggregation occurs (or does not occur) *in vivo*.

Figure 5.7 Zymogram of *P. furiosus* proteinases from a 10% non-denaturing polyacrylamide gel after substrate diffusion.

Lanes represent : 1, native standard molecular weight markers; 2, cell extract (50µl); and 3, culture supernatant (75µl).

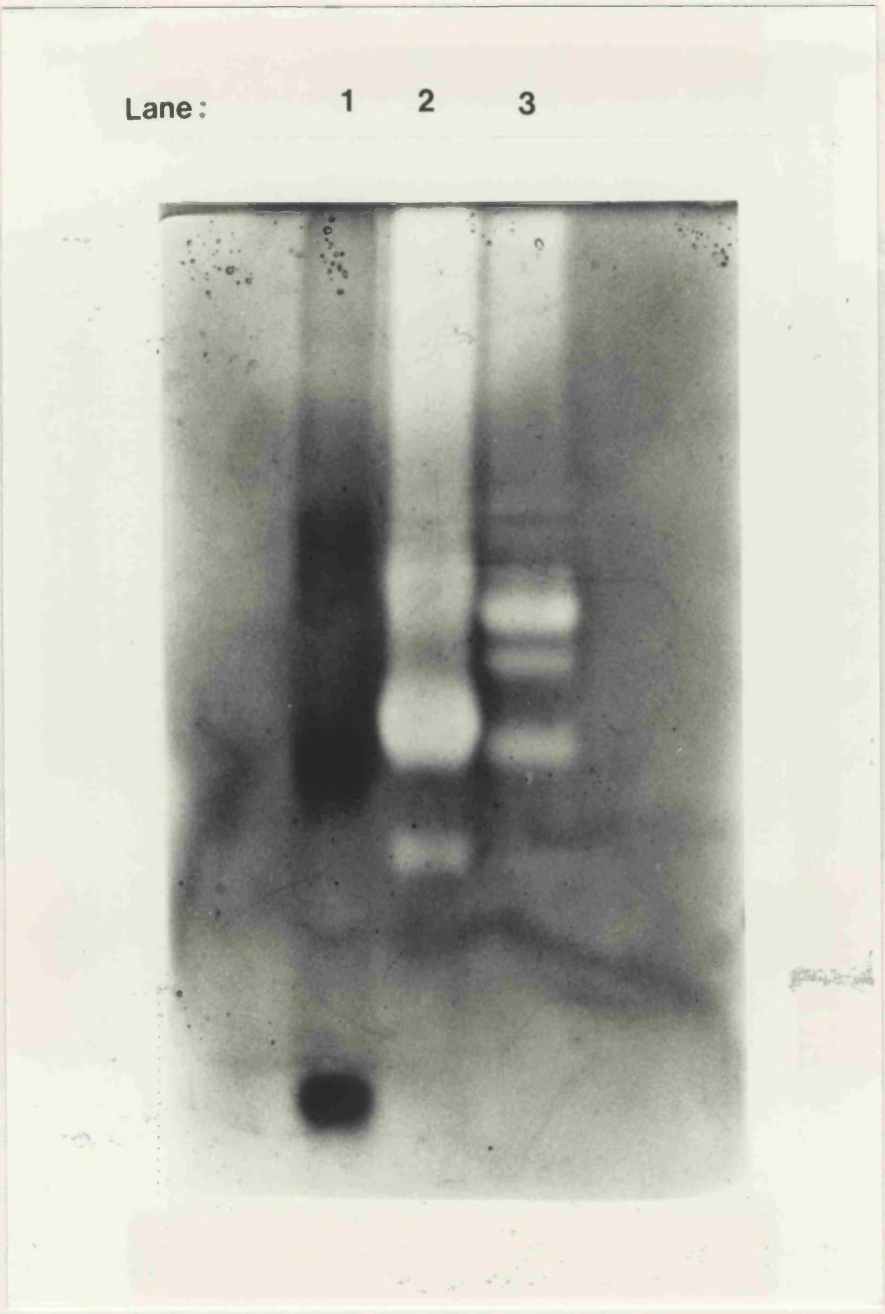
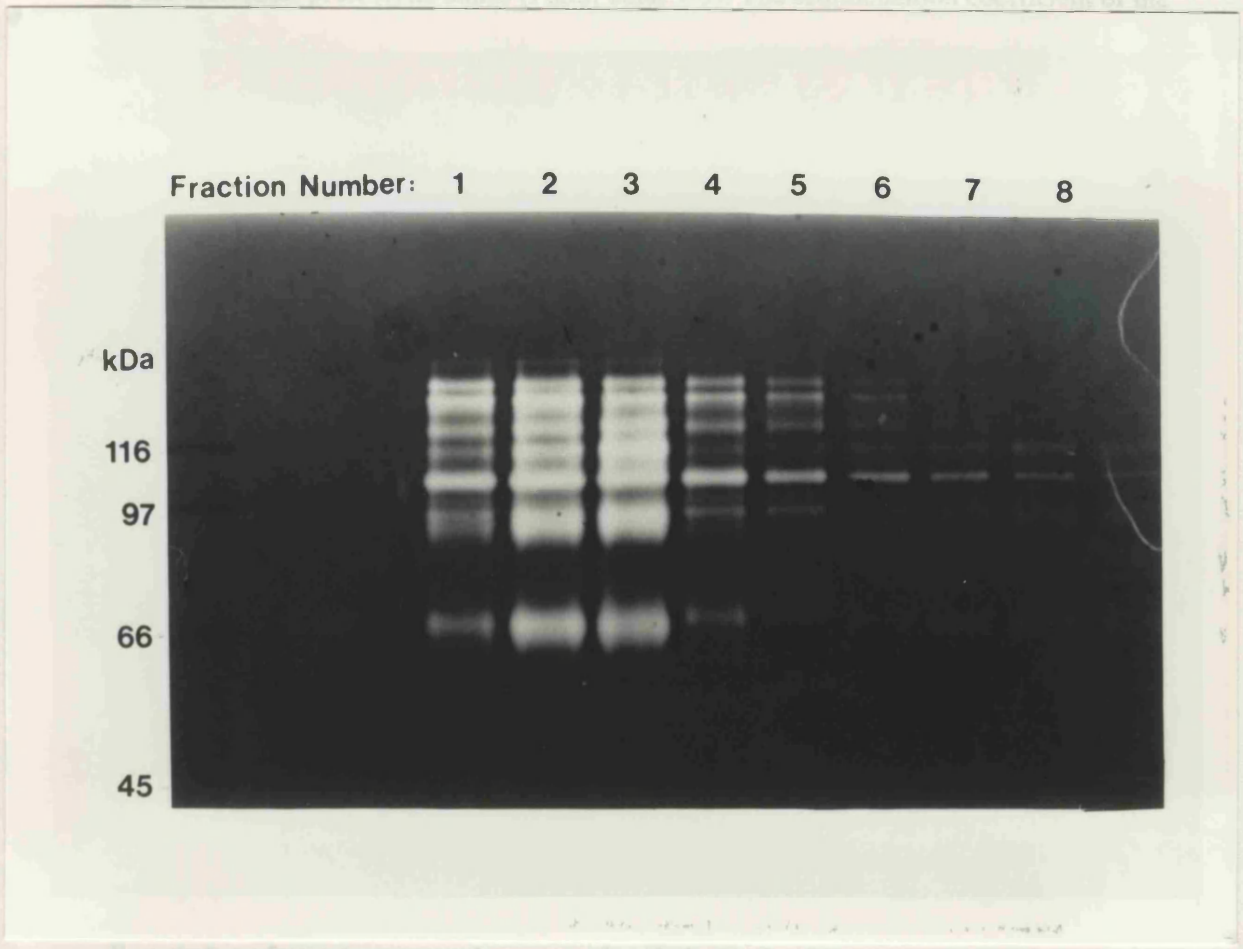


Figure 5.8 Sucrose density gradient fractions analysed using Gelatin SDS PAGE

Cell extract (500ml, 1.35mg/ml protein) was applied to a 6-30% sucrose gradient. Fractions were collected after centrifugation and electrophoresed with standard SDS markers on substrate PAGE. Lanes 1 to 8 represent the fraction number of the gradient.



5.6 Sucrose density centrifugation

Sucrose density gradient centrifugation of *P. furiosus* cell extracts and culture supernatants were performed as described in Section 2.9. Gel electrophoresis using substrate PAGE of the fractions collected from a 6-30% sucrose gradient is shown in Fig. 5.8.

Analysis of the fractions obtained from a 6-30% sucrose density gradient centrifugation revealed the multiple band pattern of proteinases in all active fractions, with no separation of the individual proteolytic bands (Fig.5, lanes 1-3). The sedimentation coefficient of the proteinase aggregate was calculated to be in the range of 9.5S-10S when compared to the standard molecular weight markers. This value corresponded to a molecular mass range of 185-200kDa. When considering the number of proteinases revealed by native PAGE, the calculated molecular weights strongly suggest that the proteinases exist as high molecular weight aggregates.

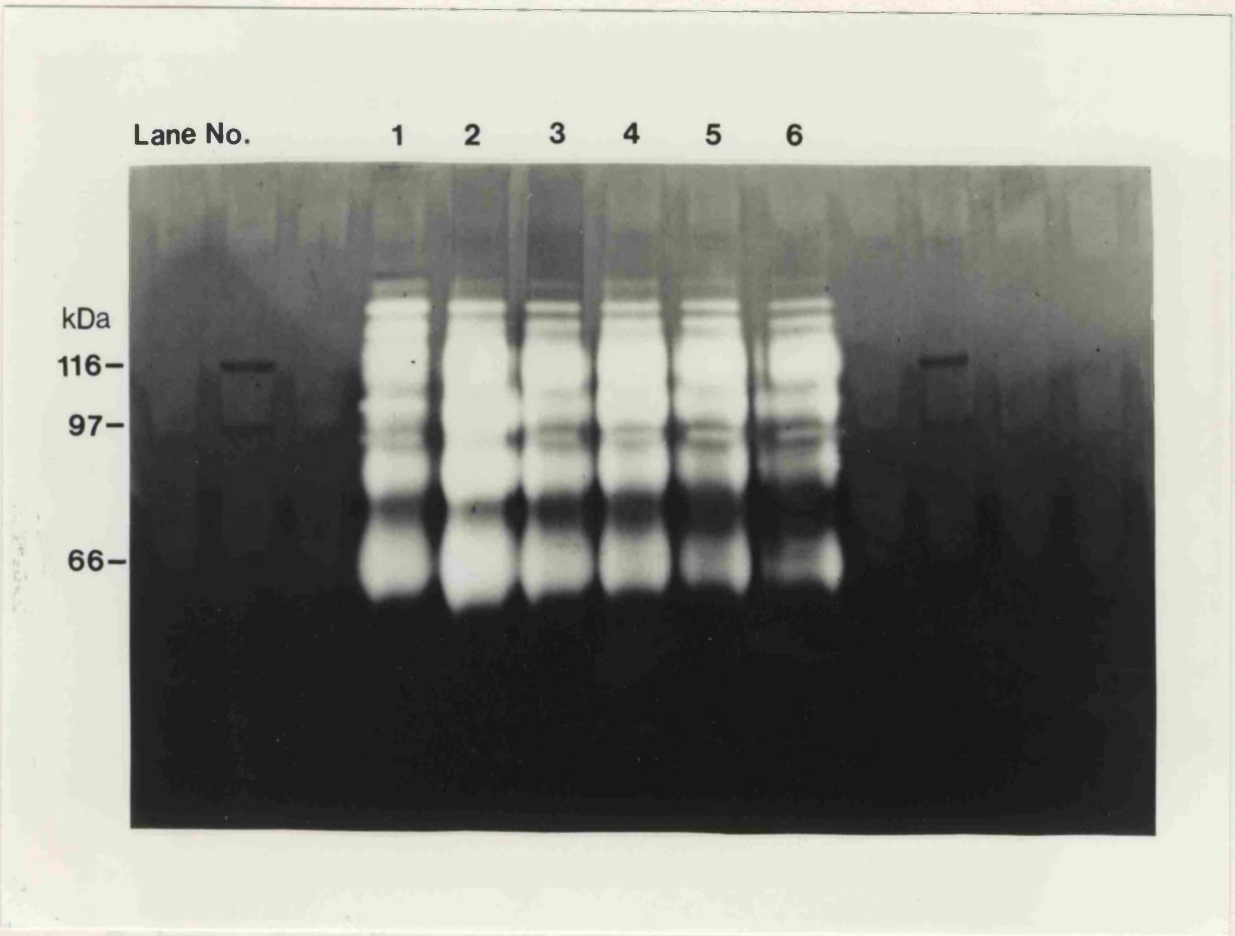
5.7 Hydrophobic interaction chromatography

The dissociation of oligomeric or multimeric structures into their individual subunits can be induced under conditions of extreme pH, temperature and ionic strength. To determine whether high ionic strength and/or high temperature could induce dissociation of the high molecular weight proteinase aggregate, hydrophobic interaction chromatography was performed on crude *P. furiosus* cell extracts (Section 2.12.1). Analysis of active fractions using gelatin SDS PAGE is shown in Fig. 5.9.

The observation of similar multiple band patterns in all active fractions suggests that dissociation of proteinase complexes into their individual polypeptides was not achieved in the presence of high concentrations of ammonium sulphate (50% saturation). This phenomenon was also observed when hydrophobic interaction chromatography was performed at 80°C in high salt. These observations, in addition to the data obtained from a variety of analytical techniques, further support the existence of high molecular weight aggregates in *P. furiosus* cell extracts and culture supernatants.

Figure 5.9 Gelatin SDS PAGE of active fractions from hydrophobic interaction chromatography using hydrogen bond.

Lane numbers correspond to fractions collected at the end of the chromatography, where activity was eluted with a low salt buffer (see text). Standard SDS molecular weight markers are denoted on the left .



RESULTS AND DISCUSSION

CHAPTER 6

PURIFICATION OF THE PROTEINASE COMPLEX

6.1 Aims

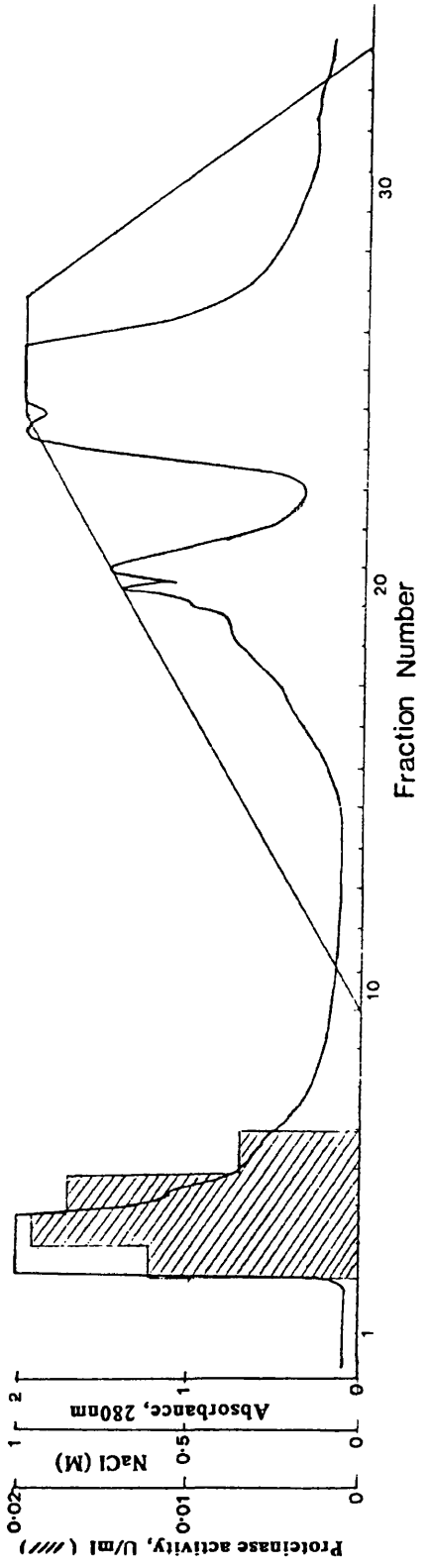
The purpose of this study was to attempt to purify the aggregate of proteinases as a whole from *Pyrococcus* cell extracts. The reasons for purifying the proteinase 'complex' (where the term 'complex' in this study implies the aggregate of proteinases) were as follows: Attempts to separate the proteinases into their individual polypeptides were not successful. Although dissociation of the 66kDa proteinase from the other high molecular mass active polypeptides was achieved using low pressure Sephacryl S300 chromatography under denaturing conditions, further separation of these proteinases was not successful using any of the conventional chromatographic methods or the commercially available analytical columns used. In addition, it was not appropriate to continue work on the 66kDa proteinase since the purification and characterisation of this protein had already been reported by Blumentals *et al.* (1990).

Since it was shown that the proteinase aggregate was observed in both intracellular and extracellular extracts, it was decided to utilise cell extracts for the purification of the complex. Cell pastes from continuous culturing of *Pyrococcus* were used. This eliminated the problem of processing large amounts of high salt culture medium.

6.2 Ion-exchange chromatography

Crude preparations of *Pyrococcus* cell extract (prepared as described in section 2.6.1) were applied to small volumes of Q-Sepharose pre-equilibrated with Na_2HPO_4-NaH_2PO_4 buffer of varying ionic strength (10-50mM) and pH (6-8). The column was washed with the appropriate buffer before eluting bound protein with a 0-1M NaCl gradient. Fractions (1ml) were collected, monitored for protein and assayed for proteinase activity using azocasein as the proteolytic substrate (section 2.7.1).

Figure 6.1 Elution profile of *P. furiosus* cell extract after Q-Sepharose.



The analysis of fractions using the azocasein assay demonstrated that the proteinase complex adsorbed to Q-Sepharose at pH 7-8 in the presence of 10 to 40mM sodium phosphate buffer. A large proportion of the contaminating protein was also bound and eluted in the fractions containing the proteinase activity. However, when crude cell extract was applied to a column equilibrated with 50mM sodium phosphate buffer at pH 7.5, a large proportion of activity was not adsorbed (approximately 80%) whereas a major proportion of contaminating protein had adsorbed. Fig. 6.1 illustrates the elution profile obtained after Q Sepharose chromatography of a *Pyrococcus* cell extract preparation in 50mM sodium phosphate buffer, pH 7.5 (20ml loading, 8ml fractions collected). Analyses of fractions showed that approximately 75-80% of the proteinase activity had passed through the column without binding. The most probable explanation is that the ionic strength of the loading buffer was too high to allow binding of the proteinase complex (the ionic strength of the cell extract had not been taken into account). However, since a large proportion of the contaminating non-enzyme protein had bound (approximately 68%). It was decided to use this anion-exchanger procedure as part of the purification sequence.

Table 6.1. Recovery of enzyme activity after batch trials on Q-Sepharose.

Concentration of buffer (mM)	Non-bound protein (mg)	Activity (U) Non-bound	Bound	% Yield (Non-bound)
(Cell extract)	(37)	(5.7)	-	100
10	2	0.7	4.8	12
20	3	0.9	4.6	16
30	5	1.6	3.3	28
40	9	2.5	2.6	44
50	12	4.4	1.0	77

6.3 Purification using ammonium sulphate fractionation

Precipitation by the addition of neutral salts such as ammonium sulphate is probably the most commonly used method for fractionating proteins by precipitation. This involves a salting-out step where the increase in ionic strength on addition of salt to a protein sample

exposes the hydrophobic patches of the protein. Intermolecular interactions of protein molecules with exposed hydrophobic patches lead to aggregation and precipitation. Thus, proteins with larger or more exposed hydrophobic patches will aggregate and precipitate before those containing fewer hydrophobic areas, resulting in fractionation.

Preliminary studies were performed using ammonium sulphate fractionation as described in section 2.16. Table 6.2 summarises the results obtained after ammonium sulphate fractionation of proteinase samples partially purified by ion-exchange.

Table 6.2 Precipitation of the proteinase complex by ammonium sulphate

$[(NH_4)_2SO_4]$	% of initial proteinase activity	
% saturation	Precipitate	Supernatant
40	8	115
60	23	34
90	13	9

From the activity data, it was evident that a large proportion of activity (about 80%) was lost when the sample was saturated to 90% with ammonium sulphate. The increased activity value at 40% saturation (115%) suggests that either enzyme activation had occurred or that the proteolytic substrate was more susceptible to hydrolysis due to the effects of a combination of high salt and high temperature (during assay incubation). It was concluded that ammonium sulphate fractionation was not a suitable method for concentrating the active pool of proteinases.

Where enzyme activity is retained after addition of relatively high levels of ammonium sulphate (as in Table 6.2 above), the resulting high ionic strength solution is suitable for subsequent hydrophobic interaction chromatography. One such chromatography system tested was Whatman Hydrogen-Bond (HB) cellulose. The gel performs in a manner analogous to more commonly used hydrophobic interaction gels (Phenyl-Sepharose and

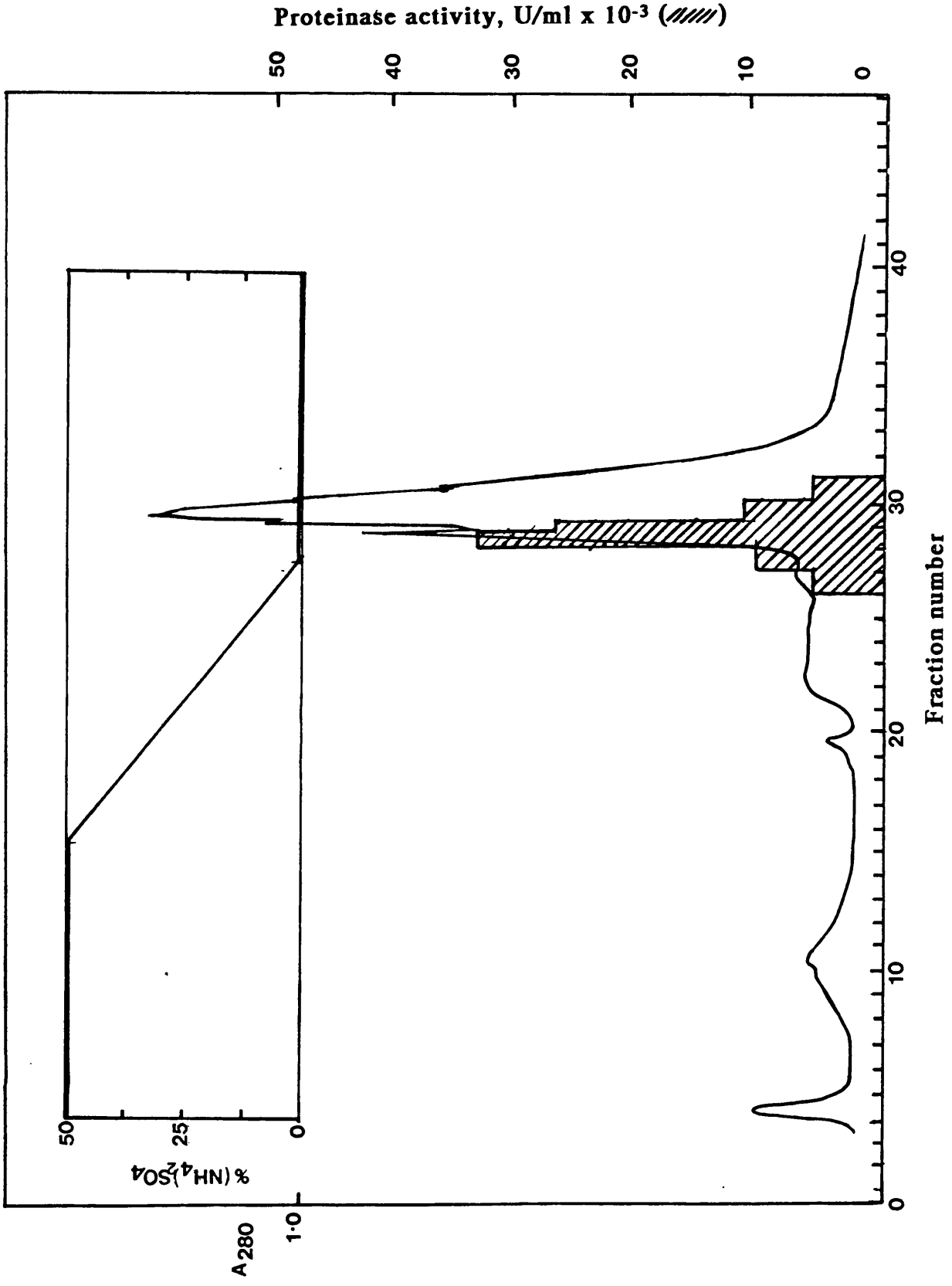
Octyl-Sepharose) but is thought to bind protein via weaker hydrophobic interactions.

In initial adsorption trials, partially pure proteinase samples were saturated with ammonium salt to give final concentrations of 20%, 40%, 50% and 60% respectively. These were applied to 2ml columns of Whatman HB cellulose pre-equilibrated in the appropriate high salt , sodium phosphate buffer, pH 7.0 before eluting with a low salt buffer (generally dilute buffer). Fractions were collected, monitored for protein and assayed using the azocasein assay.

Table 6.3 summarises the results obtained after batch trials using Hydrogen Bond chromatography. Maximum binding of proteinase activity was achieved when the hydrogen bond matrix was saturated with 50% ammonium sulphate with the eluted protein showing the highest specific activity of 0.45U/mg. Partial binding of the proteinase complex was observed at saturation values lower than 50%, with very little or no adsorption of the complex at 20% (Fig. 6.2). On the basis of these results, partially pure proteinase was applied to a column containing 30ml of HB cellulose equilibrated with 50mM sodium phosphate buffer, pH 7, and 380g/l ammonium sulphate. The sample was adjusted with the same saturation of salt before application to the column. The matrix was washed in 50% saturated solution of ammonium sulphate before a gradient of decreasing ionic strength was applied to elute adsorbed protein. Fractions (6ml) were monitored for protein (A_{280nm}) and assayed for proteinase activity (section 2.7.1). An elution profile obtained after Hydrogen Bond chromatography is reproduced in Fig. 6.2.

Analysis of fractions using azocasein as the proteolytic substrate, indicated that elution of the proteinase complex had occurred towards the end of the gradient, corresponding to a very low concentration of ammonium sulphate (effectively 0M). This result suggests that the complex is a moderately hydrophobic macromolecule. A quantitative yield of enzyme with an increase in specific activity from 0.37 to 0.81U/mg was obtained after elution. This increase in yield (from 77% after Q-Sepharose to 100% with HB cellulose) suggests that some activation of the enzyme complex may have occurred or that the presence of a

Figure 6.2 Elution profile of the proteinase complex after hydrophobic interaction chromatography using hydrogen bond.



protein inhibitor interacting with the complex was removed during chromatography.

Table 6.3 Specific activity of proteinase complex after batch trials on hydrogen bond chromatography with ammonium sulphate.

$[(NH_4)_2SO_4]$	Protein (mg/ml)		Activity (U/ml x10^{-2})		Specific Act. (U/mg)	
% saturation	A	B	A	B	A	B
None	0.32		9.3		0.29	
20	0.232	0.012	9.15	0.19	0.394	0.158
40	0.204	0.115	2.8	4.7	0.137	0.408
50	0.115	0.164	0.46	7.4	0.04	0.450
60	0.016	0.232	0.3	8.8	0.088	0.376

A: pooled wash fractions

B: pooled eluted fractions

Other hydrophobic matrices were also used in attempts to further purify the proteinase complex. Both Phenyl- and Octyl-Sepharose (section 2.12.2) were found to irreversibly bind the proteinase complex at lower ammonium sulphate saturations (30-40%) with very little or no separation of contaminating protein. In contrast to the data observed with hydrogen bond chromatography, these results suggest that the proteinase complex has a very hydrophobic character.

6.4 Further purification of the proteinase complex.

Other chromatographic techniques techniques were employed to further purify the proteinase complex.

6.4.1 Affinity chromatography

Affinity chromatography, using of CBZ-Phenylalanine-TETA-Sepharose, was used in attempts to separate partially pure samples of proteinase from contaminating protein (section 2.11.4). However, separation of the complex from contaminating protein was not achieved after an acetic acid elution (specific activity was 0.09U/mg, yield appproximately

36%) since the column would bind non-specific proteins in addition to the proteinase complex.

6.4.2 Hydroxylapatite adsorption chromatography

Chromatography was performed as described in Section 2.11.3. An elution profile demonstrating the distribution of proteinase activity and protein is illustrated in Fig. 6.3. On analysis of fractions using azocasein as the proteolytic substrate, it was observed that elution of the proteinase complex occurred at 0.35M sodium phosphate. However, activity was also observed in fractions up to 2M sodium phosphate. Further analysis of these fractions using gelatin PAGE demonstrated that the binding of the proteinase complex occurred as seen previously with Mono Q (section 5.2), where proteinase multiplicity was observed in fractions from 0.35M NaCl to 1M NaCl. It was therefore considered not to be a suitable method for purification.

