THERMOSTABLE PROTEASES FROM THERMOPHILIC MICROORGANISMS

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ABSTRACT

Two metallo-proteases, EA1 and YP-T proteases from *Bacillus* st. EA1 and *B. caldolyticus* st. YP-T, respectively, differ in amino acid sequence by only one residue (Val61 = Gly61 in EA1 and YP-T respectively). Yet EA1 protease has a higher thermostability than YP-T protease. An analysis of the half lives of the two proteases at 85°C at different Ca^{2+} concentrations shows that at low Ca^{2+} levels, autolysis is the most significant cause of loss of activity, while at higher Ca^{2+} levels denaturation is most significant.

A comparison of the sequences of the two proteases and thermolysin shows that this amino acid difference is located within the third Ca^{2+} binding site (based on the known structure of thermolysin). The residues are not directly involved in binding to the Ca^{2+}, but are located within this pocket. Molecular modelling studies of EA1 and YP-T proteases, based on the structure of thermolysin, propose that the stabilisation of EA1 over YP-T protease could be due to extra interaction/s of the side chain of Val 61 of EA1 protease with the benzene ring of Tyr 27. YP-T protease has Gly in this position. As the "side chain" of Gly is only a hydrogen, there is unlikely to be any such interaction with Tyr 27. This possible extra interaction/s could increase the bonding of the amino acids within the calcium binding pocket, which may stabilise the pocket.

Bacillus st. Ak.1 produces a thermostable serine protease (subtilisin). The 16S rRNA analysis closely groups *Bacillus* st. Ak.1 and *B. thermoglucosidasius*. However, the organisms have different growth requirements. *Bacillus* st. Ak.1 has a requirement for glutamate, while *B. thermoglucosidasius* has a requirement for a compound found in yeast extract and tryptone, but not glutamate (possibly maltose). RAPD-PCR analysis of twelve organisms show that the banding patterns of the organisms are all unique, though the closest two were between *Bacillus* st. EA1 and *B. caldolyticus*. *Escherichia coli* clone PB5517 produces Ak.1 protease constitutively at 35°C. a 10 l fermentor run was conducted, and 51 mg of the protease was purified to homogeneity.

Ak.1 protease is dramatically stabilised by Ca^{2+} ions. The half life at 70°C increases by four orders of magnitude in the presence of 5 mM Ca^{2+}, as compared to the thermostability in the absence of Ca^{2+}. As the concentration of Ca^{2+} ions increased, the degree of denaturation decreased. At high Ca^{2+} concentrations, the major cause of loss of activity was due to autolysis.

Based on the structural and enzymatic data, the extra degree of stabilisation of Ak.1 protease by Ca^{2+} above that of thermitase-Ca^{2+} could be due to the presence of an extra Ca^{2+}-binding site in Ak.1 protease. This new site is located close to Ca(1), and could therefore change the binding properties of this site also. Another possibility is that the binding constants of one or more of the Ca^{2+}-binding sites could be higher for Ak.1 protease, as compared to thermitase.

Lanthanide ions stabilised the protease, though to a much smaller degree than Ca^{2+}. Like Ca^{2+}, they stabilised the protease by the prevention of denaturation. Other cations stabilised the protease to a small degree, especially Sr^{2+}. Different cations had different effects on the stability of the enzyme. Other significant stabilisers were 90% solutions of sorbitol, trehalose and glycerol. At 105°C, 90% sorbitol increased the thermostability of the protease from <<1 minute to 104 minutes. It did so by the prevention of autolysis.

The protease has a limited substrate specificity, preferring to cleave substrates containing neutral or hydrophobic amino acids, such as valine, alanine or phenylalanine, at the P_1 site. It has a preference for proline at the P_2 site, and alanine at the P_1-P_4 sites. It also has esterase activity, being able to cleave methyl, ethyl and p-nitrophenyl esters. Studies with Suc-Ala_n-pNA substrates (n=2-5) shows that the specific activity of the protease increases with increasing chain length, though such a substrate containing 5 alanine residues appears to be being cleaved significantly at more than one site. A comparison of the K_m and V_{max} of the protease to the substrates Suc-Ala-Ala-Pro-Xaa-pNA, where Xaa = Phe, Leu or Ala, shows that the larger and more hydrophobic the P_1 amino acid is, the higher the specificity of the protease for that substrate.