6.4.3 Q-Sepharose

As a final attempt to further purify the complex of proteinases, ion-exchange chromatography with Q-Sepharose using a lower ionic strength buffer was employed. Partially pure samples of proteinase obtained from hydrogen bond chromatography were dialysed against 4 litres of 10mM sodium phosphate buffer, pH 8 for 6h at room temperature. The dialysate was then applied to a Q Sepharose column pre-equilibrated with the above buffer. The column was washed with sodium phosphate buffer before applying a 0-1M NaCl gradient. Eluted fractions were monitored for protein and assayed for proteinase activity using azocasein as the proteolytic substrate. A typical elution profile demonstrating the distribution of proteinase activity and protein is reproduced in Fig. 6.4.

The distribution of activity indicated that the proteinase complex bound to Q Sepharose, and was eluted as a complex at approximately 0.8M-1M NaCl. Purification of the proteinase complex (but not the individual components) was achieved with a specific activity increase of 6-fold. The yield was calculated to be 35-40% of the starting activity. This loss in activity was not due to non-adsorption (since activity was not found in the

Figure 6.3 Elution profile demonstrating the distribution of proteinase activity after hydroxylapatite adsorption chromatography.

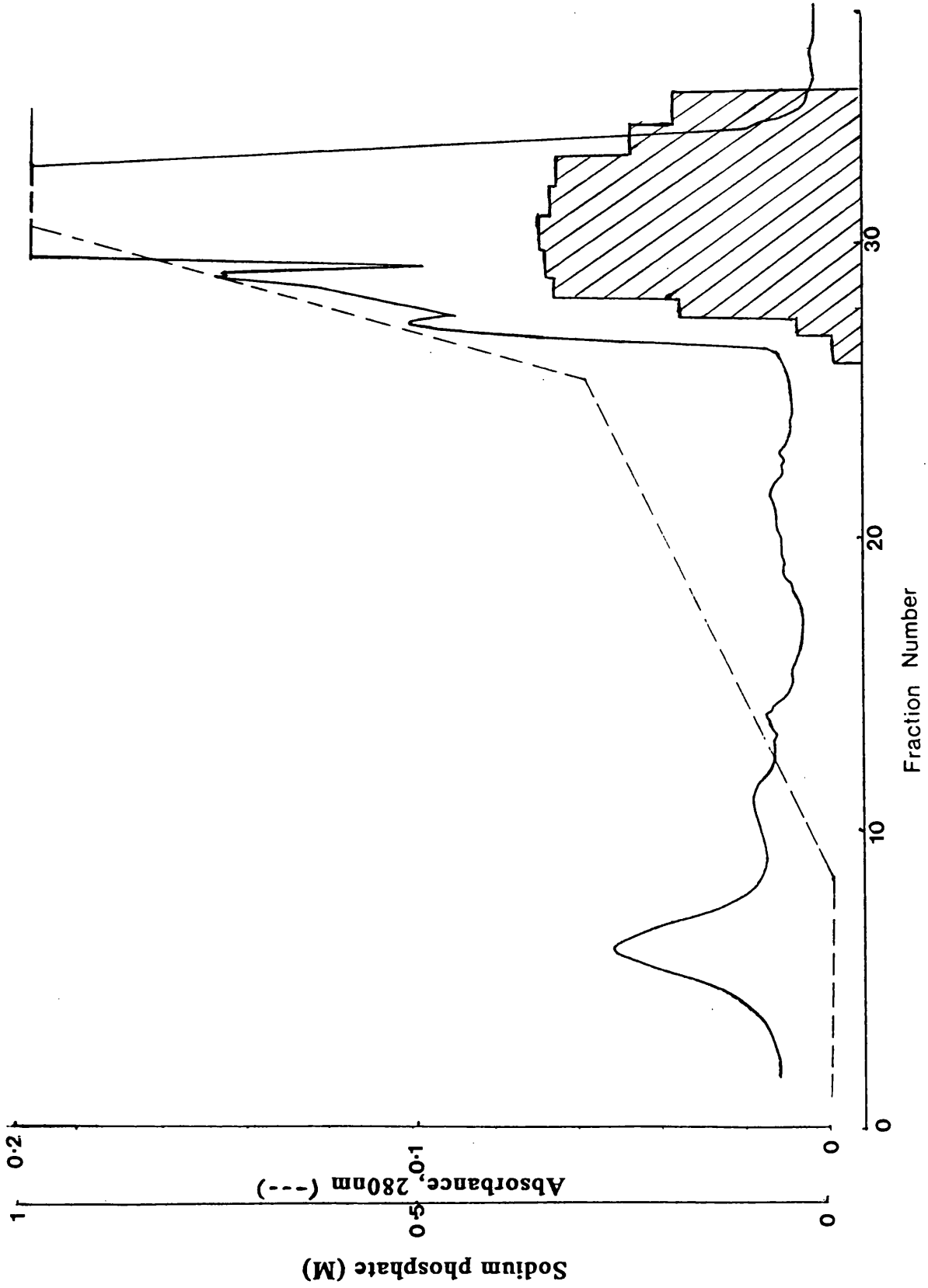
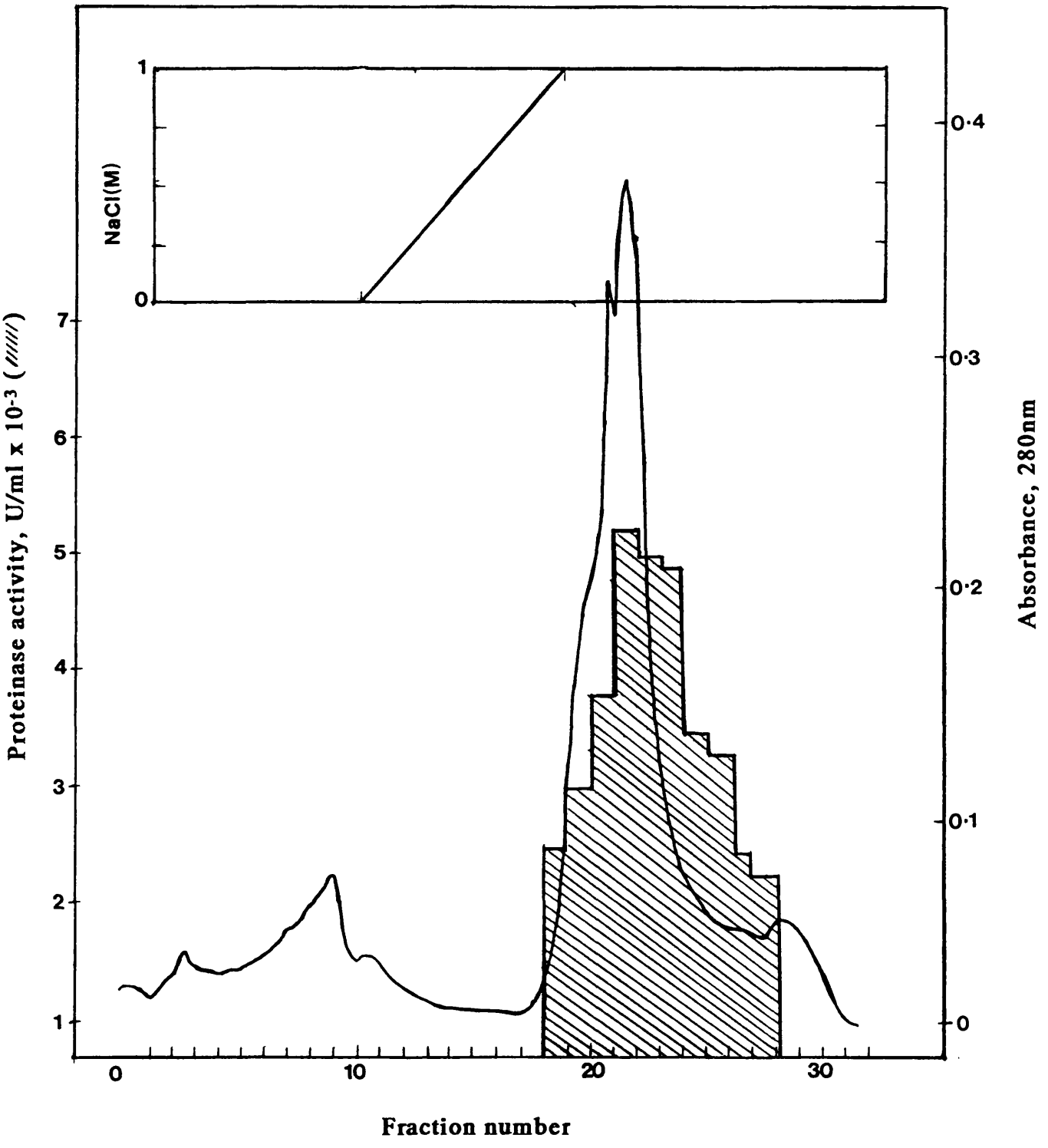


Figure 6.4 Elution profile of the proteinase complex after Q Sepharose chromatography.

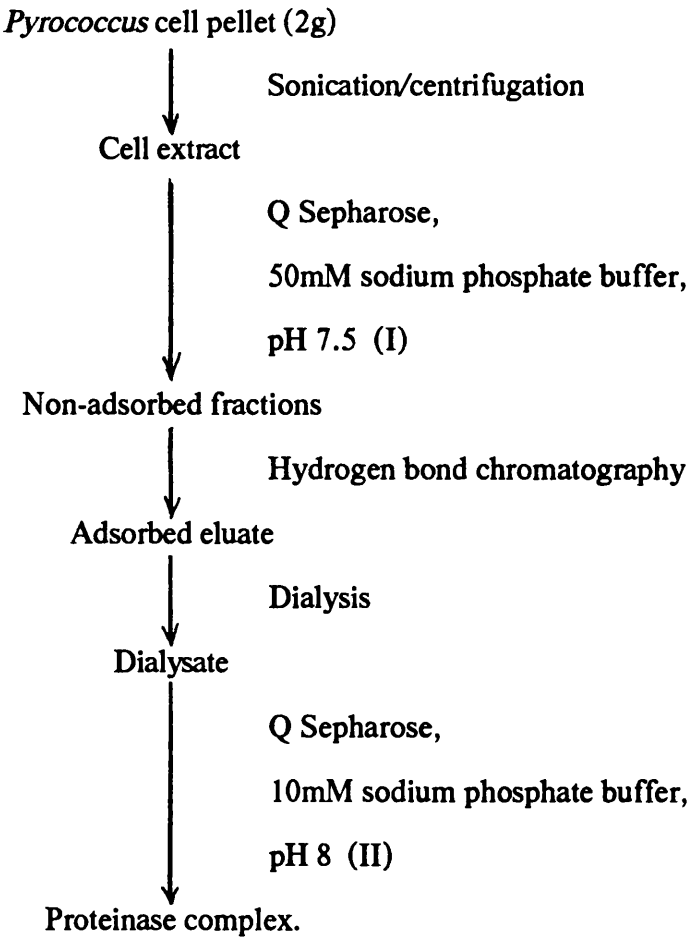


initial wash fractions) but might be attributed to the ionic strength of the buffer (see Section 7). It has been shown during the development of the purification protocol that the use of high ionic strength sodium phosphate buffer (which prevents adsorption of proteinases to the Q Sepharose matrix) does not result in the loss of activity (section 6.2).

6.5 Standardised Purification Protocol

The following standardised sequence was used routinely for the purification of the proteinase complex. The design of this protocol was based on the chromatography procedures outlined above.

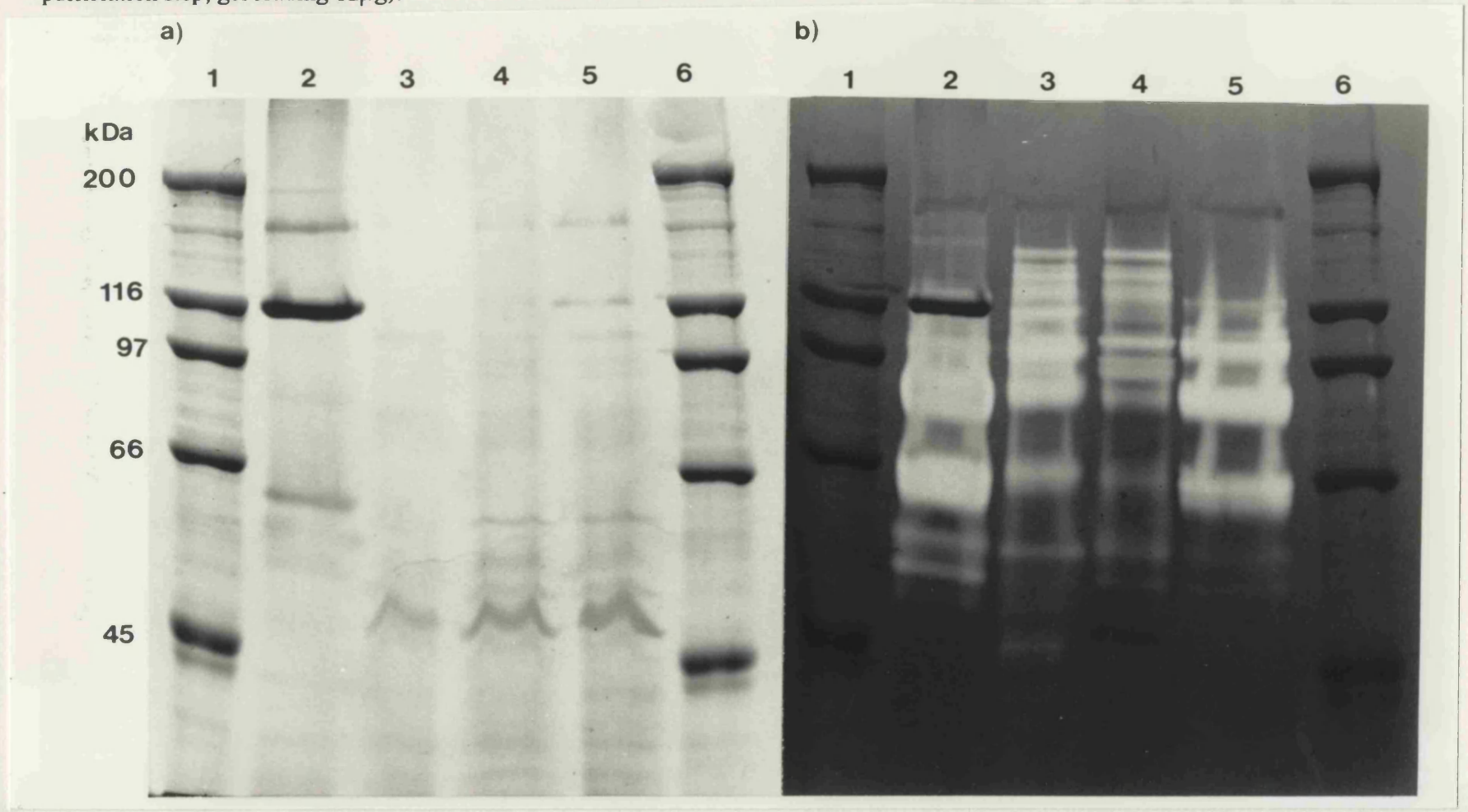
Figure 6.5 Steps in the purification of *Pyrococcus* proteinase complex.



Quantitative data derived from the purification sequence is presented in Table 6.4. The

Figure 6.6 SDS polyacrylamide gel electrophoresis (a), and activity gel analysis (b) of the proteinase complex.

Samples of proteinase were obtained at each step of the purification protocol and analysed using SDS PAGE and gelatin-containing SDS PAGE. Lanes 1 and 6 represent standard SDS markers; lane 2, crude *P. furiosus* cell extract (1.8mg/ml, gel loading 70μg); lane 3, Q Sepharose (10mM, final purification step, gel loading 50μg); lane 4, hydrogen bond chromatography (40μg); and lane 5, Q Sepharose (initial purification step, gel loading 12μg).



demonstration of purity by SDS PAGE and gelatin PAGE is shown in Fig. 6.6a and b.

Approximately 35% of the activity present in cell extracts was recovered, with an increase in specific activity of 11-fold. The greatest loss in activity occurred in the final stages of purification, possibly during dialysis. It might be speculated that the low specific activity value could be attributed to interactions with other proteinaceous material (i.e as a form of lipo- or glycoproteins) which may be co-chromatographing during with purification. Despite the low values obtained and the difficulties encountered in the separation of the complex from contaminating protein, the above sequence was evaluated to be the best possible approach for purification of the complex.

Table 6.4 Purification of the proteinase complex

Purification step	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	20	0.28	5.7	1.85	37	0.15	100	1
Q Sepharose I	40	0.11	4.4	0.30	12	0.37	77	2.4
Hydrogen Bond	36	0.16	5.8	0.20	7.2	0.81	101	5.3
Q Sepharose II	30	0.07	2.0	0.04	1.2	1.7	35	11

RESULTS AND DISCUSSION

CHAPTER 7

CHARACTERISATION OF THE PROTEINASE COMPLEX

7.1 Effect of Proteinase Inhibitors

The reactions of proteinases with well-defined classes of inhibitors generally form the basis for initial characterisation (section 1.1.3). For the classification of the *Pyrococcus* proteinase complex, its activity was measured in the presence of different proteinase inhibitors outlined in Table 7.1. Assays were performed as described in Section 2.17.

The proteinase complex was almost completely inhibited by the serine-type proteinase inhibitor phenyl methyl sulphonyl fluoride (PMSF) suggesting that the activities of the complex of proteinases predominantly belong to the class of serine enzymes. No inhibition was detected in the presence of the chymotrypsin-type, metal ion and cysteine-type proteinase inhibitors (Table 7.1). In fact, a degree of activation was observed with all of the metal-ion chelators at 10mM, suggesting that metal ions were not required for enzyme activity or stability at 95°C. Slight inhibition (about 6%) was observed with the aspartate proteinase inhibitor pepstatin A.

The data obtained from these inhibiton studies support the observations made by Blumentals *et al* (1990) and Eggen *et al* (1990) who both classify *Pyrococcus* proteinases as serine-type enzymes. This conclusion is also supported by inhibition studies of individual proteolytically active bands observed in gelatin PAGE, where all but one of the 13 proteinase bands from *Pyrococcus* showed almost complete inhibition by 10mM PMSF (Fig. 7.1). The PMSF-insensitive band (130kDa) was eventually inhibited using a much higher concentration of PMSF (100mM). Complete inhibition of the PMSF-insensitive band by the metal ion chelator EDTA (10mM), which was added to the assay buffer after the Triton X-100 wash, was also observed (Fig. 7.2). This result suggests a possible requirement of metal ions for activity. It is therefore likely that the 6% remaining activity with azocasein (after incubation with 10mM PMSF) may be due to the PMSF-insensitive

Table 7.1. Inhibition of proteinase-complex activity.

Inhibitor (class)	Inhibitor	Concentration of Inhibitor	% Remaining Activity + % SEM
	None	-	100
Serine Proteinase Inhibitors	PMSF[a]	10mM	6 + 0.5
		5mM	36 + 4.5
		1mM	40 + 3.7
Chymotrypsin Inhibitors	Chymostatin	0.1mg/ml	100 + 0.5
	TPCK[b]	5mM	100 + 4.8
Metal ion Inhibitors	EDTA	10mM	119 + 2.3
	EGTA	10mM	118 + 1.8
	o-Phenanthroline	10mM	110 + 1.8
Heavy metal ions	$CuCl_2$	10mM	100 + 4.2
		5mM	135 + 3.0
Cysteine Proteinase Inhibitors	Iodoacetic acid	10mM	100 + 0.5
		5mM	122 + 0.8
	p-Chloromercuric benzoic acid	10mM	100 + 3.2
Aspartate Proteinase Inhibitors	Pepstatin A	0.1mg/ml	94 + 3.5

[a]PMSF: phenylmethanesulphonyl fluoride; [b]TPCK: tosyl-L-phenylalanyl-chloromethyl ketone.

Figure 7.1 Effect of proteinase inhibitors on proteinase-complex activity.

Proteinase samples were incubated with specific proteinase inhibitors before analysis using gelatin SDS PAGE. Lane 1 represents standard SDS markers; lane 2, *P. furiosus* proteinase sample (undiluted, 15μg); lane 3, proteinase sample (diluted 1 in 5); lane 4, proteinase (1 in 5 dilution) with 10mM PMSF; lane 5, with 100mM PMSF; lane 6, with 10mM iodoacetamide.

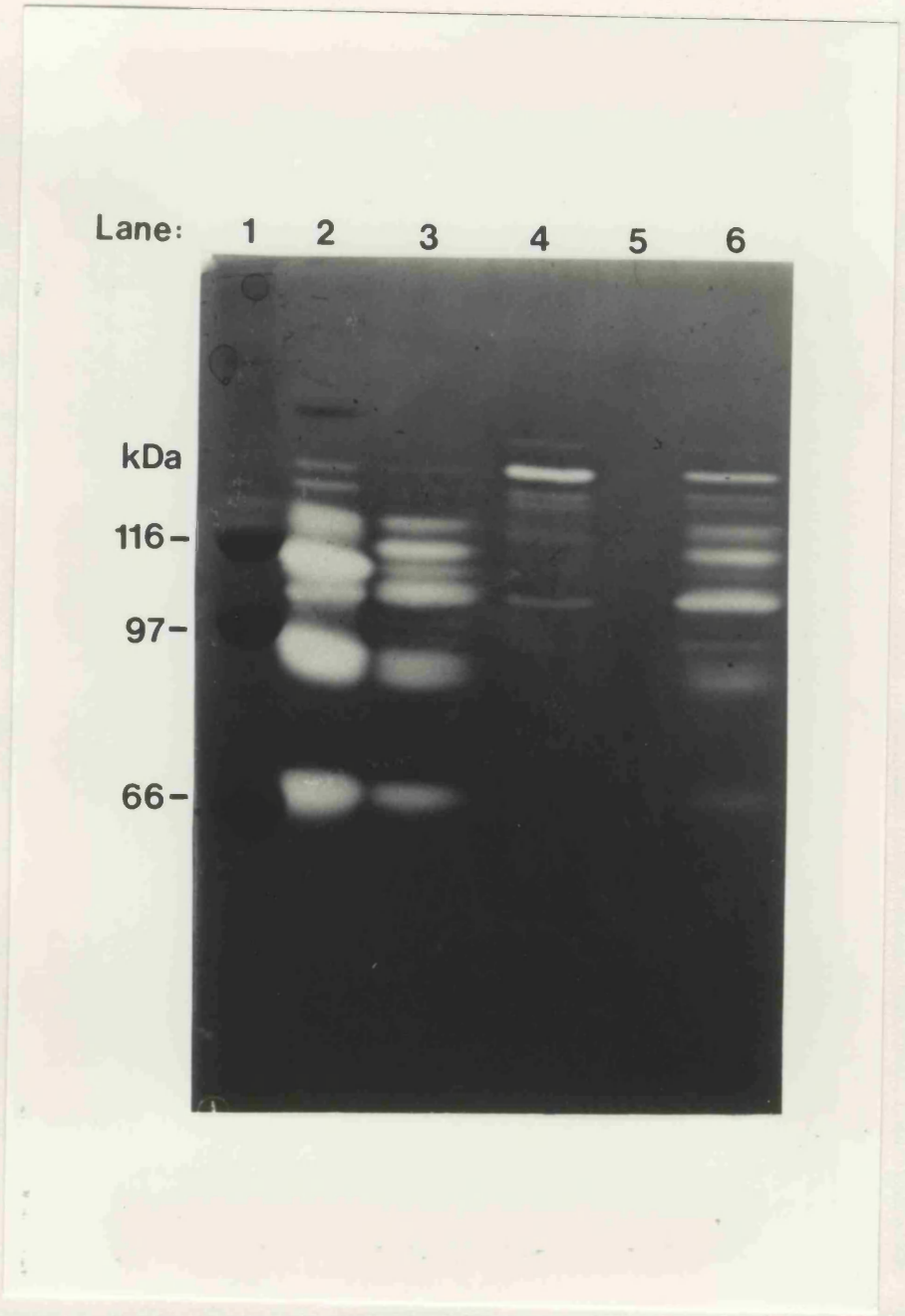
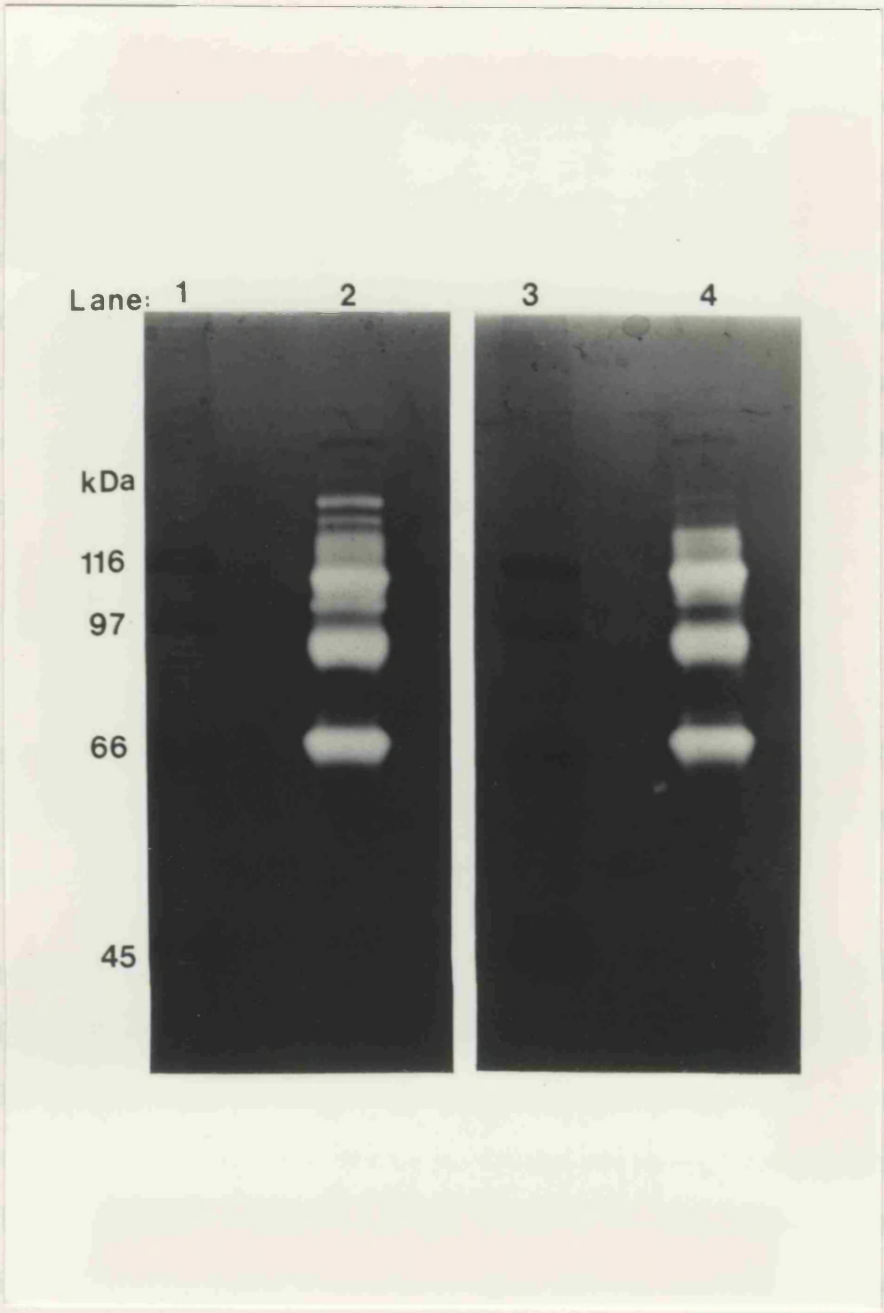


Figure 7.2 Inhibition of the PMSF-insensitive band by EDTA.

Proteinase samples (approximately 10μg) were applied to gelatin SDS polyacrylamide gels. Protein bands in the gels were renatured *in situ* and incubated in the presence and absence of 10mM EDTA in the assay buffer at 90°C for 1h. Lanes 1 and 3 represent standard SDS markers, lanes 2 and 4, proteinase sample in the absence and presence of EDTA, respectively.



band observed in gelatin SDS gels.

7.2 Influence of pH on activity

Enzyme activity was determined at different pH values as described in Section 2.19. The proteinase complex was shown to be active over the pH range of 6.5 to 8.5 with the optimum pH at 8 for the substrate azocasein (Fig. 7.3). When pH values were greater than 8.5, a sudden decrease in the rate of hydrolysis was observed. This may be possibly due to pH-induced denaturation of the "complex", or a change in ionisation of critical active site residues.

The broad pH range of activity exhibited by the *Pyrococcus* proteinase complex is in agreement with the values obtained by Blumentals *et al* (1990) and Eggen *et al* (1990). Blumentals and coworkers reported proteolytic activity over a wide pH range from 5.8 to 8.5 with a pH optimum of 7.0. Eggen *et al* (1990) reported at least 50% maximal activity between pH 6.5 and 10.5 using casein as the assay substrate.

7.3 Substrate specificity

7.3.1 Protein hydrolysis

Solutions or suspensions of proteins and dye-linked proteins (0.5%w/v) in 0.1M HEPES buffer, pH 8.3 (20°C) were assayed as described in Section 2.20.1. Results of protein hydrolysis, monitored at specified wavelengths, are presented in Table 7.2.

Of the chromogenic substrates tested, the enzyme complex showed little activity towards azocoll compared to azocasein. In the case of the native protein substrates, albumin and the insoluble protein fibrin demonstrated the highest degree of susceptibility to proteolysis relative to casein. The other insoluble proteins, collagen and elastin, were not hydrolysed at 95°C but haemoglobin (which precipitated rapidly at the assay temperature) showed significant hydrolysis by the proteinase complex. Activity towards gelatin was weak compared to casein, which is in contrast to the observations recorded with substrate gels (section 4.-). A possible explanation is that the dye-binding capacity of the two substrates

Figure 7.3 Influence of pH on proteinase activity

(a) In a universal buffer mixture
(b) In separate buffers.

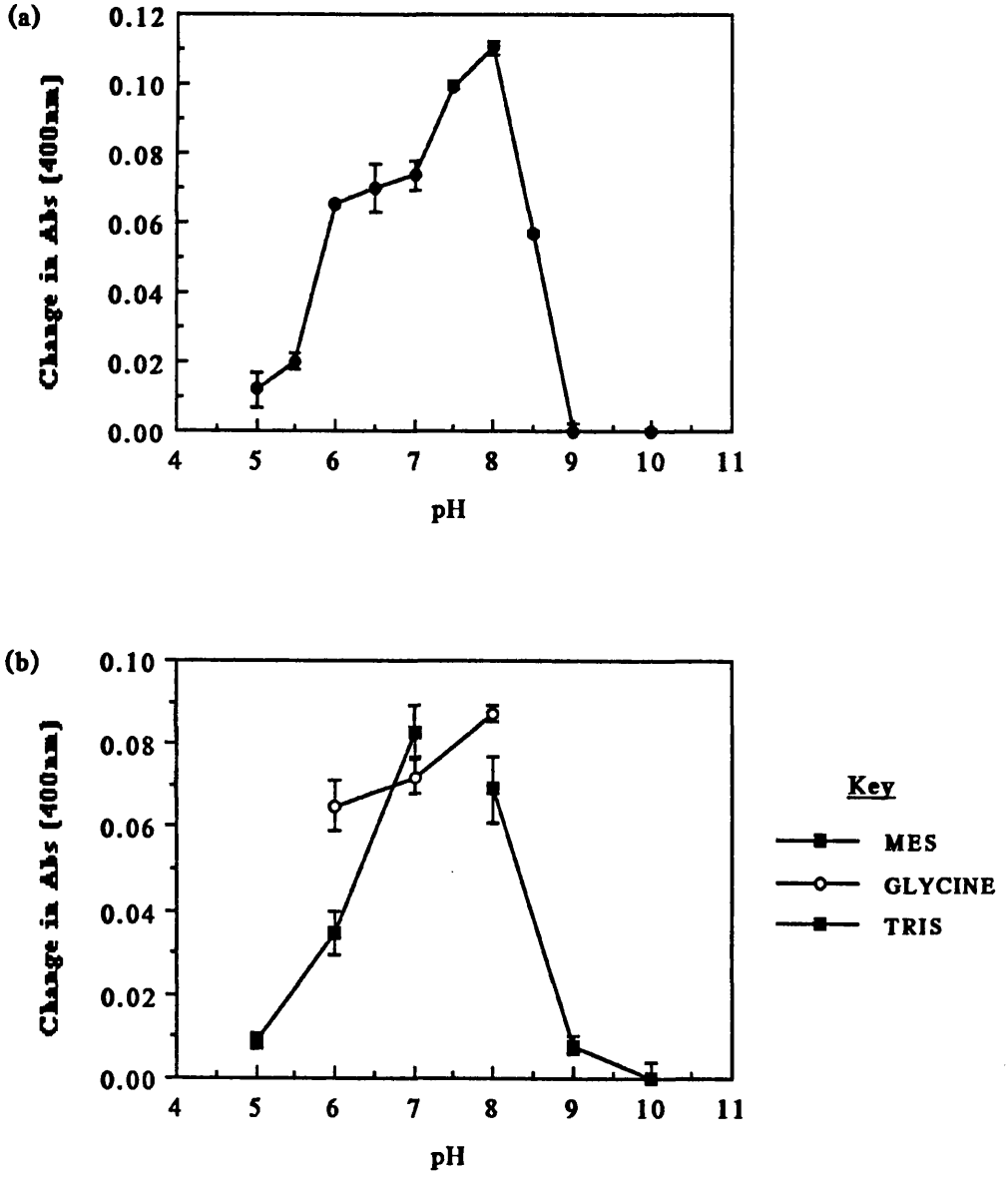


Table 7.2 Hydrolysis of protein substrates by the proteinase-complex

Substrate	Wavelength (nm)	Relative rate of hydrolysis (% ± S.E.M)
Azocasein	400	100 ± 2.7
Azocoll	400	14 ± 0.8
Casein	280	100 ± 1.2
Albumin (Bovine)	280	233 ± 3.2
Collagen	280	0
Elastin	280	0
Fibrin	280	233 ± 2.5
Gelatin	280	34 ± 0.7
Haemoglobin	280	133 ± 5.0

Table 7.3 Hydrolysis of synthetic peptides and low molecular weight substrates

Substrate	Concentration	Rate of hydrolysis (U/ml)
N-CBZ-gly-gly-leu-pNA	10mM	0
CBZ-L-leu-pNE	10mM	0
CBZ-L-phe-pNA	10mM	0.313
CBZ-L-arg-pNA	10mM	0.45
N-Benzoyl-L-arg-ethyl ester	1mM	0.017
L-lys-pNA	1mM	0.03
N-β-L-tyr-ethyl ester	10mM	0

U, unit of activity, is defined as 1μmole of product released per minute.
The chromogenic products, p-nitroanilide and p-nitrophenyl ester recorded at wavelength 440nm. Ethyl ester products were measured at 253 nm.

may be responsible for the differences in results obtained by spectrophotometry and by the gel technique. Assuming the rate of hydrolysis for a protein to be similar in both the liquid assay and in gels, then the degree of staining of gelatin in gels would be less than that of casein. Since gelatin is a partially hydrolysed protein (collagen) preparation, further hydrolysis may rapidly generate small fragments which have a low staining capacity. Since casein is a much larger and more complex protein substrate, the same rate of hydrolysis may generate larger fragments which are still stainable with Coomassie Blue dye, resulting in smaller "clear zones" and less apparent activity. However, the spectrophotometric assay can measure the absorbance of the end product of hydrolysis, thus giving a "true" indication of the rate of proteolysis by the *Pyrococcus* proteinase complex.

7.3.2 Peptides and low molecular weight substrates

The action of *Pyrococcus* proteinases on chromogenic peptides and low molecular weight substrates was determined spectrophotometrically (section 2.20.2). Aliquots of the proteinase complex solution were added to stock solutions of the substrates specified in Table 7.3. The progress of reaction was continuously monitored at the specified wavelengths. The rates of substrate hydrolysis are also shown in Table 7.3.

The proteinase complex appeared to readily hydrolyse single amino acid p-nitroanilide substrates, particularly those containing phenylalanine and arginine. This suggests that the enzyme complex may be specific towards non-polar amino acids, although there is insufficient data for any firm conclusions to be drawn. The *Pyrococcus* complex was also capable of hydrolysing N-benzoyl-L-arginine-ethyl ester, thus demonstrating esterase activity. The failure to hydrolyse the tripeptide N-CBZ-gly-gly-leu-p-nitroanilide indicated that the proteinase complex possibly lacked specificity towards the neutral aliphatic amino acid leucine in the P_1 position. This was also supported by the fact that no activity towards the low molecular weight substrate CBZ-leu-p-nitrophenyl ester was observed.

7.4 Influence of temperature on activity

The relationship between enzyme activity and temperature was determined by monitoring

Figure 7.4 **Time course of azocasein hydrolysis by the proteinase complex at various temperatures.**

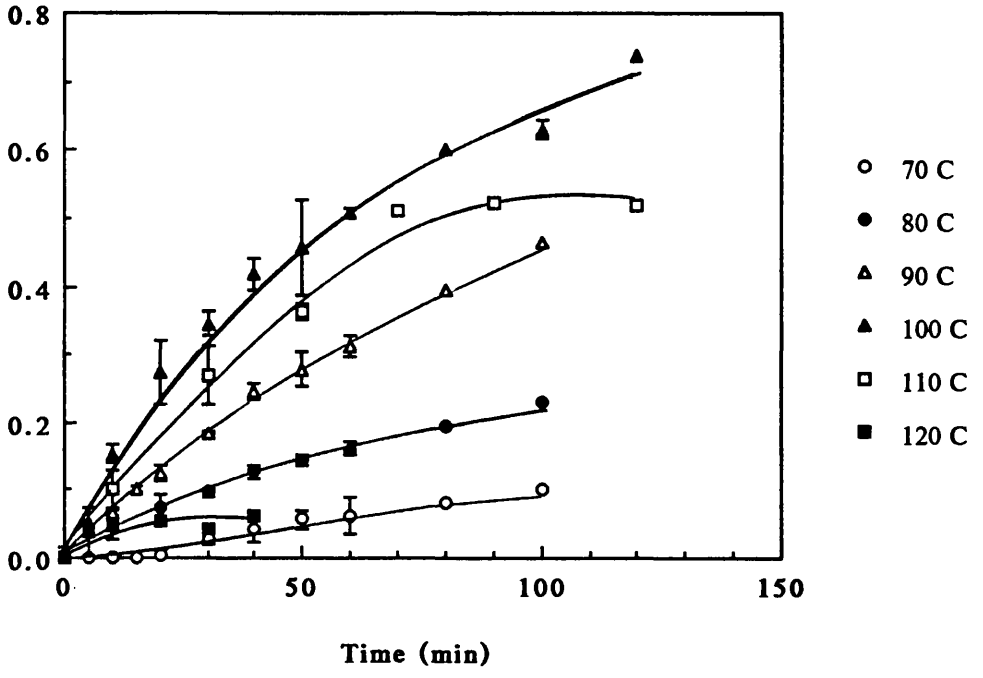
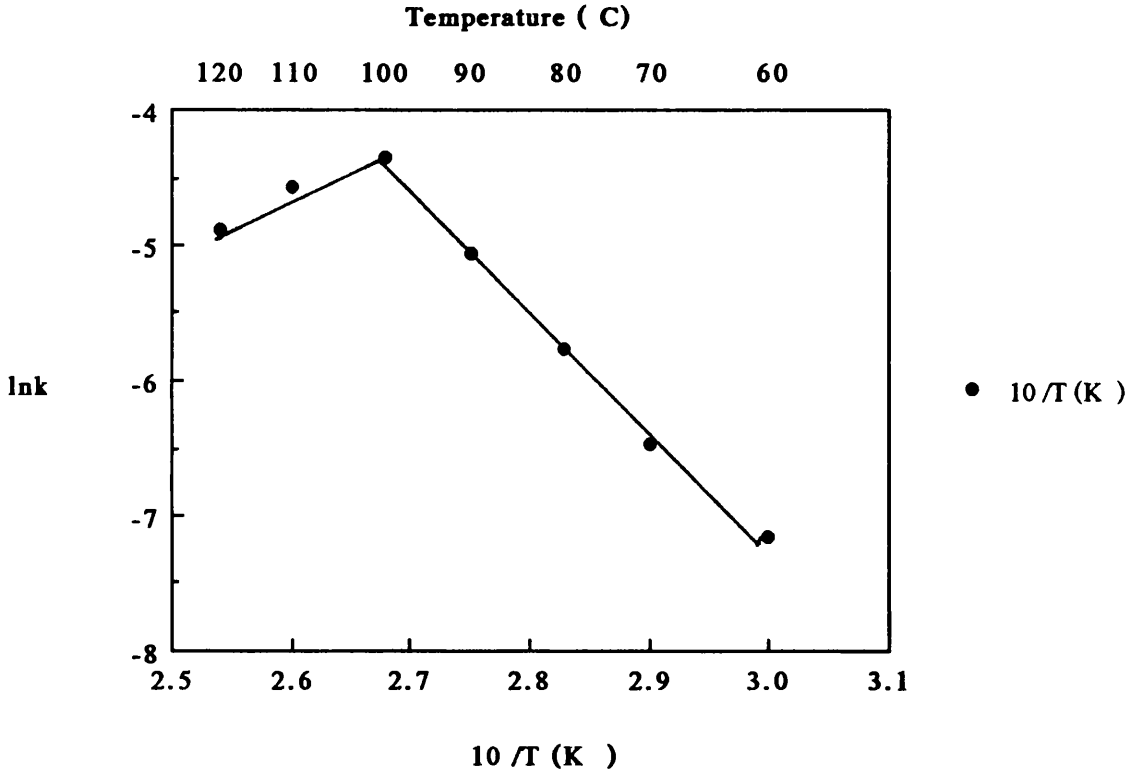


Table 7.4 Temperature dependence of the rate of azocasein hydrolysis by *P. furiosus* proteinase-complex.

Temperature °C	Initial rate, v (ΔA_{400}/min x 10^{-3})
60	0.78
70	1.54
80	3.15
90	6.4
100	12.8
110	10.5
120	7.6

Figure 7.5 Arrhenius plot for the hydrolysis of azocasein by the proteinase complex.



T_{opt} = 100°C (for the reaction conditions used).

the amount of product released in a given time at a range of temperatures. The functional optimal temperature for activity, that is, the point of compromise between maximal kinetics of hydrolysis and minimum kinetics of denaturation was determined for partially pure preparations of *Pyrococcus* proteinase complex (section 2.18). The influence of temperature on activity is illustrated in Figure 7.4.

The data displayed in Fig. 7.4. suggests that under the specified conditions employed (that is, 30 minutes incubation up to 90°C, 10 minutes from 100°C onwards), the functional optimal temperature for activity was approximately 100-105°C. Temperatures in excess of 110°C resulted in a rapid decrease in activity, attributed to thermal denaturation of the enzyme.

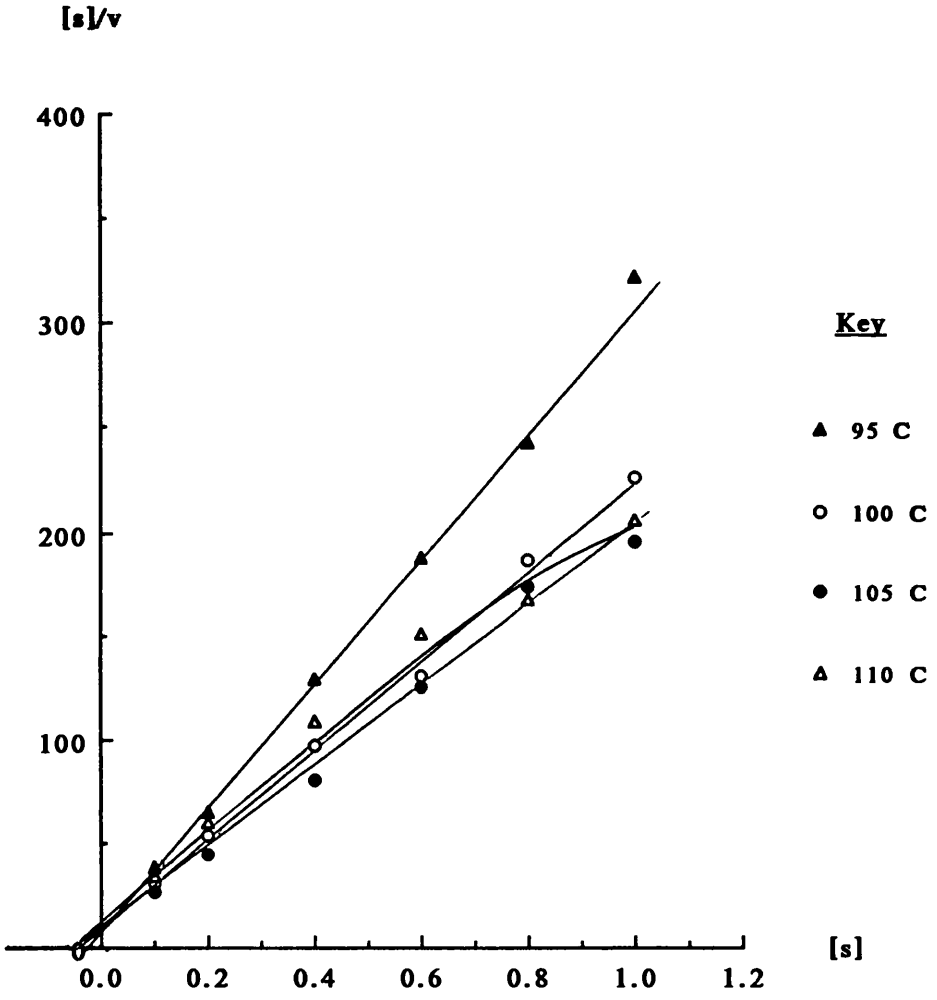
The Arrhenius plot of azocasein calculated from the initial rates of an azocasein hydrolysis time course at different temperatures (Fig. 7.4. and Table 7.4) exhibited a sharp discontinuity at around 100°C (Fig. 7.5). The activation energy calculated from the slope of the Arrhenius plot was measured to be approximately -11kJ/mol. The average Q_{10} value between 60°C to 100°C was calculated to be approximately 1.97. The result obtained from initial rates probably gives a true indication of the melting temperature of the *Pyrococcus* proteinase complex. This observed optimal temperature, however, was not in accordance with the temperature quoted by Eggen *et al* (1990) who reported the functional optimal temperature of activity to be 115°C.

7.5 Influence of temperature on K_m

Kinetic data for the *Pyrococcus* proteinase complex (reaction velocity versus substrate concentration) were determined at different temperatures using azocasein at substrate concentrations ranging from 0.1% to 1%. Hanes plots ([s/v] versus [s]) of the data are presented in Figure 7.6. The K_m and V_{max} values for the proteinase complex at different temperatures are presented in Table 7.5.

It was observed that the affinity of the enzyme for the substrate azocasein was higher at

Figure 7.6 Hanes plot describing effect of temperature on K_m and V_{max}.



95°C than at temperatures above this. It is likely that at temperatures above 95°C, conformational changes of the protein substrate and the enzyme occur, therefore affecting the binding ability of the enzyme with substrate.

Table 7.5 K_m and V_{max} values for *Pyrococcus* proteinase complex at different temperatures (± SEM)

Temperature (°C)	K_m (% azocasein)	V_{max} (ΔA/min x 10^{-3})
95	0.022 + 0.002	3.3 + 0.2
100	0.043 + 0.005	4.6 + 0.4
105	0.043 + 0.003	5.2 + 0.3
110	0.060 + 0005	4.8 + 0.4

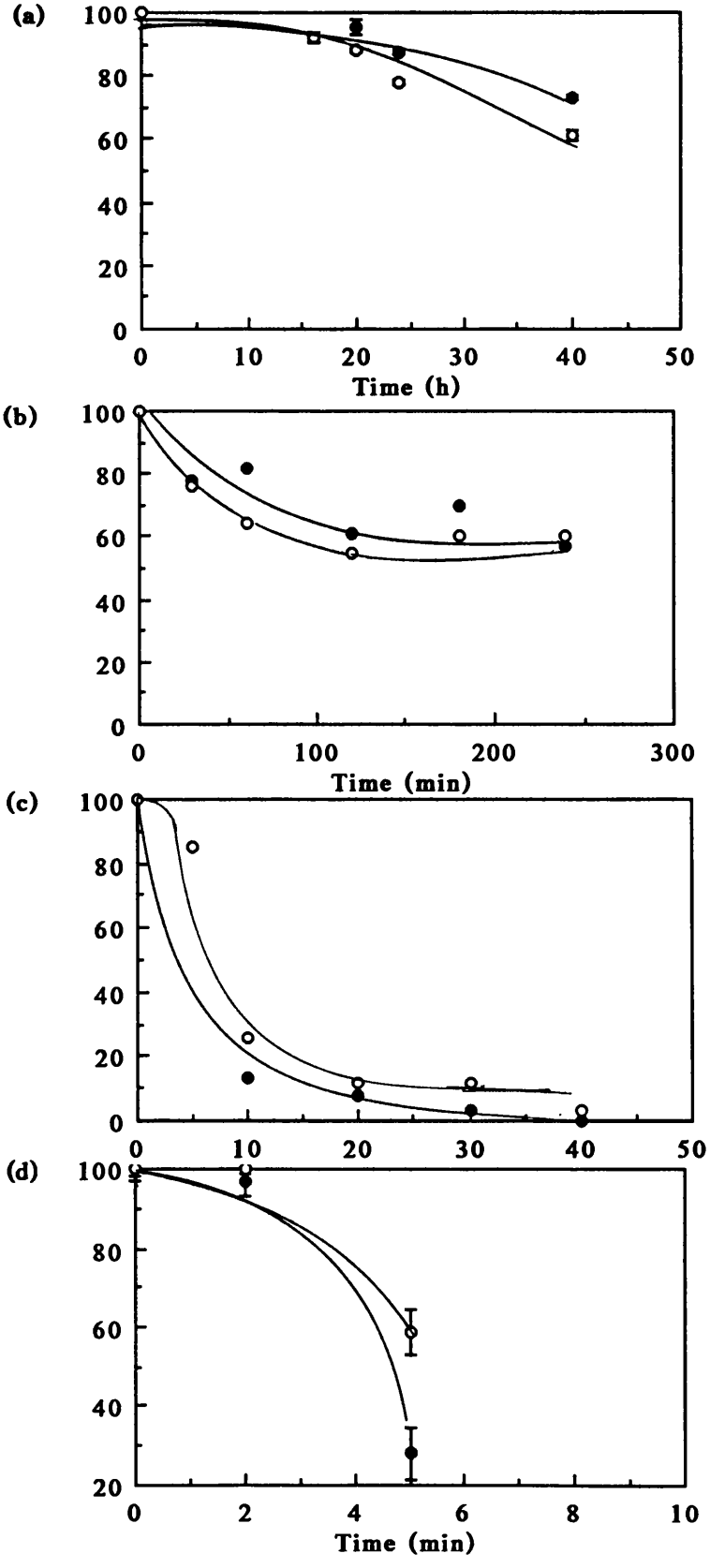
7.6 Thermostability of the proteinase complex.

The thermostability of the proteinase complex was determined as described in Section 2.21.1. Samples of enzyme (3-4ml), pretreated with 10mM EDTA, were incubated at given temperatures in the presence and absence of calcium ions. Certain metal ions are known to enhance the stability of some thermophilic proteinases. It has been shown that calcium ions can bind to exposed heat-labile sections of a thermophilic protein, thus making the structure more rigid and more thermostable (see Section 1.2). The stability profiles derived from incubations between 95°C and 125°C are presented in Fig.7.7. Half-life values of the enzyme complex (i.e., the time taken for the loss of 50% of the initial activity under the conditions specified) are presented in Table 7.6.

At 95°C, the proteinase complex demonstrated a high degree of stability with a half-life of approximately 69 hours. The addition of calcium chloride, however, did not significantly affect the stability of the complex at this temperature. Above 100°C, destabilisation of proteinase complex was evident. However, stability of the enzyme complex at 115°C and 125°C appeared to be slightly (but not significantly) higher in the presence of calcium

Figure 7.7 Thermostability profiles for *P. furiosus* proteinase complex at various temperatures

Proteinase samples were incubated at a) 95°C, b) 105°C, c) 115°C, and d) 125°C, with (○) and without (●) the addition of 10mM calcium chloride. Residual activity was determined by the azocasein assay.



chloride.

The stability of the proteinase complex from *P. furiosus* apparently exceeds that of other known extremely thermophilic proteinases such as archaelysin [$t_{1/2}$ (95°C), 70-90 minutes, Cowan et al., 1987] making it the most thermostable proteinase reported to date.

Table7.6. Half-life values of the proteinase-complex at various temperatures with and without 10mM calcium chloride.

Temperature	Half life	
°C	Without $CaCl_2$	With 10mM $CaCl_2$
95	69h	65h
105	112min	93min
115	6.8min	9.5min
125	4.2min	6.1min

7.7 Effect of chaotropic agents on the stability of the proteinase complex

The ability of a number of proteinases to function in the presence of chaotropic agents has been well documented (Suzuki *et al*, 1976; Cowan and Daniel, 1982; Veronese *et al*, 1984). The effect of denaturants such as urea and guanidinium chloride and reducing agents such as dithiothreitol on the stability of the proteinase complex was determined as described in Section 2.12.2. Stability profiles of the proteinase complex in the presence of the above chaotropic agents and their corresponding half-life values, both at 95°C and 100°C, are shown in Fig.7.8 and Table 7.7 respectively.

It was observed from stability profiles that the proteinase complex activity was initially enhanced in the presence of dithiothreitol. One possible mechanism for the apparent activation effect would be the protection of free thiol groups from oxidation. At high

Figure 7.8 Stability profiles of the proteinase complex in the presence of chaotropic agents at 100°C.

Symbols represent :
DDT (●), urea (○), guanidinium chloride (■), guanidinium chloride/DDT (□), and no addition (△).

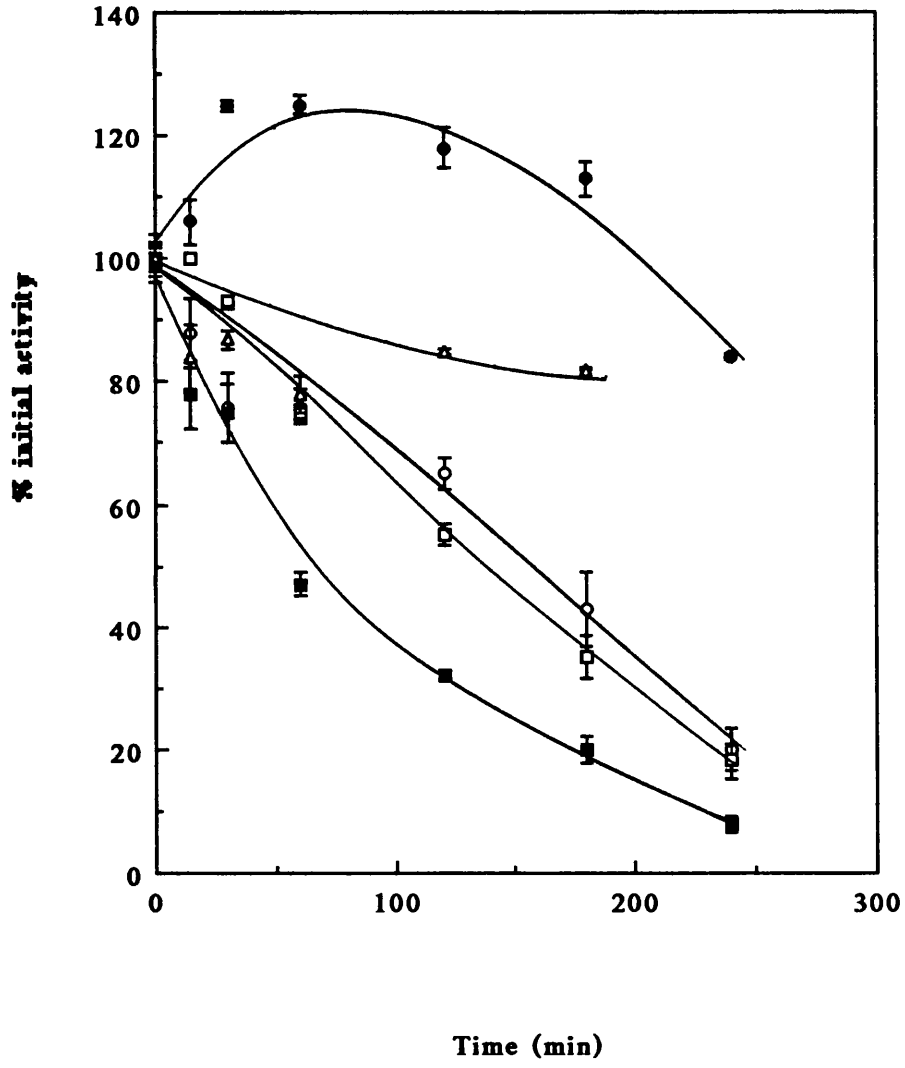
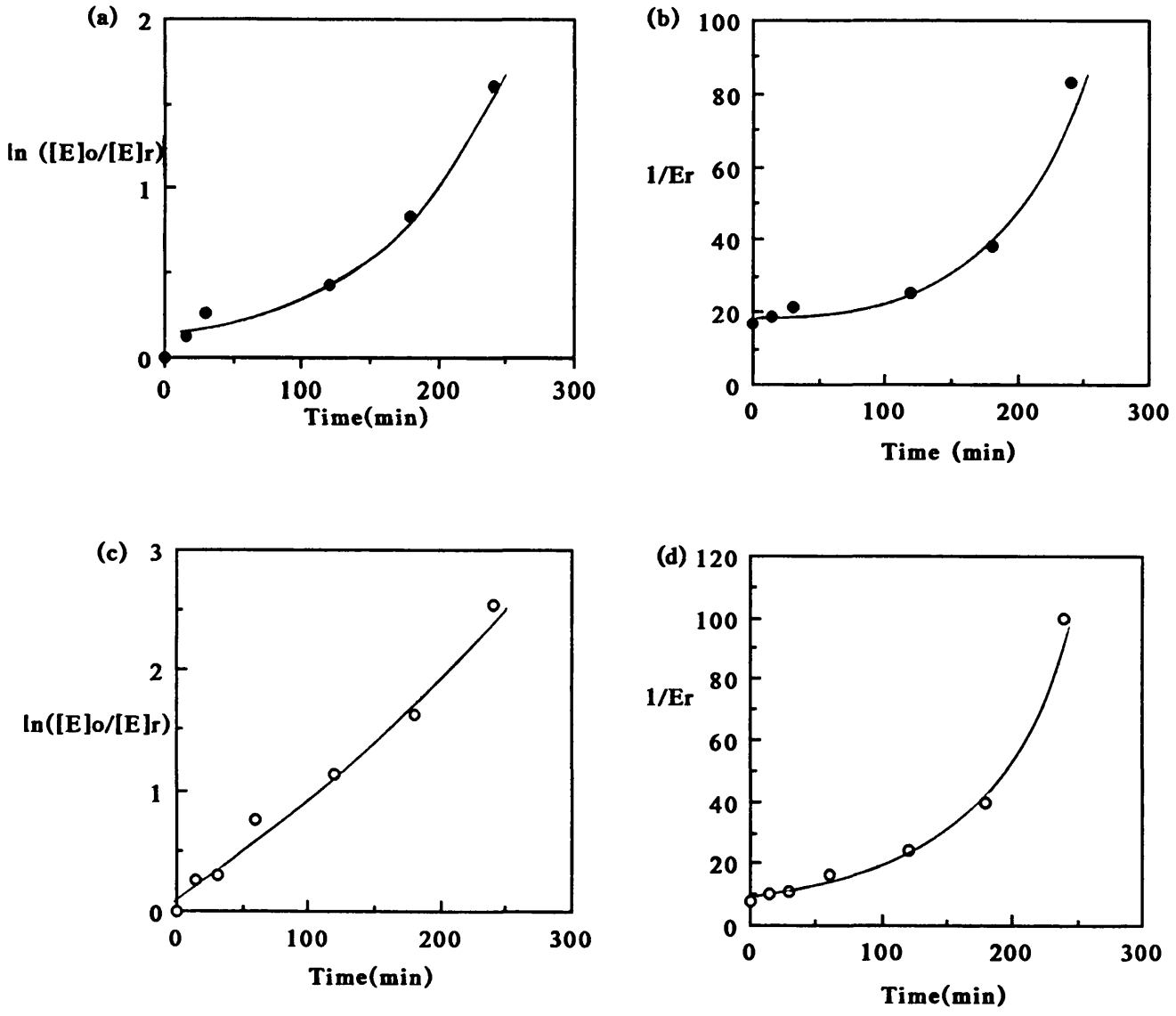
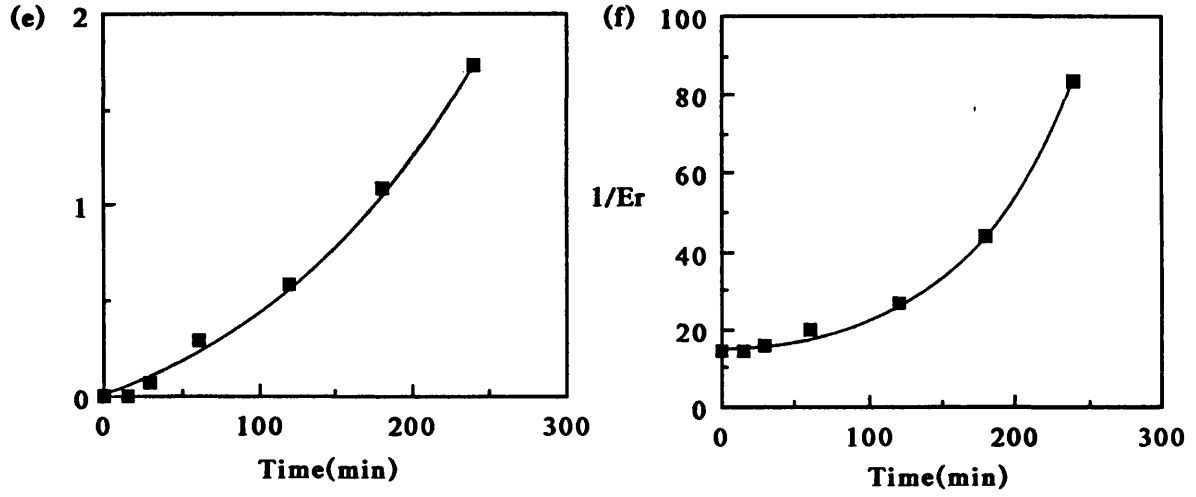


Figure 7.9 Kinetic profiles of the proteinase complex in the presence of chaotropic agents at 100°C.