An analysis of the effect of temperature on the K_m and V_{max} of Ak.1 protease with several substrates revealed that the specificity of the protease (V_{max}/K_m) changes with temperature. The K_m and V_{max} decreased with decreasing temperature, but not to the same degree with all substrates. If the protease is assayed with substrates in the presence of 50% methanol, the K_m tends to increase dramatically. This is due to hydrophobic partitioning of the solvent. The V_{max} of the protease decreases under these conditions.

The active site is a cleft, composed of hydrophobic amino acids in the substrate-binding cleft. It is similar to other subtilisins, but differs in the presence of a disulphide bond. The space-filling model of the protease with the substrate SAAPFpNA in the active site shows the cleft 'bends' at the P_2 site.

This is easily accomidated for by proline at this position, as proline causes a bend in the substrate. This can explain the preference for proline at the P_2 site.

The sequence of Ak.1 protease indicates the presence of two cysteine residues, separated by only one amino acid. The 3D structure showed that these cysteine residues exist in a disulphide bond. Tests (e.g. Ellman assay) confirmed the presence of not only two cysteine residues, but that in native conditions (i.e. in solution) these residues exist as a disulphide bond.

Reductants, such as dithiothreitol (DTT) typically reduce disulphide bonds into their constituent cysteine residues. The lower thermostability in the presence of DTT indicates that it appears to have opened a disulphide bond as disulphide bonds are known to increase the thermostability of proteins. It is proposed that the reduction of the disulphide bond causes a localised opening of the substrate binding cleft at the P_4 site, due to the location of the disulphide bond in this position. This was supported by the results that showed that the larger the substrate, the greater the effect of DTT on the K_m of the protease with that substrate. For example, the K_m was unchanged with DTT with a substrate occupying only sites P_2-P_1', while a substrate which occupies sites P_5-P_1' showed a significant increase of the K_m, suggesting it binds weaker to the binding cleft.

Heavy metals such as Hg^{2+} and Pb^{2+} bind to Ak.1 protease, causing a decrease in the specific activity of the protease. The effects of the heavy metals on activity is much smaller than with DTT. In general, the K_m and V_{max} were only changed to a small degree. Fluorescein mercuric acetate (FMA) binds to Ak.1 protease, causing the inherent fluorescence of FMA to increase. The presence of DTT caused a decrease in the thermostability of Ak.1 protease of 9.3 fold at 85°C.

In conclusion, the disulphide bond has a dual role, that of maintaining the integrity of the substrate-binding cleft and increasing the thermostability of the protease.

PREFACE

Chapter 1.2 was published as part of a review entitled "Thermostable Proteases" by Daniel, R.M., Toogood, H.S. and Bergquist, P.L. (1995) published in *Biotechnology and Genetic Engineering Reviews* **13**, 51-100. The section is included here in its entirety for completeness. Although I made a contribution to the review as a whole, I carried the major responsibility only for this section. A complete copy of the review is located in Appendix 2.

Overall, much of the work in this thesis is the biochemical element of a collaboration with molecular geneticists and protein crystallographers.

The cloning, sequencing and expression of the proteases EA1 and YP-T into *Escherichia coli* was done by Dr Dave Saul at the University of Auckland. Some of my data on these proteases (Chapter 3) was published in the paper:; Saul, D.J., Williams, L.C., Toogood, H.S., Daniel, R.M. & Bergquist, P.L. (1996) "Sequence of the gene encoding a highly thermostable neutral proteinase from *Bacillus* sp. strain EA1: expression in *Escherichia coli* and characterisation." *Biochimica et Biophysica Acta* **1308**, 74-80. This paper is included in Appendix 2.

The cloning, sequencing and expression of the protease Ak.1 into *E. coli* was done by Dr Bryce MacIver and Dr Dave Saul at the University of Auckland (*Applied and Environmental Microbiology* **60**(11), 3981-3988). The properties of Ak.1 protease determined in this project are currently being prepared for publication.