Graphs represent: first order plots, ln ($[E]_o/[E]_r$) (a, c, e and g); and second plots, $1/[E]r$ (b, d, f and h). Denaturants are represented by graphs (a) and (b), urea; (c) and (d), guanidinium chloride; (e) and (f), guanidinium chloride/DDT; (g) and (h), no addition.

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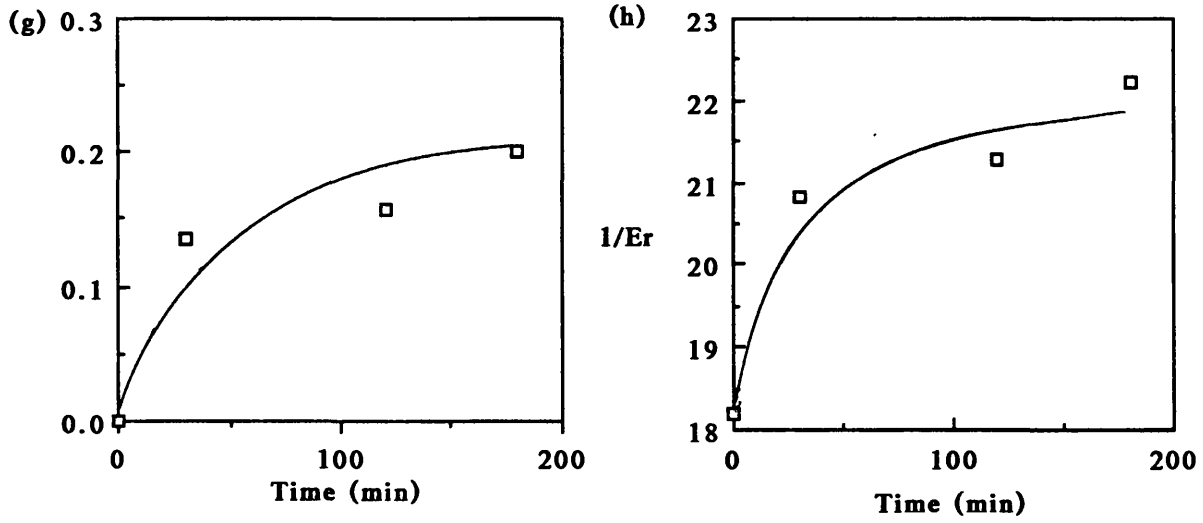
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temperatures, thiol residues are rapidly oxidised, resulting in the formation of charged sulphonate groups. It is possible to speculate that the insertion of charged groups might destabilise the tertiary structure by inducing unfavourable electrostatic interactions. The prevention of such oxidation processes would therefore give an apparent stabilisation. The stabilisation/activation induced by DTT is also observed in the presence of guanidinium chloride, a potent protein denaturant. The half-life of activity of the *Pyrococcus* proteinase at 95°C was 29-30 hours in the presence of 4.4M guanidinium chloride, but increased to 47 hours on addition of 10mM DTT.

Whatever the mechanism of DTT-induced stabilisation/activation, these results support previous suggestions that disulphide bonds are either absent from the proteinase structure, or provide no contribution to the stability of the functional components.

Table 7.7. Effect of chaotropic agents on the stability of the proteinase-complex

Chaotropic agent	Concentration	Half life	
		95°C	100°C
None	-	67h	370 min
Urea	6.2M	>60h	164 min
Dithiothreitol	10mM	>60h	350 min
Guanidinium chloride	4.4M	29.7h	71 min
Guanidinium chloride/dithiothreitol	4.4M/10mM	47.4h	156 min

The proteinase complex followed first order kinetics suggesting that denaturation processes, rather than autolysis, predominated (Fig. 7.9). However, in the presence of dithiothreitol, the kinetics were observed to be complex, largely due to the initial activation seen in the stability profiles.

7.8 Effect of detergents on stability

The effect of detergents on the stability of the proteinase complex was determined as described in Section 2.21.3. Residual enzyme activity was determined using the azocasein assay. Stability profiles illustrating the effect of detergents on enzyme activity at 105°C and half-life data at 95°C and 105°C are shown in Fig. 7.10 and Table 7.8 respectively.

Table 7.8 Effect of detergents on the stability of the proteinase-complex at 95°C and 105°C.

Detergent	Concentration	Half life	
		95°C	105°C
None	-	67h	58min
SDS	0.1%	210min	3min
	1%	18min	-
Triton X-100	0.1%	>60h	103min
	1%	>60h	114min
CTAB[a]	0.1%	19min	8min
	1%	14min	9.4min

[a] CTAB: cetyltrimethlyammonium bromide.

The stability of the proteinase complex at 105°C was enhanced almost 2-fold by the extremely hydrophobic non-anionic detergent Triton X-100 (half-life calculated as 103 mins at 0.1%) whereas, at the same concentration, the anionic detergent SDS resulted in a large destabilisation of the structure (half-life, 3 minutes). This suggests a possible

Figure 7.10 **Stability profiles demonstrating the effect of detergents on the proteinase complex at 105°C.**

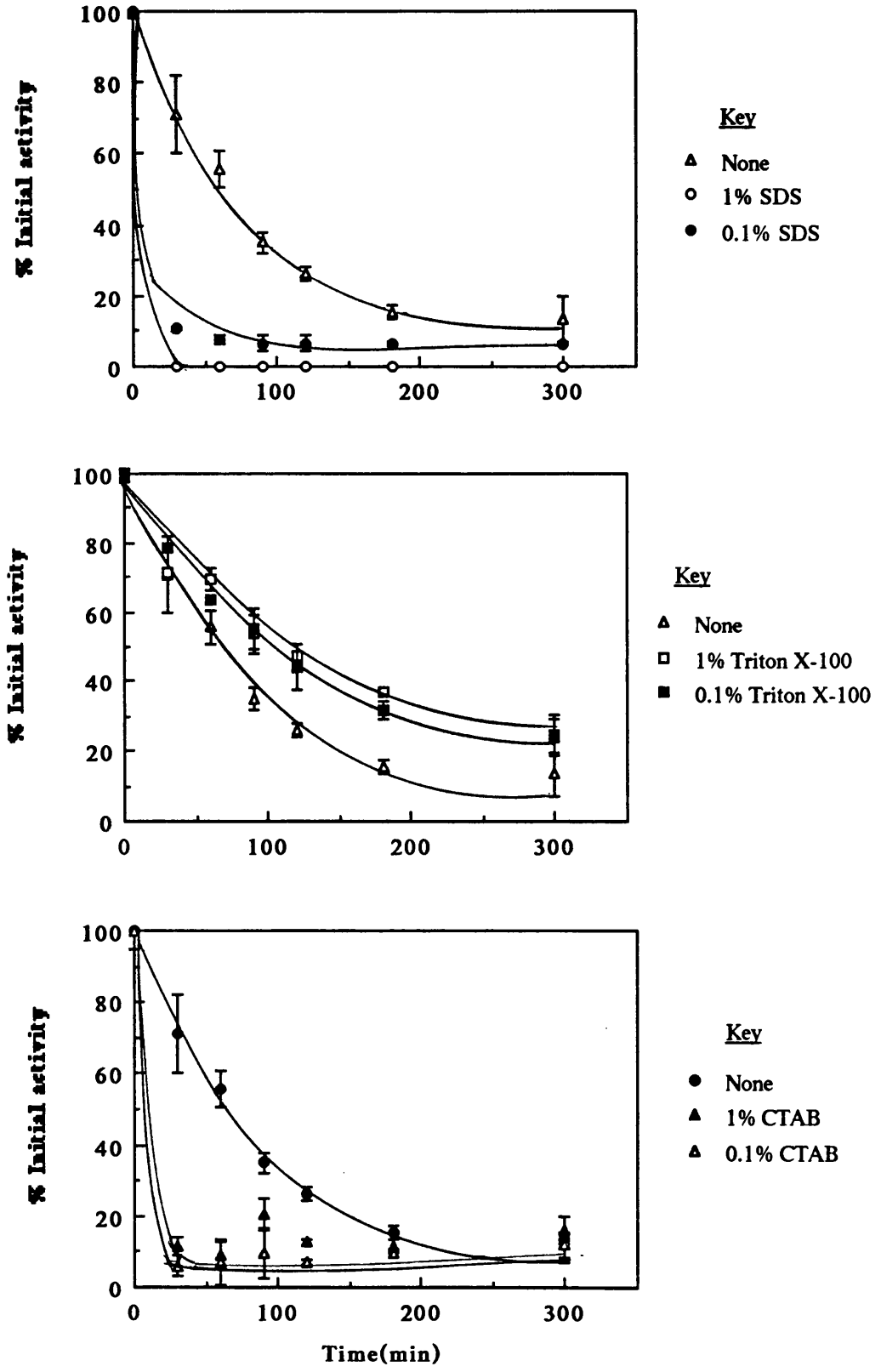
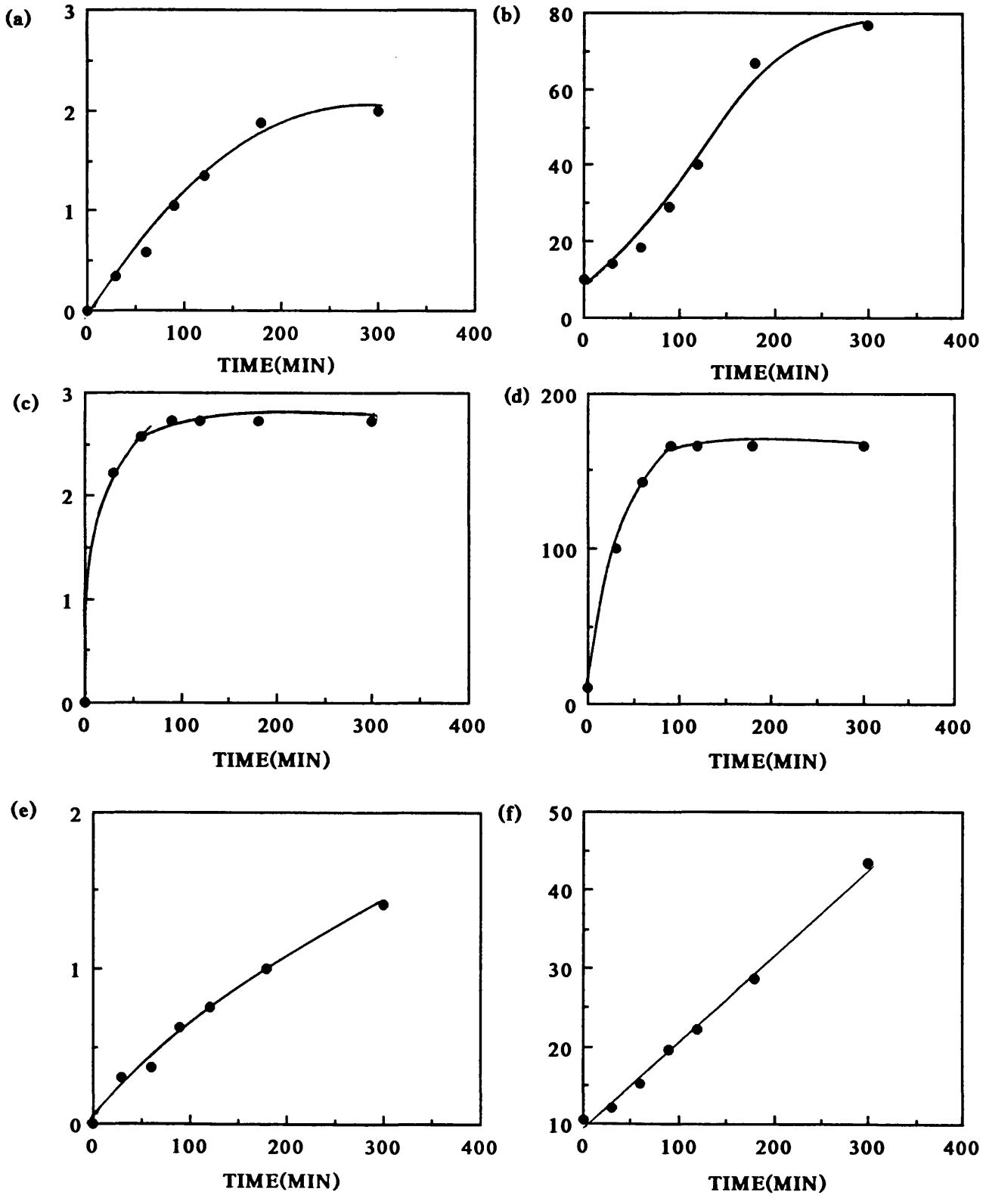
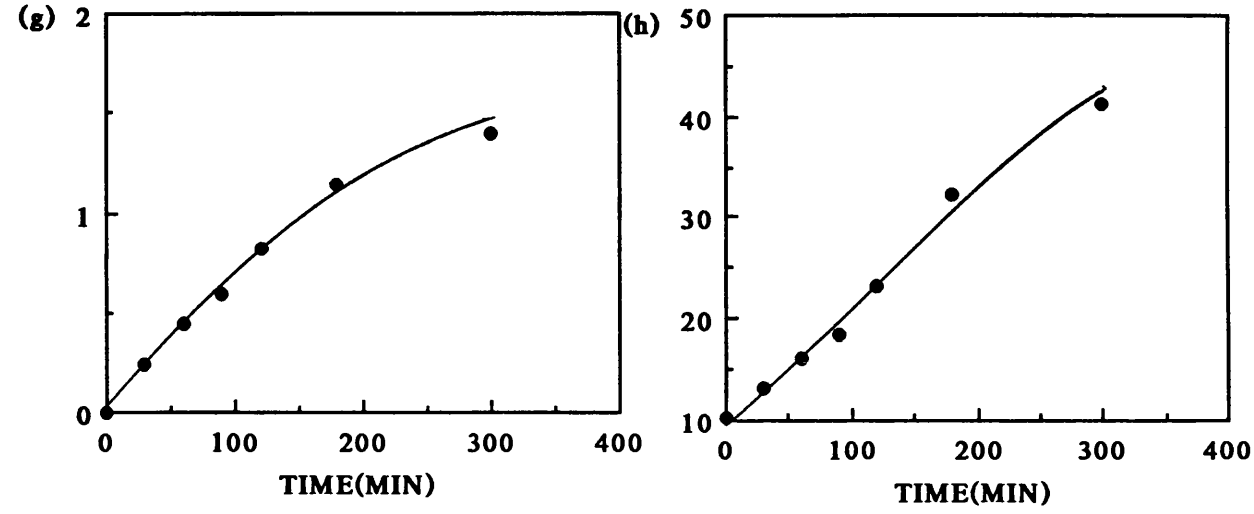


Figure 7.11 **Kinetic profiles of the proteinase complex in the presence of detergents.**

Graphs represent: a, c, e, g, i and k, first order plots (ln ($[E]_o/[E]_r$)); and b, d, f, h, j and l, second order plots ($1/[E]_r$). Detergents are represented by graphs (a) and (b), no addition; (c) and (d), 0.1% SDS; (e) and (f), 1% Triton X-100; (g) and (h), 0.1% Triton X-100; (i) and (j), 1% CTAB; (k) and (l), 0.1% CTAB.

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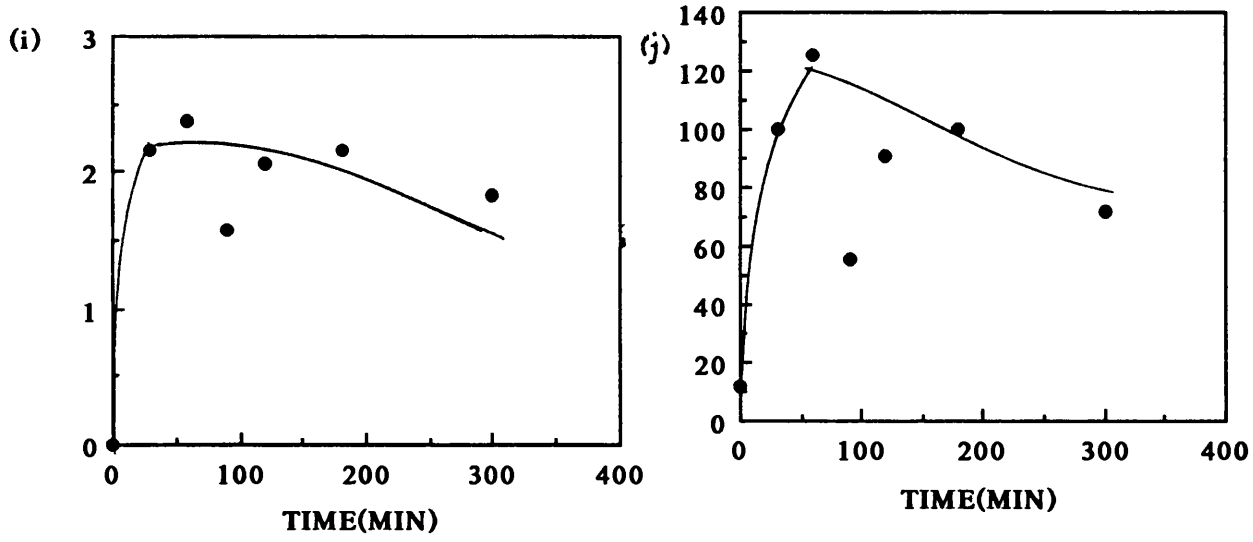
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TIME(MIN)
(j)
140
120
100
80
60
40
20
0
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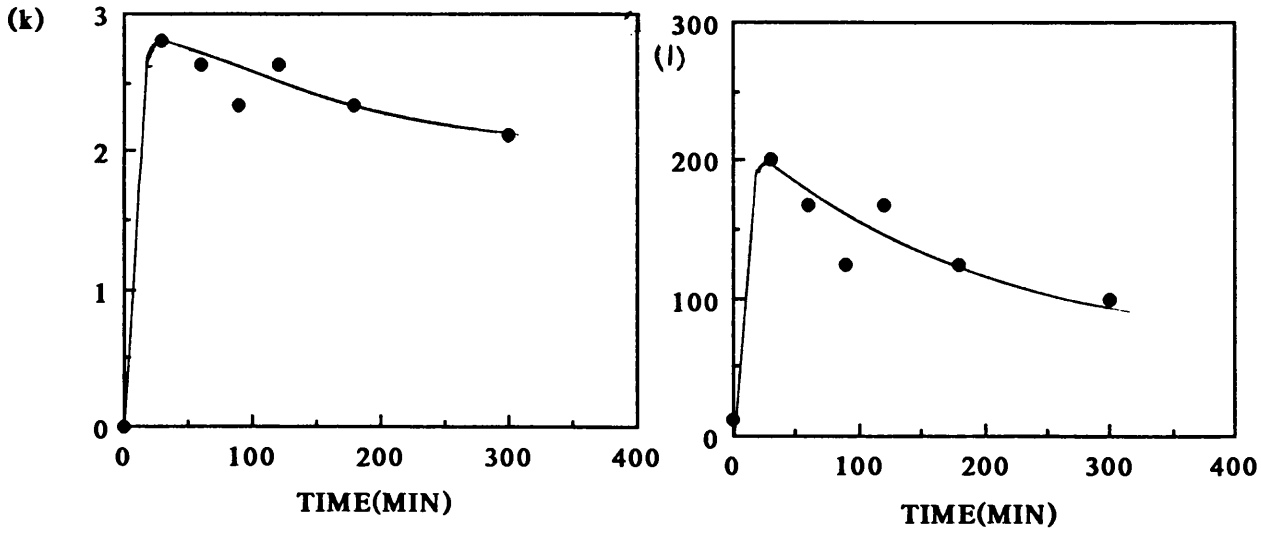
100
200
300
400
TIME(MIN)


(k)
3
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300
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correlation between protein thermostability and hydrophobicity of the detergent. This correlation is based on the hydrophile-lipophile balance, HLB values, of the detergents (Furth, 1980). The more hydrophobic the detergent, the lower the HLB value. The kinetics of activity loss for both Triton X-100 (HLB value 13.5) and SDS (HLB value 40) were shown to be first order (Fig. 7.11).

The cationic hydrophilic detergent CTAB resulted in a large destabilisation irrespective of concentration, with half-life values of approximately 14-19 minutes at 95°C and 8-9 minutes at 105°C. The kinetics of activity loss were found to be complex in the presence of this detergent (Fig. 7.11).

7.9 Effect of organic solvents on stability

It has been well documented that thermophilic proteins demonstrate enhanced thermostability and resistance to denaturation in the presence of some organic solvents with low water activity (Zaks and Klibanov, 1988). The effect of single phase aqueous:organic solvent mixtures on the stability of the proteinase complex was determined as described in Section 2.21.4. Stability profiles of the proteinase complex in the presence of miscible organic solvents and the corresponding activity half-life values at 100°C are shown in Fig.7.12 and Table 7.9 respectively.

At 100°C, the stability of the complex was markedly reduced in the presence of 10% ethanol, pyridine and acetone, with half-life values ranging from 6.5 to 16 minutes. The stability of the complex in 10% dimethylformamide was much higher, giving a half-life value of 44 minutes. On the basis of log P (where P is the partition coefficient for a given solvent between octanol and water, reflecting the hydrophobicity of the solvent (Laane *et al*, 1987)), dimethylformamide is the most hydrophilic of the solvents tested. The stability of the proteinase complex could not be compared with stabilities of other reported thermophilic proteinases, such as subtilisin, since a low water activity system was not used in this study.

Figure 7.12 **Stability profiles illustrating the effect of miscible organic solvents on the proteinase complex at 100°C.**

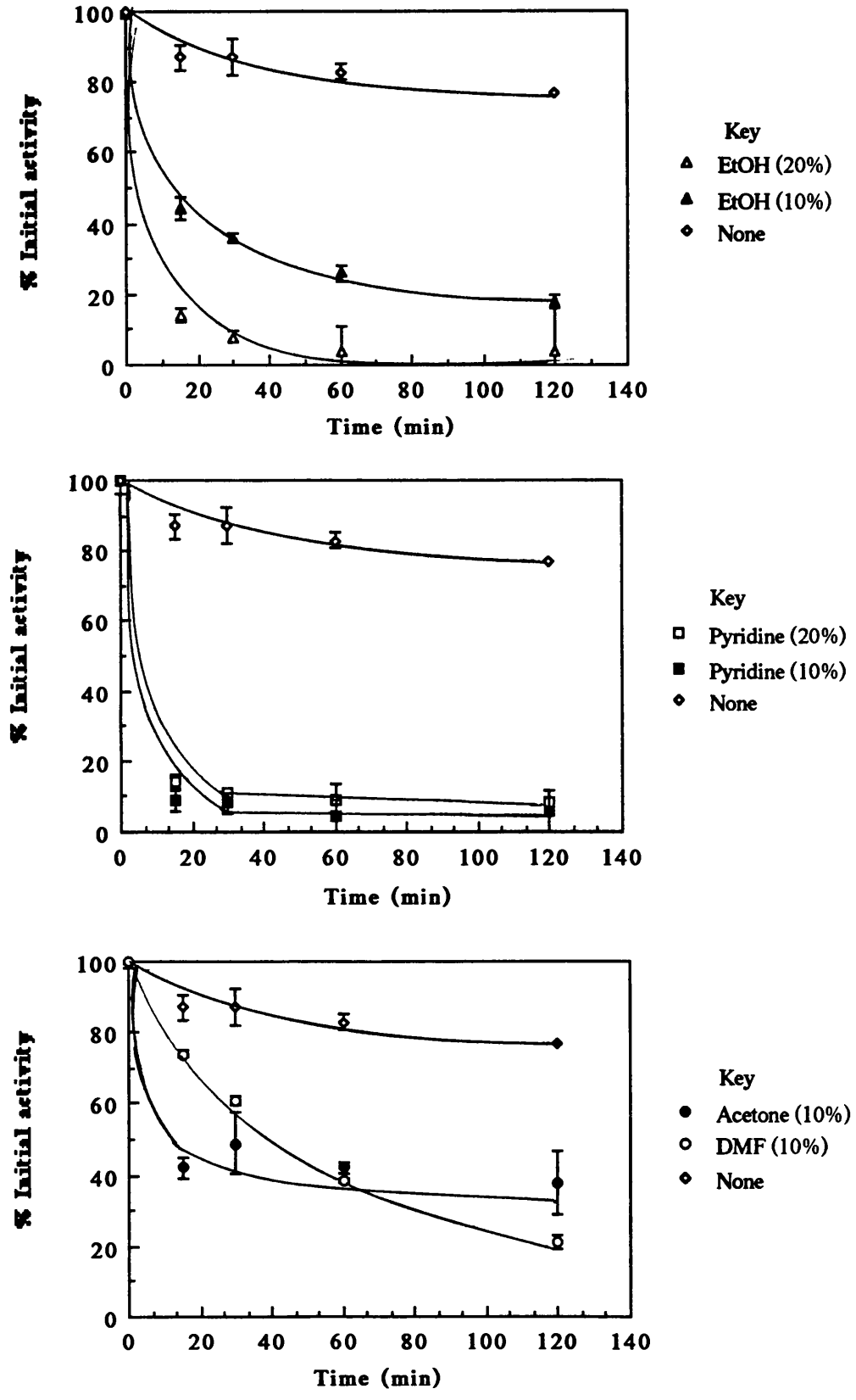


Table 7.9. Effect of miscible organic solvents on the stability of the proteinase-complex

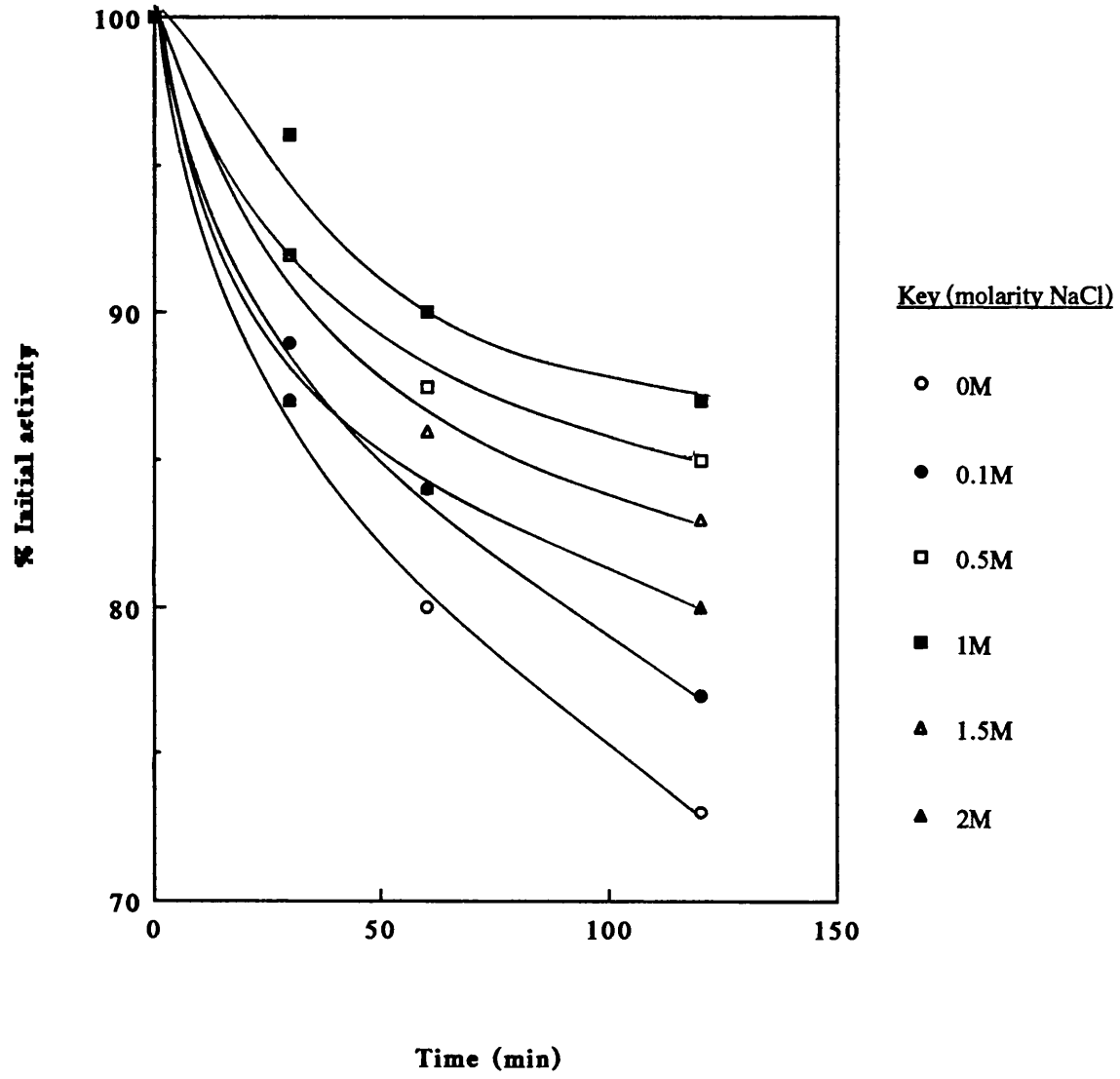
Solvent	Concentration (% v/v)	Half-life (min) at 100°C
None	-	346
Ethanol	20	2.3
	10	16.0
Acetone	10	10.8
Pyridine	20	3.0
	10	6.5
Dimethylformamide	10	44.0

7.10 Effect of ionic strength on stability

Determinations of the thermostability of the proteinase complex as a function of increasing ionic strength were carried out as described in Section 2.21.5. Thermostability profiles of the proteinase compex in the presence of various concentrations of NaCl are illustrated in Fig. 7.13.

It was observed that the stability of the proteinase complex was considerably enhanced at 100°C when the ionic strength was increased up to 1M NaCl (Fig. 7.13.), suggesting that high ionic strength may be required to stabilise the proteinase complex. This result supports the observations made in earlier studies that low ionic strength destabilises the proteinase complex. In addition, this conclusion presupposes that the functional components of the

Figure 7.13 **Stability profiles of the proteinase complex with respect to ionic strength.**



proteinase are less stable in "free" form than in the complexed state. At ionic strengths greater than 1M, the complex demonstrated a decrease in thermal stability. This destabilisation effect could be attributed to ionically-induced denaturation or dissociation of internal salt bridges during incubation of the complex at 100°C.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The hyperthermophile *Pyrococcus furiosus* is one of the few organisms, isolated in the last five years, capable of growing at temperatures at and above 100°C. Its ability to grow and perform biological functions at normally denaturing temperatures has fuelled considerable interest in attempting to understand the physiology and biochemistry of this organism. In this thesis, an investigation of the role of proteolysis in *P. furiosus* was undertaken, to gain some insight into the nutritional characteristics of this organism. In addition, the isolation, purification and characterisation of the proteinases associated with *Pyrococcus* have been carried out, to develop a better understanding of the mechanisms involved in the basis of protein stabilisation at high temperatures.

It has been shown (Table 3.2) that *P. furiosus* can grow on a variety of complex substrates such as peptone or tryptone as well as on simpler carbon substrates, such as maltose (section 3.5.3). These observations are in agreement with previous results obtained by Fiala and Stetter (1986). Poor growth, however, was observed with the protein hydrolysates, and when the protein substrates were substituted with a mixture of amino acids, in the presence of 0.1% yeast extract. No growth was observed on single amino acids or a cocktail of all amino acids without yeast extract, suggesting that *P. furiosus* can grow on peptides and proteins as sole carbon, energy and nitrogen sources and not on single amino acids. It is possible that *P. furiosus* is unable to transport specific free amino acids which are required for cell growth (Payne, 1980). It is also likely that amino acids in the form of polypeptides are less susceptible to chemical and thermal degradation. This is particularly important for very labile amino acids such as glutamine and asparagine which are deamidated at high temperatures (Volkin and Klibanov, 1990).

The fact that *P. furiosus* may be auxotrophic for one or more amino acids is further supported by the observation that the oligosaccharide maltose could be utilised as a carbon source, providing the culture medium was supplemented with a protein substrate. This

observation is in agreement with evidence provided by Snowden *et al.*, (1992) who reported similar findings and demonstrated that no growth was observed using maltose as the sole carbon source, despite the presence of metabolic pathways for the utilisation of sugars (Mukund and Adams, 1991). This suggests that certain substrates influence the growth of *P. furiosus* at high temperatures. For instance, it has been shown that *P. furiosus* cannot grow on glucose but can proliferate in the presence of oligosaccharides such as maltose, and polysaccharides such as starch (Fiala and Stetter, 1986). In addition to the data obtained from amino acid studies (section 3.5.2), this suggests that substrate stability may be an important parameter for growth at elevated temperatures.

Growth on different culturing media was found to have a significant effect on proteinase production from *P. furiosus*. Extracellular proteinase activity was induced when cultures were grown on peptone but appeared to be largely repressed when grown on maltose. For instance, growth on peptone in the presence and absence of maltose produced similar cell densities (approximately 1.2 x 10^8 cells/ml). However, proteinase production profiles were significantly different. Specific proteinase production for *P. furiosus* grown in media containing maltose and peptone was found to be lower than when the organism was cultured in the absence of maltose (45.5 U/g and 91.4 U/g protein respectively). This suggests that inhibition by maltose probably involves a catabolite repression mechanism. However, since growth was observed on both carbohydrate and protein substrates together, it can be assumed that maltose does not completely inhibit proteinase production. Snowden *et al.* (1992) reported that growth on both maltose and tryptone (a similar substrate to peptone) enabled *P. furiosus* to utilise the carbohydrate rather than the protein as a carbon and energy source when the former is available. This was supported by the fact that enzymes of the novel glycolytic pathway described in *P. furiosus* (Mukund and Adams, 1991) were induced by maltose even when high concentrations of peptide are present (Snowden *et al.*, 1992). It is only in nutrient-limited conditions, such as in the absence of maltose or low concentrations of protein substrates, when enhanced rates of proteolysis are observed and the results in this thesis suggest this to be the case for *P.*

furiosus. In its natural nutrient-limited environment, *P. furiosus* is probably able to survive possibly by increased rates of proteolysis. This element of response is generally observed during starvation and in stringent-response mechanisms by other mesophilic bacteria (Nyström *et al.*, 1990).

The identification of a number of intracellular and extracellular proteinases from *P. furiosus* (Skaja *et al.*, 1989; Blumentals *et al.*, 1990; Eggen *et al.*, 1990; Connaris *et al.*, 1991), suggest that the role of proteolysis must therefore be significant in this hyperthermophile. However, the exact number of proteolytic enzymes from this organism has not been confirmed. In this study, up to thirteen bands were identified from intracellular and extracellular extracts, using a gelatin-containing SDS PAGE method, whereas studies by Blumentals *et al.* (1990) and Eggen *et al.* (1990) demonstrated 5 bands using activity gels. Despite extensive investigations of the possible origins of band multiplicity in substrate gels, it was concluded that the activity bands observed in substrate gels were probably discrete polypeptides. This was supported by the following observations:

(i) The formation of bands was observed at the beginning of growth phase to late stationary phase, increasing in intensity when approaching peak growth of the organism, with no alteration in band patterning;

(ii) A consistent multiple band pattern was reproduced in both casein and gelatin-containing polyacrylamide gels, indicating that the bands were not artefacts resulting from heterogeneous associations with the substrate;

(iii) Activity of individual polypeptides was still observed even after treatment with different concentrations of SDS, suggesting that these bands were not artefacts resulting from incomplete unfolding;

(iv) A reproducible multiple band pattern was observed when cell extracts and culture supernatants were incubated at different temperatures (4°C to 95°C), suggesting that the bands were not active processing products generated from autolysis;

(v) the bands were not artefacts produced from thiol-interchange mechanisms, which can generally occur during the refolding process.

Despite the relative ease in which these proteinases renature after removal of SDS with Triton X-100, these observations present some discrepancies with regards to the nature of these proteinases when compared with data obtained by Blumentals *et al.* (1990). The demonstration that two of the five identified proteinases from *P. furiosus* by Blumentals *et al.* (1990) exhibiting SDS-resistance suggests that the proteinases were not fully denatured in the presence of SDS. However, the evidence presented in this thesis does not corroborate the existence of SDS-resistant proteinases since all thirteen bands observed in gelatin gels were found to be distinct polypeptides with no apparent resistance to SDS.

Interestingly, the formation of multiple bands is not unique to *P. furiosus*. Klingeberg *et al.* (1991) reported that a number of anaerobic hyperthermophilic bacteria, including *Thermococcus celer, Thermococcus AN1, Thermococcus litoralis, Thermobacteroides proteolyticus* and *Staphylothermus marinus* all demonstrated multiple clear zones of proteolytic activity in gelatin gels, with apparent molecular masses ranging from 30kDa to 300kDa. Whether this phenomenon is due to aggregation of proteins, proteolysis or due the formation of various protein species has not yet been established.

The formation of multiple enzyme bands in activity gels is not only restricted to proteinases from thermophiles. Antranikian (1990) reported a large number of amylolytic bands from polysaccharide-degrading thermophilic bacteria. In all cases, the formation of multiple band patterns was not due to proteolysis or protein aggregation during electrophoresis, since *in vivo* experiments indicated that these bands were present from the beginning of the growth phase until the end of stationary phase. Throughout the growth cycle, no alteration of the protein pattern was observed. This phenomenon appears to be a general feature of anaerobic bacteria degrading polysaccharides (Antranikian, 1989; Coleman *et al.*, 1987; Melasniemi, 1987; Spreinat and Antranikian, 1989). One possible explanation for the existence of a multiplicity of amylolytic enzymes may be due to post translational

modification of proteins by glycosylation (Coleman *et al.*, 1987). This may also be a possibility for the multiplicity of proteinases described from *P. furiosus*.

The appearance of multiple proteolytic bands from analysis of *P. furiosus* cell extracts and culture supernatants using gelatin SDS PAGE, suggests that dissociation of the proteinases under denaturing conditions can be accomplished. However, attempts to dissociate and separate the proteinases into individual components by other means (for example, by various chromatographic methods) proved to be largely unsuccessful. Only the dissociation of the lowest proteolytic active species (66kDa) was achieved by gel permeation chromatography using Sephacryl S300 under denaturing conditions. In all other cases, however, the appearance of the multiple band pattern of proteinases was evident, strongly suggesting that these proteinases either exist *in vitro* or *in vivo* as an aggregate or as a multimeric complex. This is further supported by gel filtration studies which indicated the apparent native molecular weight of the proteinases to be around 10^6 Da. It is therefore possible that the formation of aggregates or the existence of a proteinase complex in *P. furiosus* may represent a selective advantage in that the association of proteinases allows the stabilisation of the functional tertiary structure of each species, particularly in the extreme environments to which *P. furiosus* is exposed.

Due to the problems of dissociating the proteinases into their individual species, attempts to purify the proteinase as a 'complex' was carried out. This was accomplished by a combination of anion-exchange chromatography and hydrophobic interaction chromatography using ammonium sulphate. The purification of the proteinase complex to near homogeneity was achieved in three stages, thus allowing the subsequent characterisation of the structural and catalytic properties of the complex.

The proteinase complex exhibited maximum activity at about 100°C above which thermal denaturation occurred. It is interesting to note that the initial rates of azocasein hydrolysis by the proteinase complex up to this temperature followed the Arrhenius law, where a doubling in reaction rate was observed with every 10°C rise in temperature. This suggests

that the proteinases are behaving co-ordinately. This is further supported by the sharp discontinuity in the Arrhenius plot observed at 100°C suggesting that all the proteinases are denaturing co-ordinately. It might be assumed that the proteinases exist as a defined high molecular weight complex and not as a random aggregate of proteinases since it would be expected that an aggregate of independent proteinases might possibly have different temperature optima and thus produce irregularities around the point of discontinuity in the Arrhenius plot.

The proteinase complex exhibited a half life at 95°C of approximately 69h, making this the most thermostable proteinase reported to date. Metal ion chelation did not appear to be implicated in the stability of the proteinase at high temperatures, suggesting that molecular stabilisation was an intrinsic property of the protein structure. This is in contrast to some other characterised extremely thermophilic proteinases, such as the *Thermoplasma* proteinase, where a low concentration of calcium ions enhanced its thermostability 16-fold (Dahlmann *et al.*, 1989). However, archaelysin, the extremely thermostable proteinase from *Desulfurococcus*, had a half life of 90 minutes at 95°C (Cowan *et al.*, 1987) and did not require metal ions for the stabilisation of the protein structure at high temperature.

In the presence of chaotropic agents, the proteinase complex was quite stable at 95°C, exhibiting half life values of greater than 60h in the presence of 6M urea and 10mM DTT. However, at 100°C, urea and guanidinium chloride had a large destabilising effect on the protein structure, implying that hydrophobic interactions are a major contribution to the conformational stability of the proteinase complex. The apparent stabilisation/activation induced by DTT, particularly in the presence of guanidinium chloride, further suggests that disulphide bonds are either absent from the structure or, if present, do not contribute to the stability of the functional components within the protein.

The proteinase complex also exhibited considerable stability in the presence of the non-ionic detergent Triton X-100 (1% v/v, $t_{1/2}$ (95°C) ≥ 60h), whereas the anionic detergent SDS and the cationic detergent cetyltrimethylammonium bromide, at the same concentration

and temperature, produced a large destabilising effect on the structure with half life values of approximately 14-18 minutes. This suggests that hydrophobic bonding may be a significant intramolecular stabilising feature for the *P. furiosus* proteinase complex. Similar findings on the effects of detergents on the stability of the extremely thermostable proteinase archaelysin from *Desulfurococcus* are reported by Cowan *et al.* (1987).

The results of this study indicate that *P. furiosus* proteinases may possess a number of different catalytic functions. For instance, inhibitor studies revealed that the majority of the proteinases observed in gelatin SDS gels (and using the azocasein assay) were inhibited by the serine proteinase inhibitor, PMSF. However, analysis of gelatin gels revealed a PMSF-insensitive high molecular mass proteolytic band (130kDa) and another of molecular mass 128kDa, which were inhibited by the metalloproteinase inhibitor EDTA. This suggests the existence of at least one catalytic site for metalloproteinase activity within the aggregate or complex of proteinases. However, the addition of calcium to the assay buffer during the refolding process did not reactivate the EDTA-inhibited proteinases, suggesting that these proteinases are not dependent on calcium ions for activity, but possibly by other metal ions.

To date, the only proteinases from extremely thermophilic archaea to be characterised in detail are archaelysin from *Desulfurococcus* strain $Tok_{12}S_1$ (Cowan *et al.*, 1987), the thermostable acid proteinase thermopsin from *Sulfolobus acidocaldarius* (Fusek *et al.*, 1990) and the multicatalytic proteinase from *Thermoplasma acidophilum* (Dahlmann *et al..*, 1989). Preliminary characterisation studies on proteinases from other hyperthermophilic bacteria such as *Thermococcus celer, T. litoralis, Thermococcus AN1, Staphylothermus marinus* and *Thermobacteroides proteolyticus* have also been carried out (Klingeberg *et al.*, 1991). In all cases, all proteinases were found to be extremely thermostable. Inhibitor studies on all proteinases listed above with the exception of thermopsin, revealed that almost complete inhibition was observed with the serine inhibitors DFP and PMSF. However, the proteinases isolated from the hyperthermophiles studied by Klingeberg *et al.* (1991) and the proteinase from *Thermoplasma* (Dahlmann *et al.*, 1989) exhibited partial inhibition by the metalloproteinase inhibitor EDTA suggesting that possibly other distinct

catalytic sites may be present. It can be speculated that the heterogeneity of proteinases observed in hyperthermophiles studied by Klingeberg *et al.*, (1991) and proteinases isolated from *P. furiosus* may be indicative of a high molecular weight multicatalytic proteinase complex as seen in *Thermoplasma* (Dahlmann *et al.*, 1989).

The specificity of the proteinase complex from *P. furiosus* has not yet been fully determined. From the limited range of substrates tested, the complex indicated a preference for small basic amino acids, particularly with substrates containing an arginyl residue adjacent to the leaving group. In addition, the complex readily hydrolysed substrates containing phenylalanine adjacent to the leaving group. The ability to hydrolyse these particular specific amino acids has also been observed with mammalian multicatalytic proteinase complexes which contain distinct catalytic sites for the hydrolysis of peptides bonds adjacent to basic (usually Arg-X) or hydrophobic (usually Leu-X, Phe-X or Tyr-X) residues (Wilk and Orlowski, 1980). In contrast to mammalian multicatalytic proteinases, the *Thermoplasma* proteinase does not hydrolyse peptide substrates with an arginyl residue adjacent to the leaving group but readily hydrolyses those peptide substrates with a phenylalanine residue attached to the leaving group (Dahlmann *et al.*, 1989). The *P. furiosus* proteinase complex also hydrolyses a number of proteins including the insoluble protein fibrin. The ability to hydrolyse proteins and specific peptide substrates supports observations made by Snowden *et al.* (1992) who demonstrated the existence of independent regulatory mechanisms from *P. furiosus* for the hydrolysis of different protein substrates.

The existence of a high molecular weight multimeric proteinase complex from *P. furiosus* cannot, therefore, be dismissed. Recent findings by Snowden *et al.* (1992) demonstrated that the 66kDa proteinase from *P. furiosus* cross reacted with antibodies against the bovine pituitary multicatalytic proteinase complex (MPC). It was therefore suggested that the 66kDa protein and bovine MPC are immunologically related and that the proteinases may be associated with a proteosome-like complex in *P. furiosus*.

Undoubtedly, this discovery would lead to discussions on the evolutionary importance of MPCs in the archaea. It is well known that the MPC is a multimeric high molecular weight proteinase ubiquitous among eukaryotes (Rivett, 1990). A simpler version consisting of two polypeptides, α- and β-subunits, has been described in the archaeobacterium *Thermoplasma acidophilum* (Dahlmann *et al.*, 1989). MPCs can contain up to 25 different polypeptides (Martins de Sa *et al.*, 1986; Rivett and Sweeney, 1991), each representing distinct catalytic and/or regulatory functions. Recent evidence has shown that an evolutionary relationship may exist between the eukaryotic and the archaeal proteinase complexes from alignments of the N-terminal sequences of proteosomal subunits from both domains (Zwickl *et al.*, 1992). An overall sequence homology of approximately 47% was obtained after comparisons of the amino acid sequences of the α- and β-subunits of both the eukaryotes and *Thermoplasma*, suggesting that the genes encoding the two subunits of the *Thermoplasma* proteosome arose from a common ancestor. In evolutionary terms, the conservation of the proteosome quaternary structure from *Thermoplasma* to higher eukaryotes such as rat (Baumeister *et al.*, 1988) is quite remarkable since it imposes strong structural constraints on subunit divergence. Further studies by Zwickl *et al.* (1992) indicated the possible functions of the two subunits; the α-subunit is thought to be responsible for the regulatory and targeting functions, whereas the β-subunit is responsible for the catalytic functions. Structural studies of both subunits indicated that although the β-subunit did not exhibit the sequence motif characteristic for serine proteinases, it was found to contain the essential amino acid residues for the formation of the catalytic triad.

From the results presented in this thesis, there is insufficient evidence to confirm whether the multiplicity of proteinases from *P. furiosus* form part of a proteosome-type complex as observed with *Thermoplasma*. Although current results from this thesis and recent findings by other research groups indicate a strong possibility, it would be interesting to determine whether a relationship exists between the *Thermoplasma* multicatalytic proteosome and the proteinase 'complex' from *P. furiosus*. A comparison of amino acid sequence between

these enzymes and those from other extremely thermophilic archaea would provide preliminary evidence on their evolutionary relationships.

REFERENCES

Achenbach-Richter, L., Gupta, R., Stetter, K.O. and Woese, C.R. (1987) Were the original eubacteria thermophiles? *Systematic and Applied Microbiology* **9**, 34-39.

Adams, M.W.W. (1990) The metabolism of hydrogen by extremely thermophilic, sulfur-dependent bacteria. *FEMS Microbiology Reviews* **75**, 219-238.

Aiyappa, P.S. Traficante, L.J. and Lampen, J.O. (1977) Penicillinase releasing protease of *Bacillus licheniformis:* Purification and general properties. *Journal of Bacteriology* **129**, 191-197.

Alexandrov, V,Ya. (1978) *Cells, Macromolecules and Temperature.* Springer-Verlag, Berlin.

Allen, M.B. (1953) The thermophilic aerobic sporeforming bacteria. *Bacteriological Reviews* **17**, 125.

Allsopp, A. (1969) Phylogenetic relationships of the procaryota and the origin of the eucaryotic cell. *New Phytology* **68**, 591-612.

Amelunxen, R.E. (1966). Crystallization of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus. Biochimica et Biophysica Acta* **122**, 175.

Amelunxen, R.E. and Lins, M. (1968). Comparative thermostability of enzymes from *Bacillus stearothermophilus* and *Bacillus cereus. Archives in Biochemistry and Biophysics* **125**, 765.

Amelunxen, R.E. and Murdock, A.L. (1978) Mechanisms of thermophily. *CRC Critical Reviews in Microbiology* 343-393.

Anfinsen, C.B. (1972) The formation and stabilisation of protein structure. *Biochemical Journal* **128**, 737-749.

Anfinsen, C.B. and Haber, E. (1961) Studies on the reduction and reformation of protein disulfide bonds. *The Journal of Biological Chemistry* **236**, 1361-1363.

Antranikian, G. (1990) Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. *FEMS Microbiology Reviews* **75**, 201-218.

Aono, S., Bryant, F.O. and Adams, M.W.W. (1989) A novel and remarkably thermostable ferrodoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus. Journal of Bacteriology* **171**, 3433-3439.

Argos, P., Rossman, M.G., Grau, U.H., Zuber, H., Franf, G. and Tratschin, J.D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5698.

Balch, W.E. and Wolfe, R.S. (1979) Transport of coenzyme M (2-mercaptoethanesulfonic acid) in *Methanobacterium ruminantium. Journal of Bacteriology* **137**, 264-273.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. (1979) Methanogens: Reevaluation of a unique biological group. *Microbiological Reviews* **43**, 260-296.

Barett, A.J. (1977) Proteinases in mammalian cells and tissues. *Research Monographs in Cell and Tissue Physiology*, Vol. 2. North-Holland, Amsterdam, New York, and Oxford.

Barett, A.J. and Rawlings, N.D. (1991) Proteinases. Types and families of endopeptidases. *Biochemical Society Transactions* **19**, 707-715.

Barker, H.A. (1956) *Bacterial fermentations.* John Wiley and Sons, Inc., New York.

Barnes, L.D. and Stellwagen, E. (1973) Enolase from thermophile *Thermus X-1. Biochemistry* **12**, 1559.

Bauman, A.J. and Simmonds, P.G. (1969) Fatty acids and polar lipids of extremely thermophilic filamentous bacterial masses from two Yellowstone hot springs. *Journal of Bacteriology* **98**, 528-531.

Baumeister, W., Dahlmann, B., Hergerl, R., Kopp, F., Kuehn, L. and Pfeifer, G. (1988) Electron microscopy and image analysis of the multicatalytic proteinase. *FEBS Letters* **241**, 239-245.

Belehradek, S. (1937) Le mechanisme physico-chemique de l'adaptation thermique. *Protoplasma* **12**, 406.

Bendzko, P. and Hintsche, R. (1978) Study on the conformational stability of the thermophilic alkaline serine protease thermitase. *Studies in Biophysics* **72**, 61-68.

Blow, D.M., Birktoft, J.J. and Hartley, B.S. (1969) Role of a buried acid group in the mechanism of action of chymotrypsin. *Nature* **221**, 337-340.

Blumentals, I.I., Robinson, A.S. and Kelly, R.M. (1990) Characterization of SDS-resistant proteolytic activity in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *Applied and Environmental Microbiology* **56**, 1992-1998.

Boccu, E., Veronese, F.M. and Fontana, A. (1976) Isolation and some properties of enolase from *Bacillus stearothermophilus* in *Enzymes and Proteins from Thermophilic Micro-organisms, Experentia Supplementum* 26, Zuber, H. Ed., Birkhauser Verlag, Basel, 229.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the prnciple of protein-dye binding. *Analytical Biochemistry* **72**, 248.

Brady, L., Brzozowski, A.M., Derewend, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenbull, J.P., Christia, L., Hugejens, B. and Norskov, L. (1990) A serine proteinase triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **343**, 767-770.

Bridgen, J., Kolb, E. and Harris, J.I. (1973) Amino acid sequence homology in alcohol dehydrogenase. *FEBS Letters* **33**, 1-3.

Brock, T.D. (1967) Life at high temperatures. *Science* **158**, 1012.

Brock, T.D., Brock, K.M., Belly, R.T. and Weiss, R.L. (1972) *Sulfolobus*: A new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Archives in Mikrobiologie* **84**, 54-68.

Brown, S.H. and Kelly, R.M. (1989) Cultivation techniques for hyperthermophilic archaebacteria: Continuous culture of *Pyrococcus furiosus* at temperatures near 100°C. *Applied and Environmental Microbiology* 55, 2086-2088.

Brown, S.H., Constantino, H.R. and Kelly, R.M. (1990) Characteriation of amylolytic enzyme activities associated with the hyperthermophilic archaebacterium *Pyrococcus furiosus. Applied and Environmental Microbiology* **56**, 1985-1991.

Brown, T.L., Yet, M., and Wold, F. (1982) Substrate-containing gel elctrophoresis: Sensitive detection of amylolytic, nucleolytic, and proteolytic enzymes. *Analytical Biochemistry* **122**, 164-172.

Bryant, F.O. and Adams, M.W.W. (1989) Characterization of hydrogenase from the hyperthermophilic archaebacterium, *Pyrococcus furiosus. The Journal of Biological Chemistry* **264**, 5070-5079.

Buckingham, J.A., Stanbury, P.F. and LeRoux, N.W. (1989) Effect of molybdenum on the efficiency of tetrathionate utilisation by chemostat cultures of *Sulfolobus* BC. *Biotechnology Letters* **11**, 99-104.