The 3-dimensional structure of Ak.1 protease was determined by Dr Clyde Smith and Professor Ted Baker at Massey/Auckland Universities. The purified Ak.1 protease for crystallography was supplied from the batch of purified enzyme prepared as described in this thesis. The structural data was obtained during a later stage of this work. The structure, and a few of the properties of the protease are currently being written in the following paper; Smith, C.A., Toogood, H.S., Daniel, R.M. & Baker, E.N. (1998). "The Structural Analysis of the Heat-Stable Serine Protease from *Bacillus* sp. Ak.1 at 1.75a Resolution." Permission was given for the inclusion of structural data and photographs in this thesis.

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CHAPTER 1

INTRODUCTION

1.1 PROTEIN STABILITY

1.1.1 Introduction

The mechanisms of protein stability, especially thermostability, have been investigated for a number of years. Recently, however, interest in using enzymes in industrial processes has increased. Such enzymes must function under the conditions within the industrial process; exhibiting stability towards heat, detergents, oxidising/reducing agents and/or organic solvents. The study of the mechanisms of protein stability may enable the design or modification of enzymes, to make them more suitable for industrial purposes.

The molecular determinants of protein stability of thermophilic proteins, as compared to mesophilic proteins, has provided important incites about the mechanism of thermostability (e.g. Zuber, 1978). Advances in DNA recombination techniques allow us to either randomly mutate proteins, or to specifically target certain amino acids in a protein and determine what effect these changes have on stability.

There are three general mechanisms whereby proteins lose activity with temperature. First, increasing the temperature results in a greater flexibility of the protein, increasing the likelihood of unfolding (denaturation) and hence loss of activity. The second mechanism is by irreversible covalent modification reactions, such as deamidation of asparagine residues (Ahern & Klibanov, 1985). Proteases have a third mechanism. They can undergo autolysis (self-cleavage) which fragments the protease. As the temperature increases, the autolysis rate increases.

As a general rule, enzymes tend to exhibit maximal activity around the optimal growth temperature of the source organism, though many exceptions are known. Table 1.1 shows the half lives of a variety of hyperthermophilic enzymes.

Enzyme	Source	Half Life	Reference
Amylase	*Pyrococcus furiosus*	2 h at 120°C	Koch *et al.*, 1990
Amylase	*Desulfurococcus* st. $ToK_{12}S_1$	15-25 min at 103°C	Daniel *et al.*, 1989
Aspartate Amino-transferase	*Sulfolobus solfataricus*	2h at 100°C	Cacciapucti *et al.*, 1986
ATPase Complex	*Pyrobaculum. occultulum*	35 min at 110°C	Phipps *et al.*, 1991
α Glucosidase	*Thermococcus* st. AN1	35 min at 98°C	Piller *et al.*, 1996
ß Glucosidase	*Thermococcus* st. AN1	10-15 min at 95°C	Daniel *et al.*, 1989
Citrate Synthase	*Sulfolobus acidocaldarius*	10 min at 90°C	Grossebuter & Gorisch, 1985
DNA Polymerase	*S. acidocaldarius*	0.25 h at 87°C	Prangishvili, 1986
Endonuclease	*S. acidocaldarius*	0.5h at 80°C	Prangishvili *et al.*, 1985
Glucose Dehydrogenase	*Sulfolobus solfataricus*	45h at 70°C	Giardina *et al.*, 1986
Hydrogenase	*Pyrococcus furiosus*	2h at 100°C	Bryant & Adams, 1988; Woodward *et al.*, 1996
Malate Dehydrogenase	*Methanopyrus fervidus*	9 min at 90°C	Honka *et al.*, 1990
NADH Dehydrogenase	*Sulfolobus acidocaldarius*	70% left, 0.25h 100°C	Wakao *et al.*, 1987
Protease (Archaelysin)	*Desulfurococcus* st. $Tok_{12}S_1$	70-79 min at 95°C	Cowan *et al.*, 1987

Table 1.1 Thermostability of Hyperthermostable Enzymes

Originally, thermostable enzymes were thought to have large sequence and structural differences from their mesophilic counterparts (Perutz & Raidt, 1975). However, as the net free energy of stabilisation of proteins is small, only a few extra stabilising interactions, e.g. an extra salt bridge (about 4-12 KJ/mol), can account for the extra thermostability of thermophilic proteins.