Cammarano, P., Tiboni, O. and Sanangelantoni, A.M. (1989) Phylogenetic conservation of antigenic determinants in archaebacterial elongation factors (Tu proteins). *Canadian Journal of Microbiology* 35, 2-10.

Careri, G., Fasella, P. and Gratton, E. (1975) Statistical time events in enzymes: a physical assessment. *CRC Critical Reviews in Biochemistry* **3**, 141-164.

Cavender, J.A. (1989) Mechanised derivation of linear invariants. ***Molecular and Biological Evolution*** **6**, 301-316.

Chan, H.S. and Dill, K.A. (1989) Intrachain loops in polymers. Effects of excluded volume. *Journal of Chemical Physics* **90**, 492-509.

Chatton, E. (1938) Titres et travoux scientifiques. *Sete*, Sottano, Italy.

Chell, R.M., Sundaram, T.K. and Wilkinson, A.E. (1978) Isolation and characterisation of isocitrate lyase from thermophilic *Bacillus* species. *Biochemical Journal* **173**, 165-177.

Cheng, Y-S.E. and Aronson, A.I. (1977) Alterations of spore coat processing and protein turnover in a *Bacillus cereus* mutant with a defective postexponential intracellular protease. *Proceedings of the National Academy of Sciences, USA* **74**, 1254-1258.

Cho, H-Y., Tanizawa, K., Tanaka, H. and Soda, K. (1988) Thermostable dipeptidase from *Bacillus stearothermophilus*: Its purification, characterization, and comparison with aminoacylase. *Journal of Biochemistry* **103**, 622-628.

Chothia, C. and Janin, J. (1975) Principles of protein-protein recognition. *Nature* **256**, 705-708.

Clarke, J.T. (1964) Simplified 'disc' (polyacrylamide gel) electrophoresis. *Annals of the New York Academy of Sciences* **121**, 428-435.

Coleman, R.D., Yang, S-S. and McAlister, M.P. (1987) Cloning of the debranching enzyme gene from *Thermoanaerobium brockii* into *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology* **169**, 4302-4307.

Connaris, H., Cowan, D.A. and Sharp, R.J. (1991) Heterogeneity of proteinases from the hyperthermophilic archaeobacterium *Pyrococcus furiosus. Journal of General Microbiology* **137**, 1193-1199.

Consalvi, V., Chiaraluce, R., Politi, L., Vaccaro, R., De Rosa, M. and Scandurra, R. (1991) Extremely thermostable glutamate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus furiosus. European Journal of Biochemistry* **202**, 1189-1196.

Constantino, H.R., Brown, S.H. and Kelly, R.M. (1990) Purification and characterisation of an α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus,* exhibiting a temperature optimum of 105° to 115°C. *Journal of Bacteriology* **172**, 3654-3660.

Cowan, D.A. (1980) An extracellular protease from an extreme thermophile. PhD Thesis, University of Waikato, New Zealand.

Cowan, D.A. and Daniel, R.M. (1982) Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile. *Biochimica et Biophysica Acta* **705**, 293-305.

Cowan, D.A., Daniel, R.M. and Morgan, H.W. (1987) The specific activities of mesophilic and thermophilic proteinases. *International Journal of Biochemistry* **19**, 741-743.

Cowan, D.A., Smolenski, K.A., Daniel, R.M. and Morgan, H.W. (1987) An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88°C. *Biochemical Journal* **247**, 121-133.

Crabb, J.W., Murdock, A.L. and Amelunxen, R.E. (1977) Purification and characterization of thermolabile glyceraldehyde-3-phosphate dehydrogenase from the facultative thermophile *Bacillus coagulans KU. Biochemistry* **16**, 4840.

Crewther, W.G. and Lennox, F.G. (1950) Preparation of crystals containing protease from *Aspergillus oryzae*. *Nature* **165**, 680.

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R. and Baumeister, W. (1989) The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. *FEBS Letters* **251**, 125-131.

Dahlquist, F.W., Long, J.W. and Bigbee, W.L. (1976) Role of calcium in the thermal stability of thermolysin. *Biochemistry* **15**, 1103-1111.

Daniel, R.M., Cowan, D.A., Morgan, H.W. and Curran, M.P. (1982) A correlation between protein thermostability and resistance to proteolysis. *Biochemical Journal* **207**, 641-644.

Danson, M.J. (1988) Archaebacteria: The comparative enzymology of their central metabolic pathways. *Advances in Microbial Physiology* **29**, 166-231.

Darland, G., Brock, T.D. Samsonoff, W. and Conti, S.F. (1970) A thermophilic acidophilic mycoplasma isolated from a coal refuse pile. *Science* **170**, 1416-1418.

Daron, H.H. (1970) Fatty acid composition of lipid extracts of a thermophilic *Bacillus* species. *Journal of Bacteriology* **101**, 145-151.

De Soete, G. (1983) A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621-626.

Drapeau, G.R. (1978) Role of a metalloprotease in activation of the precursor of *Staphylococcal* protease. *Journal of Bacteriology* **136**, 607-613.

Eggen, R., Geerling, A., Watts, J. and M. De Vos, W. (1990) Characterization of pyrolysin, a hyperthermoactive serine protease from the archaebacterium *Pyrococcus furiosus*. *FEMS Microbiology Letters* **71**, 17-20.

Eldjarn, Z. and Pihl, A. (1957) On the mode of action of X-ray protective agents. II. Interaction between biological important thiols and disulfides. *The Journal of Biological Chemistry* **225**, 499-510.

Endo, S. (1962) Studies on protease produced by thermophilic bacteria. *Hakko Kogasu Zasshi* **37**, 353.

Farrell, J. and Campbell, L.L. (1969) Thermophilic bacteria and bacteriophages. *Advances in Microbial Physiology* **3**, 83.

Fassina, G., Vita, C., Dalzoppo, D., Zamai, M., Zambonin, M. and Fontana, A. (1986) Autolysis of thermolysin. Isolation and characterisation of a three-fragment complex. *European Journal of Biochemistry* **156**, 221-228.

Felsenstein, J. (1982) Numerical methods for inferring evolutionary tree. *Quarterly Reviews in Biology* **57**, 379-404.

Felsenstein, J. (1984) The statistical approach to inferring evolutionary trees and what it tells us about parsimony and compatibility. *In* Cladistics: perspectives on the reconstruction of evolutionary history. T.Ducan and T.F.Steussy (eds.) Columbia University Press, New York. pp. 169-191.

Fiala, G. and Stetter, K.O. (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. *Archives of Microbiology* **145**, 56-61.

Fitch, W.M. (1981) A non-sequential method for constructing trees and hierarchial classifications. *Journal of Molecular Evolution* **18**, 30-37.

Fitch, W.M. and Margoliash. E. (1967) Construction of phylogenetic trees. *Science* **155**, 279-284.

Fontana, A. (1988) Structure and stability of thermophilic enzymes. Studies on thermolysin. *Biophysical Chemistry* **29**, 181-193.

Forterre, P., Elie, C., Kohiyama, M. (1984) Aphidocolin inhibits growth and DNA synthesis in halophilic archaebacteria. *Journal of Bacteriology* **159**, 800-802.

Fox, G.E., Peckman, K.J. and Woese, C.R. (1977) Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryote systematics. *International Journal of Systematic Bacteriology* **27**, 44-57.

Fox, G.E., Stackebrandt, E., Hepsell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N. and Woese, C.R. (1980) The phylogeny of prokaryotes. *Science* **209**, 457-463.

Fruton, J.S. (1976) The mechanism of catalytic action of pepsin and related acid proteinases. *Advances in Enzymology* **44**, A. Meister, ed., Interscience, New York, pp1-36.

Furth, A.J. (1980) Removing unbound detergent from hydrophobic proteins. *Analytical Biochemistry* **109**, 207-215.

Fusek, M., Lin, X-L. and Jordan, T. (1990) Enzymic properties of thermopsin. *The Journal of Biological Chemistry* **265**, 1496-1501.

Gaucher, G.M.and Stevenson, K.J. (1976) Thermomycolin. *Methods in Enzymology* **45** (L. Lorand, ed.) Academic Press, New York, San Francisco, and London, p415.

Ghelis, C. and Yon, J. (1982) Protein Folding. *Molecular Biology. An International Series of Monographs and Textbooks.* Academic Press, London.

Gilles, A-M., De Wolf, A. and Keil, B. (1983) Amino-acid sequences of the active site sulfhydryl peptide and other thiol peptides from the cysteine proteinases α-clostripain. *European Journal of Biochemistry* **130**, 473-479.

Glenn, A.R., Both, G.W., McInnes, J.L., May, B.K. and Elliott, W.H. (1973) Dynamic state of the messenger RNA pool specific for extracellular protease in *Bacillus amyloliquefaciens*: in revelance to the mechanism of enzyme secretion. *Journal of Molecular Biology* **73**, 221-230.

Goldberg, A.L. and Dice, J.F. (1974) Intracellular protein degradation in mammalian and bacterial cells. *Annual Review of Biochemistry* **43**, 835-867.

Gouy, M. and Li, W.H. (1989) Phylogenetic analysis based on ribosomal RNA sequences supports the archaebacterial rather than the eocyte tree. *Nature* **339**, 145-147.

Granelli-Piperno, A. and Reich, E. (1978) A study of proteases and protease-inhibitor complexes in biological fluids. *Journal in Experimental Medicine* **148**, 223-234.

Gurd, F.R.N. and Rothgeb, T.M. (1979) Motions in Proteins. *Advances in Protein Chemistry* **33**, 73-165.

Haber, E. and Anfinsen, C.B. (1962) Side-chain interactions governing the pairing of half-cystine residues in ribonuclease. *The Journal of Biological Chemistry* **237**, 1839-1844.

Hachimori, A., Matsunaga, A., Shimizu, M., Samejima, T. and Nosoh, Y. (1974) Purification and properties of glutamine synthetase from *Bacillus stearothermophilus*. *Biochimica et Biophysica Acta* **350**, 461-474.

Hachimori, A., Shiroya, Y., Hirato, A., Miyahara, T. and Samejima, T. (1979) Effects of divalent cations on thermophilic inorganic phosphatase. *Journal of Biochemistry (Tokyo)* **86**, 121-130.

Hanner, M., Redl, B. and Stoffler, G. (1990) Isolation and characterization of an intracellular aminopeptidase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. *Biochimica et Biophysica Acta* **1033**, 148-153.

Hartley, B.S. (1960) Proteolytic enzymes. *Annual Review of Biochemistry* **29**, 45-72.

Hausdorf, G., Kruger, K. and Honne, W.E. (1980) Thermitase, a thermostable serine protease from *Thermoactinomyces vulgaris*. Classification as a subtilisin-type protease. *International Journal of Peptide and Protein Research* **15**, 420-429.

Heilbrunn, L.V. (1924) The colloid chemistry of protoplasm. IV. The heat coagulation of protoplasm. *American Journal of Physiology* **69**, 190.

Heinen, U.J. and Heinen, W. (1972) Characteristics and properties of a caldo-active bacterium producing extracellular enzymes and two related strains. *Archives of Microbiogy* **82**, 1.

Heinen, W. and Lauwers, A.M. (1976) Amylase activity and stability at high and low temperatures depending on calcium and other divalent cations, in *Enzymes and Proteins from Thermophilic Micro-organisms, Experientia Supplementum* 26, Zuber, H. Ed., Birkhauser verlag, Basel, 77.

Hensel, R., Zwickl, P., Fabry, S., Lang, J. and Palm, P. (1989) Sequence comparison of glyceraldehyde-3-phosphate dehydrogenases from the three urkingdoms: evolutionary implication. *Canadian Journal of Microbiology* **35**, 81-85.

Heussen, C. and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. *Analytical Biochemistry* **102**, 196-202.

Hibino, Y., Nosoh, Y. and Samejima, T. (1974) On the conformation of NADP-dependent isocitrate dehydrogenase from *Bacillus stearothermophilus*. *Journal of Biochemistry (Tokyo)* **75**, 553.

Hilpert, R., Winter, J., Hammes, W. and Kandler, O. (1981) The sensitivity of archaebacteria to antibiotics. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe* **C 2**: 11-20.

Hippe, H. (1984) Maintenance of methanogenic bacteria. In *Maintenance of Microorganisms* ed. Kirsop, B.E. and Snell, J.J.S. Academic Press, London, pp69-81.

Hocking, J.D. and Harris, J.I. (1976) Glyceraldehyde-3-phosphate dehydrogenase from an extreme thermophile, *Thermus aquaticus* in *Enzymes and Proteins from Thermophilic Micro-organisms, Experientia Supplementum* 26, Zuber, H. Ed., Birkhauser Verlag,Basel, 121.

Holmquist, R., Miyamoto, M.M. and Goodman, M. (1988) Analysis of higher-primate phylogeny from transversion differences in nuclear and mitochondrial DNA by Lake's methods of evolutionary parsimony and operator metrics. *Molecular Biology in Evolution* **3**, 1-18.

Horie, N., Fukuyama, K., Ito, Y. and Epstein, W.L. (1984) Detection and characterisation of epidermal proteinases by polyacrylamide gel electrophoresis. *Comparative Biochemistry and Physiology* **77B**, 349-353.

Iijima, S., Saiki, T. and Beppu, T. (1980) Physicochemical and catalytic properties of thermostable malate dehydrogenase from an extreme thermophile *Thermus flavus* AT-62. *Biochimica et Biophysica Acta* **613**, 1-9.

Iwabe, N., Kuma, K-I., Hasegawa, M., Osawa, S. and Miyata, T. (1989) Evolutionary relationship of archaebacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. *Proceedings of the National Academy of Sciences, USA* **86**, 9355-9359.

James, M.N.G. (1980) An x-ray crystallographic approach to enzyme structure and function. *Canadian Journal of Biochemistry* **58**, 251-271.

Kagawa, Y., Sone, N., Hirata, H. and Yoshida, M. (1979) Reconstitution of ATPase and carriers from thermophilic biomembranes. *Trends in Biochemical Sciences* **4**, 31-33

Kagawa, Y., Sone, N., Yoshida, M., Hirata, H. and Okamoto, H. (1976) Proton translocating ATPase of a thermophilic bacterium. Morphology, subunits and chemical composition. *Journal of Biochemistry* **80**, 141-151.

Kaine, B.P., Gupta, R.and Woese, C.R. (1983) Putative introns in transfer RNA genes of prokaryotes. *Proceedings for the National Academy of Sciences* **80**, 3309-3312.

Kandler, O. (1982) Cell wall structures and their phylogenetic implications. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg.Abt. 1. Orig. Reihe* **C 3**: 149-160.

Katchalski-Katzir, E. and Freeman, A. (1982) Enzyme engineering reaching maturity. *Trends in Biochemical Sciences* **7**, 737-749.

Kates, M., Park, C.E., Palaneta, B. and Joo. C.N. (1972) Synthesis of diphytanyl ether analogues of phosphatidic acid and cytidine diphosphate diglyceride. *Canadian Journal of Biochemistry* **50**, 109.

Kelleher, P.J and Juliano, R.L. (1984) Detection of proteases in polyacrylamide gels containing covalently bound substrates. *Analytical Biochemistry* **136**, 470-475.

Kelly, R.M. and Deming, J.D. (1988) Extremely thermophilic archaebacteria: Biological and engineering considerations. *Biotechnology Progress* **4**, 47-62.

Keradopoulos, D. and Wulff, K. (1974) Thermophilic alanine dehydrogenase from *Halobacterium salinarium*. *Canadian Journal of Biochemistry* **52**, 1033.

Kessel, M. and Klink, F. (1980) Archaebacterial elongation factor is ADP-ribosylated by diptheria toxin. *Nature* **287**, 250-251.

Khoo, T.C., Cowan, D.A., Daniel, R.M. and Morgan, H.W. (1984) Interactions of calcium and other metal ions with caldolysin, the thermostable proteinase from *Thermus aquaticus* strain T-351. *Biochemical Journal* **221**, 407-413.

Kjems, J. and Garrett, R.A. (1985) An intron in the 23S rRNA gene of the archaebacterium *Desulfurococcus mobilis*. *Nature* **318**, 675-677.

Klingeberg, M., Hashwa, F. and Antranikian, G. (1991) Properties of extremely thermostable proteases from anaerobic hyperthermophilic bacteria. *Applied Microbiology and Biotechnology* **34**, 715-719.

Koch, R., Zablowski, P., Spreinat, A. and Antranikian, G. (1990) Extremely thermostable amylolytic enzymes from the archaebacterium *Pyrococcus furiosus*. *FEMS Letters* **71**, 21-26.

Koffler, H. (1957) Protoplasmic differences between mesophiles and thermophiles. *Bacteriological Reviews* **27**, 227.

Kristjansson, M.M. and Kinsella, J.E. (1990) Alkaline serine proteinase from *Thermomonospora fusca* YX. Stability to heat and denaturants. *Biochemical Journal* **270**, 51-55.

Kushner, D.J., Bayley, S.T. Boring, J., Kates, M. and Gibbons, N.E. (1964) Morphological and chemical properties of cell envelopes of the extreme halophile, *Halobacterium cutiribrum*. *Canadian Journal of Microbiology* **10**, 483-497.

Laane, C., Tramper, J. and Lilly, M.D. (1987) *Biocatalysis in Organic Media.* Elsevier, Amsterdam pp 65-84.

Lacks, S.A. and Springhorn, S.S. (1980) Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. *The Journal of Biological Chemistry* **255**, 7467-7473.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lake, J.A. (1987). A rate independent technique for analysis of nucleic acid sequences. Evolutionary parsimony. *Molecular and Biological Evolution* **4**, 167-191.

Lake, J.A. (1988) Origin of the eukaryotic nucleus determined by rate-invariant analysis of ribosomal RNA sequences. *Nature* **331**, 184-186.

Lake, J.A. (1989) Origin of the eukaryotic nucleus: eukaryotes and eocytes are genotypically related. *Canadian Journal of Microbiology* **35**, 109-118.

Lake, J.A., Clark, M.W., Henderson, E., Fay, S.P., Oakes, M., Scheinman, A., Thornber, J.P. and Mah, R.A. (1985) Eubacteria, Halobacteria, and the origin of photosynthesis: The photocytes. *Proceedings for the National Academy of Sciences, USA* **82**, 3716-3720.

Langworthy, T.A. and Pond, J.L. (1986) Archaebacterial ether lipids and chemotaxonomy. *Systematic and Applied Microbiology* 7, 253-257.

Langworthy, T.A., Smith, P.F. and Mayberry, W.R. (1974) Long chain glycerol diether and polyol dialkyl glycerol triether lipids of *Sulfolobus acidocaldarius. Journal of Bacteriology* **119**, 106-116.

Larsen, H. (1962) *Family V. Halobacteriaceae.* Gibbons p. 261-267. *In* N.R. Kreig and J.G. Holt (eds.), *Bergeys manual of systematic bacteriology.* The Williams and Wilkins Co., Baltimore.

Lin, S.H., Konishi, Y., Denton, M.E. and Scheraga, H.A (1984) Influence of an extrinsic cross-link on the folding pathway of ribonuclease A. Conformational and thermodynamic analysis of cross-linked (lysine^{7}-lysine^{41})-ribonuclease A. *Biochemistry* **23**, 5504-5512.

Lin, X-L. and Tang, J. (1990) Purification, characterization, and gene cloning of thermopsin, a thermostable acid protease from *Sulfolobus acidocaldarius. The Journal of Biological Chemistry* **265**, 1490-1495.

Ljundahl, L.G., Peterson, B., Eriksson, K.E. and Wiegel, S. (1983) A yellow affinity substance involved in the cellulolytic system of *Clostridium thermocellum. Current Microbiology* **9**, 195-199.

Ljungdahl, L. and Sherod, D. (1976) Proteins from thermophilic micro-organisms. In *Extreme Environments - Mechanisms of Microbial Adaptation* (M.R. Heinrich, ed.) Academic Press, New York, Sa Francisco and London, p147.

Malik, B., Su, W-w., Wald, H.L., Blumentals, I.I. and Kelly, R.M. (1989) Growth and gas production for the hyperthermophilic archaebacterium, *Pyrococcus furiosus. Biotechnology and Bioengineering* **34**, 1050-1057.

Martin, A., Yeats, S., Janekovic, D., Reiter, W., Aicher, W. and Zillig, W. (1984) SAV 1, a temperate uv-inducible DNA virus-like particle rfom the archaebacterium *Sulfolobus acidocaldarius* isolate B12. *EMBO Journal* **3**, 2165.

Martin, R.G. and Ames, B.N. (1961) A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. *The Journal of Biological Chemistry* **236**,1372-1379.

Mathur, E.J., Adams, M.W.W., Callen, W.N. and Cline, J.M. (1991) The DNA polymerase gene from the hyperthermophilic marine archaebacterium, *Pyrococcus furiosus,* shows sequence homology with α-like DNA polymerases. *Nucleic Acids Research* **19**, 6952.

Matsumura, M., Becktel, W.J. amd Matthews, B.W. (1988) Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature* **334**, 406-410.

Matsumura, M., Katakura, Y., Imanaka, T. and Aiba, S. (1984) Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic *Bacilli* in comparison with that encoded by plasmid pUB110. *Journal of Bacteriology* **160**, 413-420.

Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwon S-T. and Ohta, T. (1988) Purification and characterization of aqualysin I (a thermophilic alkaline serine protease) produced by *Thermus aquaticus* YT-1. *European Journal of Biochemistry* **171**, 441-447.

Matthews, B.W. (1988) Structural basis of the action of thermolysin and related zinc peptidases. *Accounts of Chemical Research* **21**, 333-340.

Matthews, B.W., Weaver, L.H. and Kester, W.R. (1974) The conformation of thermolysin. *The Journal of Biological Chemistry* **249**, 8030-8044.

McCammon, J.A. and Karplus, M. (1983) The dynamic picture of protein structure. *Accounts of Chemical Research* **16**, 187-193.

McCarroll, R., Olsen, G.J., Stahl, Y.D., Woese, C.R. and Sogin, M.L. (1983) Nucleotide sequence of the *Dictyostelium discoideum* small subunit ribosomal RNA inferred from the gene sequence. Evolutionary implications. *Biochemistry* **22**, 5858-5868.

Means, G.E. and Feeny, R.E. (1971) *Chemical Modification of Proteins.* Holden-Day, Inc., San Francisco.

Melasniemi, H. (1987) Characterisation of α-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum.* Evidence for a novel thermostable amylase. *Biochemical Journal* **246**, 193-197.

Militzer, W., Sonderegger, T.B., Tuttle, L.C. and Georgi, C.E. (1949) Thermal enzymes. *Archives in Biochemistry* **24**, 75.

Morihara, K. (1974) Comparative specificity of microbial proteinases. *Advances in Enzymology* **41**, 179-243.

Mukund, S. and Adams, M.W.W. (1990) Characterisation of a tungsten-iron-sulfur protein exhibiting novel spectroscopic and redox properties from the hyperthermophilic archaebacterium *Pyrococcus furiosus. The Journal of Biological Chemistry* **265**, 11508-11516.

Mukund, S. and Adams, M.W.W. (1991) The novel tungsten-iron-sulfur protein of the hyperthermophilic archaebacterium, *Pyrococcus furiosus,* ia an aldehyde ferrodoxin oxidoreductase. Evidence for its participation in a unique glycolytic pathway. *The Journal of Biological Chemistry* **266**, 14208-14216.

Murray, R.G.E. (1974) A place for bacteria in the living world, p 4-9 In R.E. Buchanan and N.E. Gibbons (eds.), *Bergey's Manual of Determinate Bacteriology, 8th ed.* The Williams and Wilkins Co., Baltimore.

Norris, P.R., Marsh, R.M. and Lindstrom, E.B. (1986) Growth of mesophilic and thermophilic acidophilic bacteria on sulfur and tetrathionate. *Biotechnology and Applied Biochemistry* **8**, 318-329.

Nystrom, T., Flardh, K. and Kjelleberg, S. (1990) Responses to multiple-nutrient starvation in marine *Vibrio* strain CCUG 15956. *Journal of Bacteriology* **172**, 7085-7097.

O'Brien, R.T. and Campbell, L.L. (1957) Purification and properties of a proteolytic enzyme from *Bacillus stearothermophilus. Archives of Biochemistry and Biophysics* **70**, 432.

Ogasahara, K., Imanishi, A. and Isemura, T. (1970) Studies on thermophilic α-amylase from *Bacillus stearothermophilus.* II. Thermal stability of thermophilic α-amylase. *Journal of Biochemistry (Tokyo)* **67**, 77.

Ohta, Y. (1967) Thermostable protease from thermophilic bacteria. I. Studies on the stability of the protease. *The Journal of Biological Chemistry* **242**, 509-515.

Oshima, T. (1982) A pentaamine is present in an extreme thermophile. *The Journal of Biological Chemistry* **257**, 9913-9914.

Owusu, R.K. and Cowan, D.A. (1989) Correlation between microbial protein thermostability and resistance to denaturation in aqueous-organic solvent 2-phase systems. *Enzyme and Microbial Technology* **11**, 568-574.

Pace, C.N., Grimsley, G.R., Thomson, J.A. and Barnett, B.J. (1988) Conformational stability and activity of ribonuclease T1 with zero, one, and two disulfide bonds. *The Journal of Biological Chemistry* **263**, 11820-11825.

Payne, J.W. (1980) Transport and utilisation of peptides by bacteria, p. 211-256. *In* J.W. Payne (ed.), *Microorganisms and nitrogen sources.* John Wiley and sons, Chichester, Great Britain.

Pecher, T. and Bock, A. (1981) *In vivo* susceptibility of halophilic and methanogenic organisms to protein synthesis inhibitors. *FEMS Letters* **10**, 295-297.

Perry, L.J. and Wetzel, R. (1984) Disulfide bond engineered into T4 lysozyme: Stabilization of the protein towards thermal inactivation. *Science* **226**, 555-557.

Perutz, M.F. (1978) Electrostatic effects in proteins. *Science* **201**, 1187-1191.

Peterson, G.L. (1977) A simplification of the protein assay method of Lowry *et al*, which is more generally applicable. *Analytical Biochemistry* **83**, 346-356.

Pfeuller, S.L. and Elliott, W.H. (1969) The extracellular α-amylase of *Bacillus stearothermophilus. The Journal of Biological Chemistry* **244**, 48.

Polgar, L. and Bender, M.L. (1969) the nature of general base-general acid catalysis in serine proteases. *Proceedings of the National Academy of Sciences, USA* **64**, 1335-1342.

Prasad, A.R.S. and Maheshwari, R. (1978) Purification and properties of trehalase from the thermophilic fungus *Humicola lanuginosa*. *Biochimica et Biophysica Acta* **525**, 162-170.

Rao, J.K.M. and Argos, P. (1981) Structural stability of halophilic proteins. *Biochemistry* **20**, 6536-6543.

Raven, N.D.H., Marks, T.S., Ladwa, N.M/, Cossar, J.D. and Sharp, R.J. (1991) Continuous culture of *Pyrococcus furiosus*. Poster presentation at the SGM Fermentation Technology Symposium, Nottingham, September.

Ray, P.H., White, D.C. and Brock, T.D. (1971a) Effect of temperature on the fatty acid composition of *Thermus aquaticus*. *Journal of Bacteriology* **106**, 25-30.

Ray, P.H., White, D.C. and Brock, T.D. (1971b) Effect of growth temperature on the lipid composition of *Thermus aquaticus*. *Journal of Bacteriology* **108**, 227-235.

Rees, D.C. and Lipscomb, W.N. (1981) Binding of ligands to the active site of carboxypeptidase A. *Proceedings of the National Academy of Sciences, USA* **78**, 3408-3412.

Reiter, W.D., Palm, P. and Zillig, W. (1988) Analysis of transcription in the archaebacterium *Sulfolobus* indicates that archaenbacterial promoters are homologous to eukaryotic Pol II promoters. *Nucleic Acids Research* **16**, 1-19.

Ringe, D. and Petsko, G.A. (1985) Mapping protein dynamics by X-ray diffusion. *Progress in Biophysics and Molecular Biology* **45**, 197-235.

Rivett, A.J. (1989a) High molecular mass intracellular proteases. *Biochemical Journal* **263**, 625-633.

Rivett, A.J. (1989b) The multicatalytic proteinase. Multiple proteolytic activities. *The Journal of Biological Chemistry* **264**, 12215-12219.

Rivett, A.J. and Sweeney, S.T. (1991) Properties of subunits of the multicatalytic proteinase complex revealed by the use of subunit specific antibodies. *Biochemical Journal* **278**, 171-177.

Roncari, G. and Zuber, H. (1969) Thermophilic aminopeptidases from *Bacillus stearothermophilus*. I. Isolation, specificity, and general properties of the thermostable aminopeptidase I. *Protein Research* **1**, 45-61.

Saravani, G-A., Cowan, D.A., Daniel, R.M. and Morgan, H.W. (1989) Caldolase, a chelator-insensitive extracellular serine proteinase from a *Thermus* spp. *Biochemical Journal* **262**, 409-416.

Schafer, T. and Schonheit, P. (1991) Pyruvate metabolism of the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Acetate formation from acetyl CoA and ATP synthesis are catalysed by an acetyl Coa A synthetase (ADP-forming). *Archives of Microbiology* **155**, 366-377.

Schellman, J.A. (1976) The effect of binding on the melting temperature of biopolymers. *Biopolymers* **15**, 999-1000.

Schmid, G., Pecher, T. and Bock, A. (1982) Properties of the translational apparatus of archaebacteria. *Zentralbl. Bakteriologika, Mikrobiological Hyg. Abt. 1, Original.* **C3**, 209-217.

Schnabel, H., Zillig, W., Pfaffle, M., Schnabel, R., Michel, H. and Delius, H. (1982) *Halobacterium halobium* phage phi-H. *EMBO Journal* **1**, 87-92.

Schulz, G.E. and Schirmer, R.H. (1979) *Principles of protein structure*. Springer-Verlag, New York, Heidelberg, Berlin.

Searcy, D.G. and Delange, R.J. (1980) *Thermoplasma acidophilum* histone-like protein: Partial amino acid sequence suggestive of homology to eukaryotic histones. *Biochimica et Biophysica Acta* **609**, 197-200.

Searcy, D.G. and Stein, D.B. (1980) Nucleoprotein subunit structure in an unusual prokaryotic organism: *Thermoplasma acidophilum. Biochimica et Biophysica Acta* **609**, 180-195.

Sehgal, S.N., Kates, M. and Gibbons, N.E. (1962) Lipids of *Halobacterium cutiribrum. Canadian Journal of Biochemistry* **40**, 69-81.

Shinmyo, A., Okazaki, M. and Terui, G. (1968) Kinetic studies on enzyme production by microbes. 4. Some physiological basis for kinetic studies on acid protease production by *Aspergillus niger. Journal of Fermentation Technology* **46**, 733.

Sidler, W. and Zuber, H. (1972) Neutral proteases with different thermostabilities from a facultative strain of *Bacillus stearothermophilus* grown at 40° and at 50°. *FEBS Letters* **25**, 292-294.

Singleton, R., Jr., Kimmel, J.R. and Amelunxen, R.E. (1969) The amino acid composition and other properties of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus. Journal of Biological Chemistry* **244**, 1623.

Skaja, A.K., Blumentals, I.L., Brown, S.H., Lessick, R.C. Anfinsen, C.E., Robb, F.T. and Kelly, R. (1989) Characterization and thermostability analysis of proteases from the hyperthermophile *Pyrococcus furiosus.* Presented at the 1989 Annual Meeting of the American Chemical Society, Miami, Florida, September 12.

Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical taxonomy.* W.H.Freeman and Company, New York.

Snowden, L.J., Blumentals, I.I. and Kelly, R.M. (1992) Regulation of proteolytic activity in the hyperthermophile *Pyrococcus furiosus. Applied and Environmental Microbiology* 58, 1134-1141.

Sogin, S.J., Sogin, M.L. and Woese, C.R. (1972) Phylogenetic measurement in procaryotes by primary structural characterization. *Journal of Molecular Evolution* **1**, 173-184.

Spreinat, A. and Antranikian, G. (1990) Purification and properties of a thermostable pullulanase from *Clostridium thermosulfurogenes* EM1 which hydrolyses both α-1,6 and α-1,4- glycosidic linkages. *Applied Microbiology and Biotechnology* **33**, 511-518.

Stamato, F.M.L.G., Longo, E. and Yoshioka, L.M. (1984) The catalytic mechanism of serine proteases: single proton versus double proton transfer. *Journal of Theoretical Biology* **107**, 329-338.

Stellwagen, E. and Barnes, L.D. (1976) Analysis of the thermostability of enolases. In *Enzymes and Proteins from Thermophilic Micro-organisms. Experientia Supplementum* **26**, (H. Zuber, ed.) Birkhayser Verlag, Basel and Stuttgart, p223.

Stetter, K.O., Fiala, G., Huber, G., Huber, R. and Sergerer, A. (1990) Hyperthermophilic micro-organisms. *FEMS Microbiology Reviews* **75**, 117-124.

Stetter, K.O., Konig, H. and Stackerbrandt, E. (1983) *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulfur reducing archaebacteria growing optimally at 105°C. *Systematic and Applied Microbiology* **4**, 535-551.

Sundaram, T.K., Chell, R.M. and Wilkinson, A.E. (1980) Monomeric malate synthase from a thermophilic *Bacillus*. Molecular and kinetic characteristics. *Archives of Biochemistry and Biophysics* **199**, 515-525.

Suzuki, K. and Imahori, K. (1973) Glyceraldehyde-3-phosphate dehydrogenase of *Bacillus stearothermophilus*. Kinetics and physicochemical studies. *Journal of Biochemistry (Tokyo)* **74**, 955.

Suzuki, Y. and Imai, T. (1982) Abnormally high tolerance against proteolysis of an exo-oligo-1,6-glucosidase from a thermophile *Bacillus thermoglucosidus* KP 1006, compared

with its mesophilic counterpart from *Bacillus cereus* ATCC 7064. ***Biochimica et Biophysica Acta*** **705**, 124-126.

Tagaki, H., Takahashi, T., Momose, H., Inouye, M., Maeda, Y., Matsuzawa, H. and Ohta, T. (1990) Enhancement of the thermostability of subtilisin E by introduction of disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. *The Journal of Biological Chemistry* **265**, 6874-6878.

Taguchi, H., Hamaoki, M., Matsuzama, H. and Ohta, T. (1983) Heat stable extracellular proteolytic enzyme produced by *Thermus caldophilus* strain GK24, an extremely thermophilic bacterium. *Journal of Biochemistry* **93**, 7-13.

Tai, J.Y., Kortt, A.A., Liu, T-Y. and Elliott, S.D. (1976) Primary structure of streptococcal proteinase. III. Isolation of cyanogen bromide peptides: complete covalent structures of the polypeptide chain. *The Journal of Biological Chemistry* **251**, 1955-1959.

Tajima, M., Urabe, I., Yutani, K. and Okada, H. (1976) Role of calacium ions in the thermostability of thermolysin and *Bacillus subtilis* var. *amylosacchariticus* neutral protease. *European Journal of Biochemistry* **64**, 243-247.

Titani, K., Hermodso, M.A., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1972) Amino acid sequence of thermolysin. Isolation and chracterisation of fragments obtained by the cleavage with cyanogen bromide. *Biochemistry* **11**, 2427.

Van de Castelle,M., Demarez, M., Legrain, C., Glansdorff, N.(1990) Pathways of arginine biosynthesis in extreme thermophic archaebacteria and eubacteria. *Journal of General Micribiology* **136**, 1177.

Veronese, F.M., Boccu, E., Schiavon, C., Grandi, C. and Fontana, A. (1984) General stability of thermophilic enzymes. Studies on 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus and yeast. Journal of Applied Biochemistry* **6**, 39-47.

Vita, C., Fontana, A. and Jaenicke, R. (1989) Folding of thermolysin fragments. Hydrodynamic properties of isolated domains and subdomains. *European Journal of Biochemistry* **183**, 513-518.

Volkin, D.B. and Klibanov, A.M. (1987) Thermal destruction processes in proteins involving cystine residues. *The Journal of Biological Chemistry* **262**, 2945-2950.

Volkin, D.B. and Klibanov, A.M. (1990) Minimising protein inactivation, p1-24 *In* T.E. Creighton (ed.), *Protein function: a practical approach.* IRL Press, Oxford.

Wang, J.H. (1968) Facilitated proton transfer in enzyme catalysis. *Science* **161**, 328-334.

Ward, O.P. (1983) Proteinases in: *Microbial Enzymes and Biotechnology,* Fogarty, W.M. ed., Applied Science, London, New York, p251-317.

Wedler, F.C. and Hoffmann, F.M. (1974) Glutamine synthetase of *Bacillus stearothermophilus.* I. Purification and basic properties. *Biochemistry* **13**, 3207.

Wedler, F.C., Carfi, J. and Ashour, A.E. (1976) Glutamine synthetase of *Bacillus stearothermophilus.* Regulation, site ineractions and functional information. *Biochemistry* **15**, 1749-1755.

Wetzel, R., Perry, L.J., Baase, W.A. and Becktel, W.J. (1988) Disulphide bonds and thermal stability in T4 lysozyme. *Proceedings of the National Academy of Science, USA* **85**, 401-405.

White, Jr., F.H. (1969) Regeneration of native secondary and tertiary structures by air oxidation of reduced ribonuclease. *The Journal of Biological Chemistry* **236**, 1353-1360.

Wiegel, J. and Ljundahl, L.G. (1985) The importance of thermophilic bacteria in biotechnology in *CRC Critical Reviews in Biotechnology* **3**, 39-108.

Wilk, S. and Orlowski, M. (1983) Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *Journal of Neurochemistry* **40**, 842-849.

Williams, R.A.D. (1975) Caldoactive and thermophilic bacteria and their thermostable proteins. *Science Progress (Oxford)* **62**, 373.

Woese, C.R. (1983) The primary lines of decent and the universal ancestor p 209-233. *In*, D. Bendall (ed.) *Evolution from molecules to men.* Cambridge University Press, Cambridge.

Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Woese, C.R. and Fox, G.E. (1977) Phylogenetic structures of the prokaryotic domain: the primary kingdoms. *Proceeding of the National Academy of Sciences, USA* **74**, 5088-5090.

Woese, C.R. and Olsen, G.J. (1986) Archaebacterial phylogeny: perspectives on the urkingdoms. *Systematic and Applied Microbiology* **7**, 161-177.

Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings for the National Academy of Sciences, USA* **87**, 4576-4579.

Woese, C.R., Magrum, L.J. and Fox, G.E. (1978) Archaebacteria. *Journal of Molecular Evolution* **11**, 245-252.

Wolters, J. and Erdmann, V.A. (1986) A 5S rRNA tertiary structural model inspired by the known transfer RNA structure. *Endocytobiosis and Cell Research* **3**, 157-166.

Wrba, A., Jaenicke, R., Huber, R. and Stetter, K.O. (1990) Lactate dehydrogenase from the extreme thermophile *Thermotoga maritima. European Journal of Biochemistry* **188**, 195-201.

Yutani, K., Ogasahara, K., Sugino, Y. and Matsushiro, A. (1977) Effect of a single amino acid substitution on the stability of conformation of a protein. *Nature* **267**, 274-275.

Zabel, H.P., Konig, H. and Winter, J. (1984) Isolation and characterisation of a new coccoid methanogen, *Methanogenium tatii*, spec. nov. from a solfataric field on Mount Tatio. *Archives of Microbiology* **137**, 3087-315.

Zaks, A. and Klibanov, A. (1988) Enzymatic catalysis in nonaqueous solvents. *The Journal of Biological Chemistry* **263**, 3194-3201.

Zillig, W., Yeats, S., Holz, I., Bock, A., Gropp, F. and Simon, G. (1986) *Desulfurolobus ambivalens* genus nov. species nov., an autotrophic archaebacterium facultatively oxidising or reducing sulphur. *Systematic and Applied Microbiology* **8**, 197-203.

Zillig, W., Holz, I., Janekovic, D., Klenk, H-P., Imsel, E., Trent, J., Wunderl, S., Forjaz, V.H., Coutinho, R. and Ferreira, T. (1990) *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaebacterium that ferments peptides. *Journal of Bacteriology* **172**, 3959-3965.

Zillig, W., Holz, I., Klenk, H-P., Trent, J., Wunderl, S., Janekovic, D., Imsel, E. and Haas, B. (1987) *Pyrococcus woesei*, sp. nov., an ultra-thermophilic marine archaebacterium, representing a novel order, *Thermococcales. Systematic and Applied Microbiology* **9**, 62-70.

Zillig, W., Stetter, K.O. and Tobien, M. (1978) DNA dependent RNA polymerase from *Halobacterium halobium. European Journal of Biochemistry* **91**, 193-199.

Zuber, H. (1981) *Structural and functional aspects of enzyme catalysis*, (H.Eggerer and H. Huber, eds.) Springer-Verlag, Heidelberg, p114.

Zuckerkandl, E. and Pauling, L. (1965) Molecules as documents of evolutionary history. *Journal of Theoretical Biology* **8**, 357-366.

Zwickl, P., Fabry, S., Bogedian, C., Haas, A. and Heinsel, R. (1990) Glyceraldehyde-3-phospahte dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*: Characterisation of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*. *Journal of Bacteriology* **172**, 4329-4338.

Zwickl, P., Grzima, A., Puhler, G., Dahlmann, B., Lottspeich, F. and Baumeister, W. (1992) Primary structure of the *Thermoplasma* proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase. *Biochemistry* **31**, 964-972.

PUBLICATIONS

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Preservation of the hyperthermophile *Pyrococcus furiosus*

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Methods are evaluated for the preservation of the hyperthermophile *Pyrococcus furiosus*. The use of glass capillary tubes stored over liquid nitrogen with dimethyl sulphoxide appears to be the preferred method of preservation. Lyophilization resulted in loss of viability and storage at room temperature and +4°C resulted in considerable loss of viability within 4 weeks.

Procedures and problems associated with the preservation of micro-organisms have been discussed by Kirsop & Snell (1984) and Sharp (1984). The choice of preservation method depends on a number of factors including convenience, availability of equipment, the stability of the organism and its ability to survive the processes involved. Most organisms survive lyophilization although it is unsatisfactory for several strains of *Vibrio*, *Myxobacterium*, *Halobacterium*, non-sporulating *Streptomyces* and most algae and protozoa. Cryopreservation, using either liquid nitrogen or mechanical refrigeration, has become more widely used although there are obvious problems in the transport of frozen cultures.

Thermophilic micro-organisms including *Bacillus* and *Thermus* species have been successfully preserved in our laboratories by both cryopreservation and lyophilization although lyophilization has been observed to result in plasmid loss in some strains of *Thermus* (Sharp 1984). Following the isolation of a number of unidentified, extremely thermophilic archaebacteria we used similar procedures for their preservation. This proved unsatisfactory with the loss of many strains and we therefore examined alternative procedures for the preservation of extremely thermophilic archaebacterial isolates. This paper describes and compares the effectiveness of the procedures used to preserve the hyperthermophilic, sulphur-metabolizing archaebacterium *Pyrococcus furiosus*. This heterotrophic organism isolated from hydrothermal vents off the coast of Italy grows optimally at 100°C under strictly anaerobic conditions.

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Materials and Methods

PREPARATION OF CELL SUSPENSION

The hyperthermophilic archaebacterium *P. furiosus* (DSM 3638) was grown anaerobically in 20 ml serum bottles as described by Fiala & Stetter (1986). After 17 h incubation at 98°C, direct cell counts were obtained and cells were harvested by centrifugation (20 000 **g** for 15 min). Aliquots of cell pellets suspended in growth medium alone, or in growth medium containing sterile 5% (v/v) dimethyl sulphoxide (DMSO) or 10% (v/v) glycerol were prepared to give a final cell density of approximately 10^9 cells/ml of solution. These cell suspensions were treated as follows.

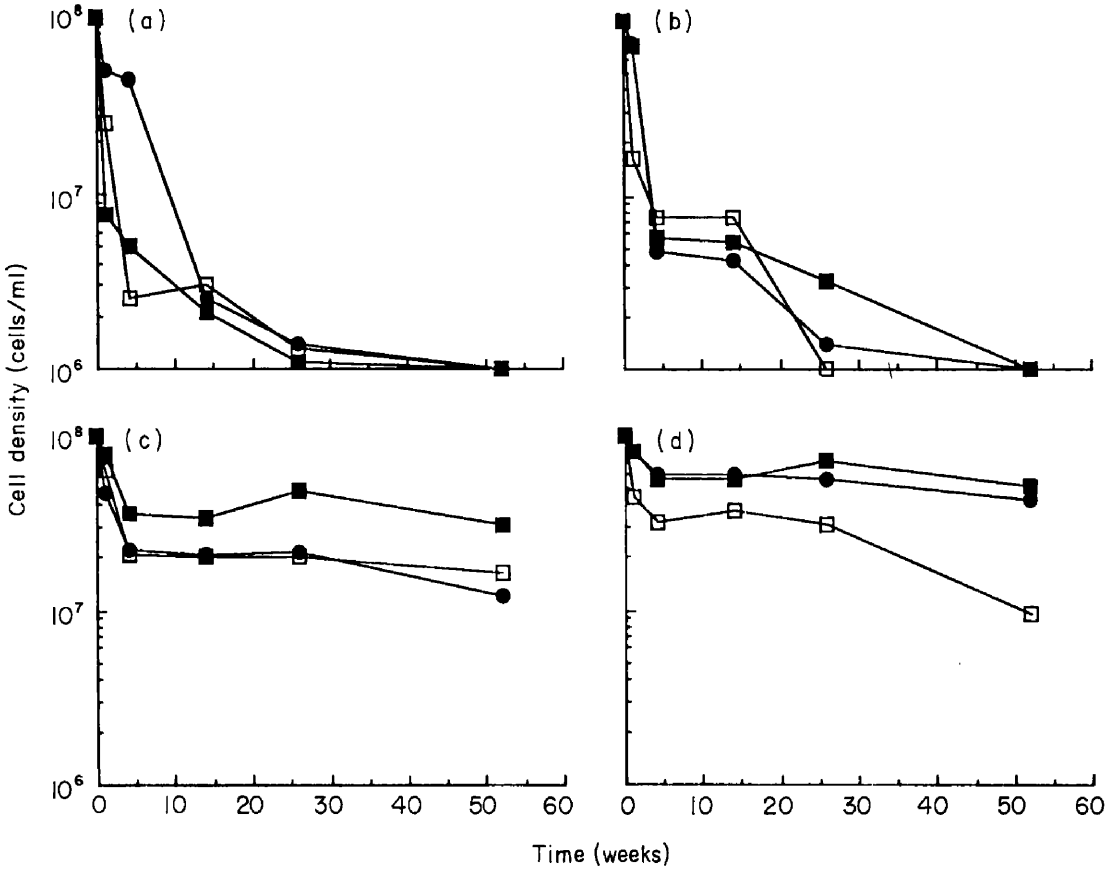

Fig. 1. Effect of storage temperature on *Pyrococcus furiosus* stored in glass capillary tubes. Viability profiles of *P. furiosus* showing cell density (cells/ml) against the number of weeks of storage at (a) room temperature, (b) 4°C, (c) −80°C and (d) liquid nitrogen vapour phase. Aliquots of culture were stored in either □, growth medium alone; or in growth medium containing ■, 5% dimethyl sulphoxide or ●, 10% glycerol. Cell viabilities were assessed following 17 h incubation at 98°C following inoculation to fresh medium.

Freezing

Cell suspensions (100 μl) were dispensed anaerobically and sealed in sterile glass capillary tubes as described by Hippe (1984). The capillaries were maintained at (a) 20°C, (b) 4°C, (c) −80°C and (d) liquid nitrogen vapour phase. Cryotubes (Sterilin Ltd) containing 1 ml of cell suspension in the appropriate cryoprotectant were also used as an alternative vessel for storage. Over a 1 year period, capillaries and cryotubes were removed and contents inoculated into fresh culture medium and grown overnight as described previously. Since it was not possible to carry out plate counts and direct counts would not differentiate between viable and non-viable cells, viability estimations were determined by direct counts following 17 h incubation.

Freeze-drying

Cell suspensions (1 ml) were added to sterile, cotton wool-plugged, labelled vials (9·9 × 6 mm, internal diameter 5 mm) and transferred to an Edwards centrifugal refrigerated freeze-dryer (model EF03/F2303) and left overnight at 4°C. After freeze-drying, vials were constricted above the level of the cotton wool plug and attached to the manifold of the freeze-drying machine for secondary drying overnight. The vials were then heat-sealed at the level of constriction and stored in the dark at 4°C. After a set period of time in storage, vials were removed and contents were inoculated into 20 ml serum bottles containing fresh culture medium. These were left for 17 h at 98°C and cell viability was determined as before.

Results and Discussion

Viability determinations of *P. furiosus* cultures taken throughout 1 year of storage indicated that freezing in glass capillary tubes was the most effective method of preserving this micro-organism. The results (Fig. 1) indicated that viability of *P. furiosus* was retained following

storage at −180°C in the vapour phase of liquid nitrogen. The use of cryoprotectants enhanced viability at −180°C although little overall difference was observed between the effectiveness of 5% DMSO or 10% glycerol.

After 1 year of storage in the presence of DMSO, viable counts were obtained equivalent to 55% and 30% of the original population when stored at −180°C and −80°C, respectively. While DMSO significantly enhanced viability at −80°C, the addition of glycerol had no apparent effect. Storage at room temperature and +4°C indicated considerable loss of viability after 4 weeks of storage although at room temperature the addition of glycerol appeared to give enhanced viability for a short time. Between 25 and 50 weeks, viabilities decreased to less than 1%. Similarly, cultures of *P. woesei* placed into storage at −80°C and −180°C using the glass capillary method were found to be viable after 1 year (data not shown).

Cultures stored in cryotubes and removed from storage in liquid nitrogen after 1 and 6 months failed to initiate growth (data not shown). Since the redox indicator resazurin indicated that some oxidation of the medium had occurred during storage, this is a likely explanation for the rapid loss of cell viability. It is possible that the oxygen permeability of the plastic cryotubes is relatively high and that this problem might be minimized by the use of glass vials. Alternatively, the freeze/thaw rate, which is slower in cryotube samples because of the larger volume, might have a significant effect on cell viability.

Freeze-drying or lyophilization is one of the most widely used methods for the preservation of micro-organisms. Since this method is routinely used in preserving thermophilic eubacteria in our laboratories, it was also examined for the preservation of *Pyrococcus*. *Pyrococcus furiosus* cells examined following lyophilization and stored for 1 and 6 months at +4°C showed no evidence of cell viability. Lyophilization has, however, proved satisfactory for the preservation of other extremely thermophilic archaebacteria such as *Desulfurococcus* and *Thermococcus* which have previously been recovered from freeze-dried cultures (D. Cowan, personal communication).

References

FIALA, G. & STETTER, K.O. 1986 *Pyrococcus furiosus* sp. nov. represents a genus of marine heterotrophic archaebacteria growing optimally at 100°C. *Archives of Microbiology* **145**, 56–61.

HIPPE, H. 1984 Maintenance of methanogenic bacteria. In *Maintenance of Microorganisms* ed. Kirsop, B.E. & Snell, J.J.S. pp. 69–81. London: Academic Press.

KIRSOP, B.E. & SNELL, J.J.S. 1984 *Maintenance of Micro-organisms. A Manual of Laboratory Methods.* London: Academic Press.

SHARP, R.J. 1984 The preservation of genetically unstable microorganisms and the cryopreservation of fermentation seed cultures. In *Advance in Biotechnological Processes*, Vol. 3. ed. Mizrah, A. & van Wezel, A.L. pp. 81–109. New York: Alan R. Liss.

WOESE, C.R., MAGRUM, L.S. & FOX, G.E. 1978 Archaebacteria. *Journal of Molecular Evolution* **11**, 345–252.

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Strain differentiation of capsule type 23 penicillin-resistant *Streptococcus pneumoniae* from nosocomial infections by pyrolysis mass spectrometry

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Sixteen isolates of penicillin-resistant *Streptococcus pneumoniae* (penicillin-resistant pneumococci, PRP) serotype 23 with identical antibiograms were examined by pyrolysis mass spectrometry (PYMS) as a possible method of rapid inter-strain comparison. Some of the isolates were from well-documented hospital outbreaks of PRP infection whilst others were sporadic isolates. The results were in good agreement with the epidemiological data and showed that PYMS can distinguish strains within a single serotype of *Strep. pneumoniae*. Pyrolysis mass spectrometry is an attractive technique for the identification and management of nosocomial infections with penicillin-resistant strains of *Strep. pneumoniae*.

Penicillin resistance in *Streptococcus pneumoniae* is still unusual in the UK, but its incidence is rising. Several presumed outbreaks of infection with penicillin-resistant pneumococci (PRP) have been described in British hospitals (Gould *et al.* 1987; Anon. 1988). It is therefore important to quickly detect the presence of PRP and to isolate the index patient in order to prevent nosocomial transmission.

An essential prerequisite for the investigation of cross-infection episodes and for devising adequate patient isolation procedures is the ability to type strains of *Strep. pneumoniae*. Conventionally this is done serologically but the existence of different antibiotic susceptibility profiles within the same capsular type of PRP suggests that distinct strains exist within a capsular serotype (Simberkoff *et al.* 1986). A method of inter-strain comparison more discriminatory than capsule typing may be of value in investigating outbreaks of PRP infections.

Pyrolysis mass spectrometry (PYMS) has been successfully applied to inter-strain comparison of a wide range of bacterial species and groups, including salmonellas (Freeman *et al.* 1990), *Strep. pyogenes* (Magee *et al.* 1989) and staphylococci (Freeman *et al.* 1991). We have assessed the usefulness of PYMS for inter-strain comparison of penicillin-resistant *Strep. pneumoniae*.

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Materials and Methods

Twelve isolates of PRP from two apparently separate outbreaks in two different hospitals in Newcastle upon Tyne were available for study. The outbreak at Hospital A has previously been reported (Gould *et al.* 1987). Six patients were infected over a period of several weeks and

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Heterogeneity of proteinases from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*

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Intracellular and extracellular samples from the extremely thermophilic archaeobacterium *Pyrococcus furiosus* showed the presence of multiple active proteinases. Using gelatin-containing SDS-PAGE, up to 13 activity bands were visualized with apparent molecular masses of between 66 and 135 kDa. Characterization studies revealed these bands to be due to discrete polypeptides, and not artefacts. Results from gel permeation chromatography, sucrose density gradient centrifugation and non-denaturing PAGE suggested that some of these proteolytic polypeptides may exist as active aggregates either *in vivo* or *in vitro* before being dissociated by SDS to active monomers.

Introduction

The ability for extreme thermophiles, most of which belong to the Third Primary Kingdom of the Archaeobacteria, to survive temperatures of around 80 °C and above is critically dependent on the structure and function of cellular components. Enzymes from these organisms are generally capable of withstanding high temperatures and exhibit a high degree of resistance to denaturing reagents, organic solvents and other deleterious influences (Veronese *et al.*, 1984; Owusu & Cowan, 1989). It is generally accepted that protein stability in these extreme thermophiles results from modifications to protein structure, such as the addition of hydrogen bonds and ionic bonds, generated by subtle differences in the amino acid sequence. Enhancement of thermal stability can also occur through improved rigidity of the more thermolabile regions of the protein structure, due to an extrinsic influence of metal ions, as with Co^{2+} for *Sulfolobus solfataricus* aminopeptidase (Hanner *et al.*, 1990) and Ca^{2+} for *Thermus aquaticus* proteinase (Cowan & Daniel, 1982). Despite these examples of extrinsic stabilization, most proteins from extreme thermophiles are of high intrinsic molecular stability [e.g. the extracellular proteinase archaelysin from the archaeobacterium *Desulfurococcus* (Cowan *et al.*, 1987), the acid proteinase from *Sulfolobus acidocaldarius* (Lin & Tang, 1990) and the hydrogenase from the hyperthermophilic archaeobacterium *Pyrococcus furiosus* (Bryant & Adams, 1989)]. Proteinases from extremely thermophilic archaeobacteria are all highly thermostable. In other respects, these enzymes show a diversity of characteristics typical of eubacterial proteinases (Cowan *et al.*, 1987).

The extremely thermophilic archaeobacterium *Pyrococcus furiosus*, isolated by Fiala & Stetter (1986), is a heterotrophic organism which grows optimally at 100 °C under strictly anaerobic conditions. Five intracellular proteinases have been previously identified from this hyperthermophile (Blumentals *et al.*, 1990; Eggen *et al.*, 1990), with an SDS-resistant proteinase (66 kDa) purified to homogeneity (Blumentals *et al.*, 1990). In this paper, we present further data on the heterogeneity of the proteinases.

Methods

Growth conditions. Pyrococcus furiosus (DSM 3638) was grown anaerobically in 1 l Duran bottles at 98 °C as described by Fiala & Stetter (1986). For the production of intracellular proteinases, cells were harvested after a 17 h incubation. Cell pellets were collected by centrifugation (50000 *g* for 15 min), washed, and resuspended in 0·01 M-NaH_2PO_4/Na_2HPO_4 buffer, pH 7·5. Cell-free extracts for gel permeation chromatography and sucrose density gradients were prepared by sonicating cells for three 1 min bursts with 30 s intervals using a Soniprep Sonicator. The cell lysate was then centrifuged for 30 min at 30000 *g* to remove cell debris. Otherwise, aliquots of culture (1 ml) were centrifuged at high speed in an MSE Microcentaur centrifuge and cell pellets were washed, resuspended in phosphate buffer (100 µl) and lysed by the addition of an equal volume of SDS sample buffer (4% (w/v) SDS/20% (w/v) glycerol/10% (w/v) mercaptoethanol with bromophenol blue). For extracellular proteinases, cell-free culture supernatants were obtained after harvesting and used directly.

Cells and supernatants were either used immediately or stored at −70 °C.

Proteinase assay. Proteinase activity was determined by the hydrolysis of azocasein (Sigma) as described by Cowan *et al.* (1987).

Substrate-containing SDS-PAGE. Discontinuous SDS-PAGE was performed according to Laemmli (1970), except that the separation gel (10%) contained 0·01% gelatin (Type I, porcine skin) or casein (purified; Sigma) and 0·1% SDS. The stacking gel (5%) also contained 0·1% SDS. Protein samples (75 µl) and known molecular mass markers (50 µl) were mixed with an equal volume of SDS sample buffer (Laemmli, 1970) and boiled for 5–10 min before loading on to slab gels (20 × 16 × 1·5 mm). Electrophoresis was performed at 45 mA constant current using an LKB Vertical Electrophoresis Unit with an LKB 2197 Power Supply.

After electrophoresis, gels were gently shaken at room temperature for 2 h in two changes of aqueous 2·5% (w/v) Triton X-100 (Horie *et al.*, 1984), then incubated for 1 h at 90 °C in 0·1 M-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer, pH 7·5 (90 °C). After incubation, gels were immediately fixed with 50% (w/v) TCA for 30–60 mins before staining with Coomassie Blue G250. Gels were then destained overnight in methanol:acetic acid:water (3:2:35, by vol.). Unstained bands, indicating proteolytic activity, were recorded by photography.

Quantitative analysis of data. Negatives of Polaroid photographs of substrate-containing gels were scanned using an Ultrascan XL Laser Densitometer (LKB Instruments). Proteinase activity was determined by integration of peak area for each band.

Native gels. Non-denaturing PAGE was performed on slab gels of different acrylamide concentrations (up to 10%) to determine the molecular masses of the proteinases. Native gels were prepared (Clarke, 1964) and electrophoresed as before. After electrophoresis, gels were immersed in a solution of 1% gelatin in 0·1 M-HEPES, pH 8·3, and gently shaken for 1 h. The gels were then incubated for 1–3 h at 90 °C before being fixed with 50% (w/v) TCA. Gels were stained and destained as before.

Sucrose gradient centrifugation. Sucrose gradients [6–30% (w/v) sucrose in distilled water] were prepared as described (Martin & Ames, 1961). Cell extract and culture supernatant samples (500 µl) were layered on top of the gradients together with known molecular mass markers (0·25 mg of β-amylase and bovine serum albumin in 50 µl of distilled water). The samples were centrifuged at 208000 *g* for 24 h at 20 °C in an L-7 Beckman Ultracentrifuge. The contents were removed by inserting a capillary tube to the bottom of each tube and withdrawing the contents using a peristaltic pump. Fractions (0·5 ml) were collected. Protein was monitored spectrophotometrically at 280 nm and proteinase activity was determined using substrate PAGE. Molecular masses were calculated using the equation $s_1/s_2 \times (mm_1/mm_2)^{2/3}$, where s_1/s_2 is the partial specific volume, mm_1 is the molecular mass of the unknown protein and mm_2 is the molecular mass of the known standard (Martin & Ames, 1961).

Fast protein liquid gel permeation chromatography. Samples of *P. furiosus* cell extracts (200 µl) were applied to a Superose 12 HR10/30 column (Pharmacia). The column was pre-equilibrated with 20 mM-triethanolamine buffer, 50 mM-NaCl, pH 7·5, before samples were loaded. Proteins were then eluted with the same buffer, and 1 ml fractions were collected. Throughout the chromatography, protein content was monitored spectrophotometrically at 280 nm. All fractions collected were assayed for proteinase activity using substrate PAGE.

Effect of inhibitors. Proteinase inhibitors (final concentrations 10–100 mM) were added to 100 µl of cell extracts and culture supernatants and incubated at room temperature for 1 h before analysis by substrate PAGE. Non-covalent inhibitors were added to the proteinase assay buffer during incubation of gels at 90 °C, at a final concentration of 10 mM.

Thermostability of proteases. Cell-free culture supernatants samples (100 µl) were sealed in capillary tubes (Barach & Adams, 1977), and immersed in a paraffin oil bath at temperatures between 95 °C and 125 °C. At appropriate time intervals, tubes were removed and immediately placed on ice. Residual proteinase activity was determined using substrate PAGE.

Results and Discussion

Static cultures of the hyperthermophilic archaeobacterium *Pyrococcus furiosus* were sampled for proteinase activity using azocasein as the proteolytic substrate. Proteinase activity was detected in both cell extracts and culture supernatants, reaching a peak at around 17–18 h after inoculation (Fig. 1*a* and *b*). Proteinase production did not appear to be growth-phase dependent, as specific proteinase production (expressed as units of activity per g of protein) was observed to be linear throughout growth (Fig. 1*c*). Since the azocasein assay yields only the total titre of proteinase activity and provides no information on enzyme multiplicity, substrate-containing PAGE was used subsequently for more detailed analysis.

Proteinase heterogeneity

Using substrate-containing SDS-PAGE, a multiple band pattern of up to 13 distinct active proteinase monomers was observed in both cell extracts and culture supernatants, where apparent molecular masses ranged from 66 kDa to 135 kDa (Fig. 2). Levels of these active hydrolases were seen to increase until late exponential phase in a manner consistent with the analysis of total proteinase titres (Fig. 1*b*).

The observation of multiple proteinase bands from *P. furiosus* confirmed data obtained by Blumentals *et al.* (1990) and Eggen *et al.* (1990) who both report a multiple band pattern with the lowest molecular weight band being around 66 kDa. However, both groups describe the presence of only 5 proteinase bands.

The difference between our observations and those of Blumentals *et al.* (1990) and Eggen *et al.* (1990) lies in the sensitivity and resolution of the detection procedures used. Blumentals *et al.* (1990) reported extensive streaking and poor resolution of bands with gelatin-SDS-PAGE and were thus forced to use a substrate gel overlay, from which only 5 bands were detected. On the other hand, Eggen *et al.* (1990) who also reported 5 bands, did not appear to have the problem of streaking in substrate gels. We can only conclude that the sensitivity and resolution of the substrate-containing SDS-PAGE technique may be dependent on details of its operation.

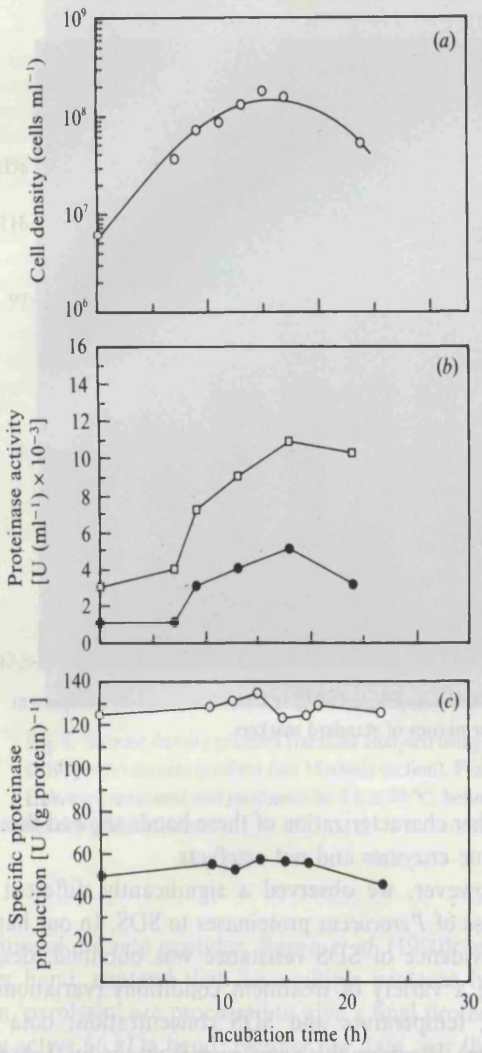

Fig. 1. Growth, proteinase activity, and specific proteinase productivity of *Pyrococcus furiosus*. (*a*) Cell density; (*b*) proteinase activity in cell extract (●) and culture supernatant (□) fractions; and (*c*) specific proteinase production in cell extract (●) and culture supernatant (○) fractions.

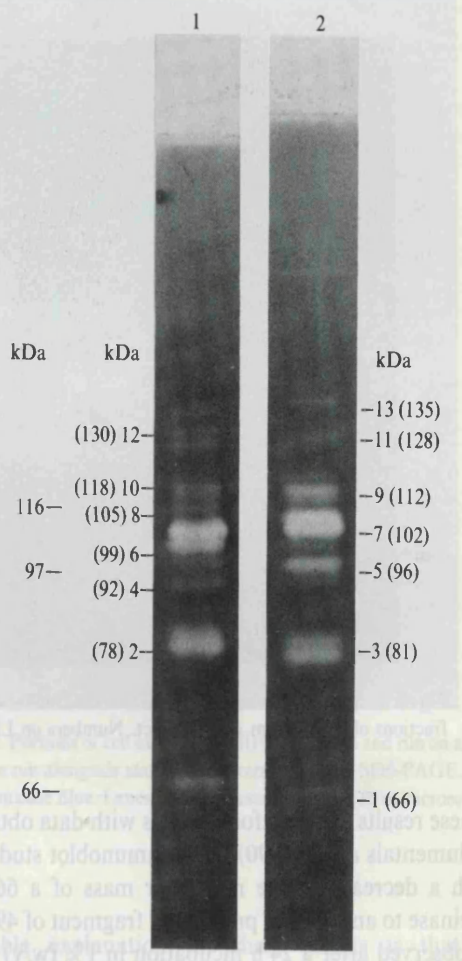

Fig. 2. Proteinase multiplicity from *P. furiosus*. Lane 1, cell extract fraction; lane 2, culture supernatant fraction. Numbers on RHS and LHS of photograph indicate band numbers in ascending molecular mass (indicated in brackets), whereas numbers on the far LHS denote molecular masses of standard markers (66 kDa, bovine serum albumin; 97 kDa, phosphorylase b; 116 kDa, β-galactosidase).

Also, the reliance of the gel overlay detection method on diffusion of the enzyme will inevitably result in substantial loss of resolution and detection. We note that the substrate concentration used by us, Blumentals *et al.* (1990) and Eggen *et al.* (1990) was found to differ as much as 50-fold.

There are a number of possible origins of a multiplicity of discrete activity bands in substrate-containing SDS-PAGE gels. These might include: (i) active processing products from larger species, such as proteolytic or autolytic degradation products; (ii) artefacts resulting from incomplete unfolding; (iii) artefacts resulting from heterogeneous associations with the substrate during electrophoresis; and (iv) discrete genetically distinct polypeptides. We shall consider each of these possible mechanisms in turn.

The generation of heterogeneous active proteinase fragments by proteolytic or autolytic degradation is known to occur (Fontana, 1988). However, preincubation of *Pyrococcus* cell-free culture supernatants and cell extract samples at different temperatures (4 °C, 20 °C, 60 °C and 95 °C) for up to 24 h before analysis by substrate PAGE, showed no activity bands of <66 kDa (data not shown). Also, banding patterns identical to those in Fig. 2 were observed with negligible loss of apparent activity for any proteinase. If significant autolysis did occur, then a gradual decrease in the higher-molecular-mass bands, possibly corresponding to an increase in the lower-molecular-mass bands, might be observed over time. Under no conditions did we observe this behaviour, suggesting that the activity bands were unlikely to be degradation products.

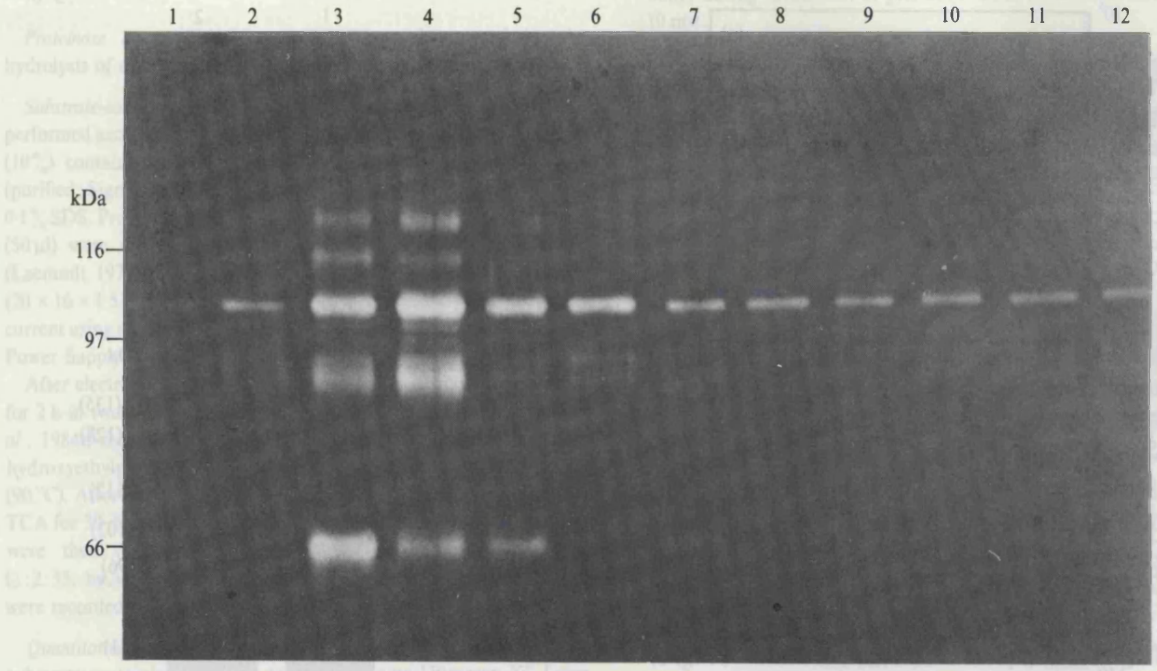

Fig. 3. Gelatin-containing SDS-PAGE of fractions from FPLC gel permeation using Superose 12 HR 10/30. Lanes 1 to 12 represent fractions obtained from a cell extract. Numbers on LHS denote molecular masses of standard markers.

These results are therefore at odds with data obtained by Blumentals *et al.* (1990) from immunoblot studies in which a decrease in the molecular mass of a 66 kDa proteinase to an inactive proteolytic fragment of 49 kDa was observed after a 24 h incubation in 1% (w/v) SDS. In the case of Eggen *et al.* (1990), preincubation of 'pyrolysin' at 95 °C in the absence of substrate at different times, followed by activity staining using gelatin PAGE, showed a concomitant increase in intensity of the lower-molecular-mass bands to 65 kDa, suggesting processing of higher-molecular-mass proteinases. However, our results, obtained under similar experimental conditions, did not show this increase in intensity of the 66 kDa band. In fact, a slight decrease in intensity of the 66 kDa band was observed after 20 h, which clearly does not support the view that this band is a proteolytic degradation product of high-molecular-mass proteins (Eggen *et al.*, 1990).

Incomplete protein unfolding prior to electrophoresis can be a major cause of artefacts in substrate gels (Horie *et al.*, 1984; Kelleher & Juliano, 1984). Partially unfolded proteins can retain activity and hence interact with the substrate during electrophoresis, retarding mobility and thereby producing multiple bands and streaking across gels. Proteinases which are resistant to SDS will also remain active and cause streaking in substrate gels. This phenomenon was observed by Blumentals *et al.* (1990) who found that of the five native proteinases identified from *P. furiosus* cell extracts, two of these had shown SDS resistance, causing streaks on gelatin-containing gels. Further characterization of these bands showed these to be true enzymes and not artefacts.

However, we observed a significantly different response of *Pyrococcus* proteinases to SDS. In our hands, no evidence of SDS resistance was obtained, despite using a variety of treatment conditions (variations of time, temperature and SDS concentration; data not shown). We draw this conclusion from the total absence of streaking and the consistently reproducible band patterns as seen in Fig. 2.

An alternative, albeit unlikely, source of artefactual banding multiplicity might arise from heterogeneous non-specific interactions, during electrophoresis, between denatured protein and gel-encapsulated substrate. However, our observation that casein-containing SDS-polyacrylamide gels produce multiple band patterns of high resolution, similar to those seen with gelatin-containing gels, is strong evidence that the apparent proteinase multiplicity does not arise from some heterogeneous interaction with the substrate during electrophoresis.

These data contribute to the conclusion that the bands observed are discrete polypeptides. However, the question as to whether these bands are genetically related still remains. From immunoblot studies, Blumentals *et al.* (1990) have shown that proteinases S66 and S102 are not related, and correspond to different enzymes. These proteinases were also found to be distinct from the other three proteinases present in *P. furiosus* cell extracts. Our data support this contention and suggest the existence of

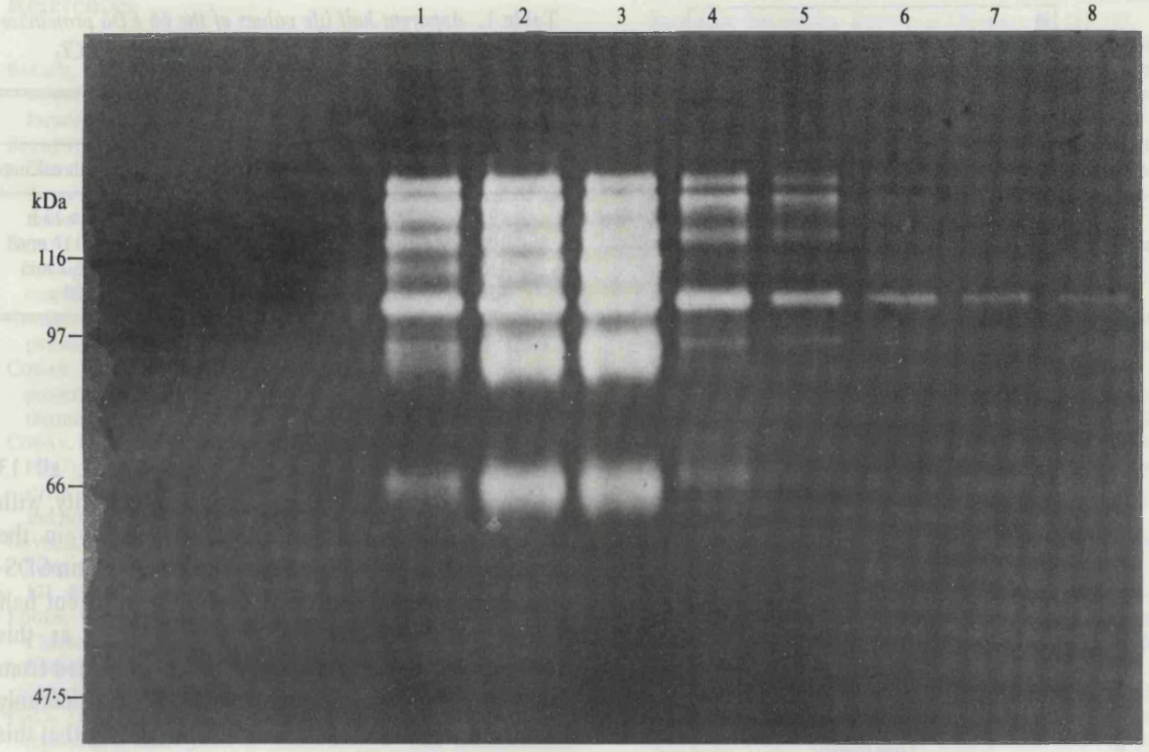

Fig. 4. Sucrose density gradient fractions analysed using gelatin-SDS-PAGE. Portions of cell extract (500 µl) were loaded and run on a 6–30% (w/v) sucrose gradient (see Methods section). Fractions collected were run alongside standard markers on gelatin-SDS-PAGE. Gels were renatured and incubated for 3 h at 90 °C, before staining with Coomassie Blue. Lanes 1 to 8 represent fractions from sucrose density gradient runs.

additional discrete peptides. Eggen *et al.* (1990), on the other hand, contend that the multiple protease bands from 'pyrolysin' are processed to give a final proteolytically active 66 kDa band. Neither our data, nor that of Blumentals *et al.* (1990), support this conclusion.

In vivo state of proteinase monomers

Non-denaturing (native) PAGE indicated the presence of more than 7 proteinases from cell extracts and culture supernatants. Analysis of native gels of varying acrylamide concentrations (5–10% acrylamide) gave apparent molecular masses of between 95 and 280 kDa (data not shown).

Separation of cell extracts by FPLC gel permeation chromatography and sucrose density centrifugation produced 2 higher-molecular-mass proteinases of approximately 185 to 200 kDa (from sucrose gradients, sedimentation coefficients of 9·5 S and 10 S respectively) and 200 to 280 kDa (FPLC) from cell extracts and culture supernatants. When subsequently analysed by substrate-containing SDS-PAGE, these high-molecular-mass active proteinases showed an identical pattern to that observed previously (Figs 3 and 4). The most probable explanation for these results is that the individual proteinases interact (either *in vivo* or *in vitro*) to form high-molecular-mass active aggregates. We have no evidence to confirm the existence of these aggregates *in vivo*, although the appearance of multiple bands on native PAGE might argue for an *in vitro* aggregation phenomenon (Figs 3 and 4).

Inhibitors

All but one of the 13 proteinase bands from *Pyrococcus* showed complete inhibition by 10 mM-phenylmethylsulphonyl fluoride (PMSF) suggesting predominance of serine-type proteinases (data not shown). There was no significant change in activity after treatment with the thiol proteinase inhibitor iodoacetamide. Partial inhibition by the metal chelator EDTA (10 mM) was observed with the PMSF-insensitive band (130 kDa), suggesting a requirement of metal ions for activity. The results of inhibition studies obtained for the additional bands support the data obtained by Blumentals *et al.* (1990) and Eggen *et al.* (1990), who both classify the 5 bands reported in their work as serine-type proteinases.

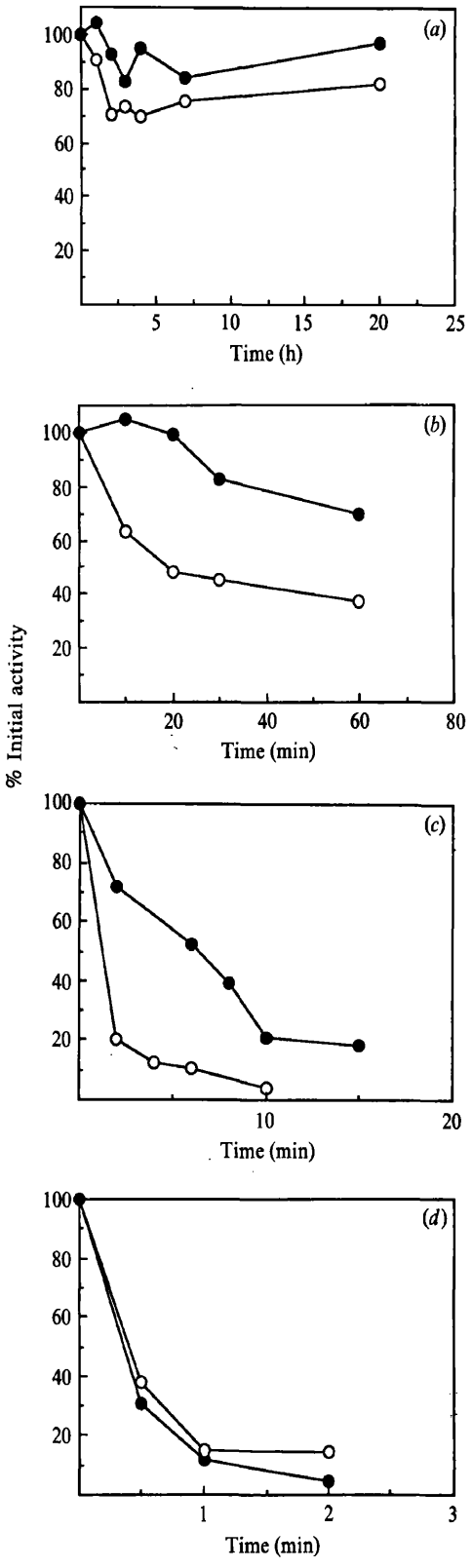

Fig. 5. Thermostability profiles for *Pyrococcus* 66 kDa proteinase at various temperatures. Culture supernatant samples (100 µl) were incubated at (*a*) 95 °C, (*b*) 105 °C, (*c*) 115 °C, and (*d*) 125 °C, with (●) and without (○) the addition of 10 mM-$CaCl_2$. Samples were removed at appropriate intervals, cooled and assayed for activity using gelatin-SDS-PAGE (see Methods).

Table 1. *Apparent half life values of the 66 kDa proteinase from P. furiosus with and without 10 mM-$CaCl_2$*

	Half life ($t_{1/2}$)	
Temperature (°C)	Without calcium	With calcium
95	>20 h	>20 h
105	15 min	117 min
115	52 s	6·3 min
125	22 s	20 s

Thermostability

At temperatures between 95 °C and 105 °C, all 13 proteinases demonstrated a high degree of stability, with some proteinases showing enhanced stability in the presence of 10 mM-$CaCl_2$ (Fig. 5). Using gelatin-SDS-PAGE as a semi-quantitative assay, the apparent half life values of the 66 kDa protein (selected as this appeared to be one of the most stable) calculated from first order constants (Moore, 1962) were considerably increased when calcium was added, suggesting that this metal ion is required to stabilize the protein structure and prevent unfolding at higher temperatures (Table 1).

The value obtained for the 66 kDa proteinase exceeds the half life values of other known archaeobacterial proteinases such as archaelysin, $t_{1/2}$ (95 °C), 70–90 minutes (Cowan *et al.*, 1987), and thermopsin, $t_{1/2}$ (80 °C), 48 hours (Lin & Tang, 1990). Blumentals *et al.* (1990) have reported that the 66 kDa proteinase from *Pyrococcus* was found to have a half life of about 33 h at 98 °C, making this the most stable proteinase to date.

Concluding remarks

In this study, up to 13 active proteolytic polypeptides from *P. furiosus* have been identified. We believe that the heterogeneity of these enzymes is valid from the evidence presented, despite conflicting evidence of 5 active bands obtained from studies by Blumentals *et al.* (1990) and Eggen *et al.* (1990). We also suggest that these active proteinases are not artefacts, but arise from the dissociation of larger multimeric proteinases.

The question that is automatically raised is why so many proteinases are produced from a hyperthermophile. It is known that multiple heterogeneous intracellular proteinases serve a wide variety of functions including protein turnover, nutritional and specific post-translational modification events. When information is available on the peptide bond specificity of individual proteinases, it may be possible to imply one or other function. However, at present the specific function for these *P. furiosus* proteinases remains unknown.

References

BARACH, J. T. & ADAMS, D. M. (1977). Thermostability at ultrahigh temperatures of Thermolysin and a protease from a psychrotrophic *Pseudomonas*. *Biochimica et Biophysica Acta* **485**, 417.

BLUMENTALS, I. I., ROBINSON, A. S. & KELLY, R. M. (1990). Characterization of SDS-resistant proteolytic activity in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *Applied and Environmental Microbiology* **56**, 1992–1998.

BRYANT, F. O. & ADAMS, M. W. W. (1989). Characterization of hydrogenase from the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. *Journal of Biological Chemistry* **264**, 5070–5079.

CLARKE, J. T. (1964). Simplified 'disc' (polyacrylamide gel) electrophoresis. *Annals of the New York Academy of Sciences* **121**, 428–435.

COWAN, D. A. & DANIEL, R. M. (1982). Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile. *Biochimica et Biophysica Acta* **705**, 293–305.

COWAN, D. A., SMOLENSKI, K. A., DANIEL, R. M. & MORGAN, H. W. (1987). An extremely thermostable extracellular proteinase from the strain of the archaebacterium *Desulfurococcus* growing at 88 °C. *Biochemical Journal* **247**, 121–133.

DAVIS, B. J. (1964). Disc electrophoresis-II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.

EGGEN, R., GEERLING, A., WATTS, J. & M. DE VOS, W. (1990). Characterization of pyrolysin, a hyperthermoactive serine protease from the archaebacterium *Pyrococcus furiosus*. *FEMS Microbiology Letters* **71**, 17–20.

FIALA, G. & STETTER, K. O. (1986). *Pyrococcus furiosus* sp. nov., represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100 °C. *Archives of Microbiology* **145**, 56–61.

FONTANA, A. (1988). Structure and stability of thermophilic enzymes. Studies on thermolysin. *Biophysical Chemistry* **29**, 181–193.

HANNER, M., REDL, B. & STOFFLER, G. (1990). Isolation and characterization of an intracellular aminopeptidase from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*. *Biochimica et Biophysica Acta* **1033**, 148–153.

HORIE, N., FUKUYAMA, K., ITO, Y. & EPSTEIN, W. L. (1984). Detection and characterization of epidermal proteinases by polyacrylamide gel electrophoresis. *Comparative Biochemistry and Physiology* **77B**, 349–353.

KELLEHER, P. J. & JULIANO, R. L. (1984). Detection of proteases in polyacrylamide gels containing covalently bound substrates. *Analytical Biochemistry* **136**, 470–475.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LIN, X.-L. & TANG, J. (1990). Purification, characterization and gene cloning of Thermopsin, a thermostable acid protease from *Sulfolobus acidocaldarius*. *Journal of Biological Chemistry* **265**, 1490–1495.

MARTIN, R. G. & AMES, B. N. (1961). A method for determining the sedimentation behaviour of enzymes: Applications to protein mixtures. *Journal of Biological Chemistry* **236**, 1372–1379.

MOORE, W. J. (1962). *Physical Chemistry*, 4th edn, pp. 260–263. London: Longman.

OWUSU, R. K. & COWAN, D. A. (1989). Correlation between microbial protein thermostability and resistance to denaturation in aqueous–organic solvent 2-phase systems. *Enzyme and Microbial Technology* **11**, 568–574.

VERONESE, F. M., BOCCU, E., SCHIAVON, C., GRANDI, C. & FONTANA, A. (1984). General stability of thermophilic enzymes. Studies on 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* and yeast. *Journal of Applied Biochemistry* **6**, 39–47.