It was suggested that the instability characteristics of proteins and DNA would determine the upper temperature limit for life (Ahern and Klibanov, 1986). Originally, this was thought to be around the boiling point of water, as around this temperature several irreversible covalent modifications of proteins occur (e.g. deamidation and disulphide bond cleavage). However, recently many species of hyperthermophilic organisms have been discovered that grow above these temperatures. It is now thought that the above irreversible reactions depend on peptide chain flexibility rather than on a specific temperature, and that such reactions do not occur significantly in hyperthermophilic proteins at temperatures above 100°C (Hensel, 1992; Daniel *et al* ., 1996).

1.1.2 Mechanisms of Protein Folding and Stability

1.1.2.1 Intrinsic Properties of Proteins

Proteins exist in a series of microstates, centered around an average state. At sufficiently high temperatures (depending on the protein), all of the molecules exist in the denatured state, while at much lower temperatures the average molecule is rigid and inflexible (Brandts, 1967). Proteins are only just stable at their 'optimum temperature'. This arises because while there are large stabilising and destabilising forces within a protein, the net free energy of stabilisation is quite small; within the range of only a few 10's of kJ/mol (Grutter *et al.*, 1979). This is important as it enables a protein to maintain its 3-dimensional structure while still allowing sufficient flexibility for it to function.

It is generally thought that the stability of proteins is intrinsic (e.g. Daniel, 1986). However, external factors can also contribute, such as the addition of Ca^{2+} ions. Therefore, the major determinants of protein stability are dependent on the secondary and tertiary structure, which is determined by the primary structure. This can be studied by changing the amino acid sequence of a protein and observing if any change in stability has occurred. Mutations that destabilise proteins do so by the elimination of, or interference of favourable interactions within the protein (Matthews, 1993). Protein structures (3°) are

generally resistant to changes in individual amino acids, except where the changes cause a loss of major stabilising or destabilising interactions, or where such substitutions exist in certain regions (e.g. the insertion of proline in an α-helix). However, even when structural changes have been minor, there still can be major changes in stability (Matthews, 1993).

No single type of interaction is the major type of contributor in all proteins, rather all the different types of forces involved are important at specific sites. In other words, the local as well as global environment is important for stability. Mutations affecting the stability of a protein are dependent on their environment and the interactions of neighbouring amino acids (Matthews, 1993). Thus, even a single amino acid change in a protein can cause many localised shifts and interactions, and could potentially affect stability significantly.

Cowan (1995) discussed the mechanisms by which hyperthermophilic proteins have an increased stability. He attributed it to factors such as increasing intramolecular packing, loss of surface loops, increase in helix-forming amino acids, stabilisation of α-helix dipoles, reduction of asparagine content and the restriction of N-terminus mobility.

1.1.2.2 Interactions Involved in Protein Folding

(A) *Hydrophobic Stabilisation*:

One of the most important stabilising interactions is *hydrophobic bonding*. Protein folding is driven by the shielding of non-polar amino acids from the solvent (water). Hydrophobic interactions of the buried amino acids stabilise the folded protein (Nosoh and Sekiguchi, 1993). Until recently, the energy of stabilisation by transferring hydrophobic amino acids from the solvent to the protein interior was thought to be about 25-30 cal/mol/A (Matthews, 1993). It is now thought that these contributions are much higher.

Brandts (1967) investigated the relationship between the energy of transfer (Δf_{sc}) of an amino acid from the protein interior to water with temperature. He found that at all temperatures, the Δf_{sc} was large and positive, but that it became much more positive as the temperature increased. Therefore, hydrophobic stabilisation of proteins will occur at all temperatures, but more so at high temperatures.

The neutral protease of *Bacillus subtilis* contains a C-terminal leucine (Leu-300) residue. It is important in maintaining the thermal stability of the protease (Eijsink, 1991). Models, based on the known 3-dimensional structure of thermolysin, suggested that this residue is located in a hydrophobic pocket. Eijsink (1991) replaced this residue with the smaller amino acid alanine, with a polar asparagine or with a sterically unfavourable isoleucine. The thermostability of all of the mutant proteases was decreased. Replacing Leu-300 with phenylalanine resulted in an *increase* in thermostability. Thus, hydrophobic interactions are important for the stability of this protease.

Since hydrophobicity increases with increased temperature, it is expected that thermostable proteins would have a higher proportion of hydrophobic amino acids than mesophilic proteins. Studies have been carried out which replaced buried hydrophilic amino acids with hydrophobic ones (e.g. Grutter *et al.*, 1979; Imanaka and Aiba, 1986). These studies showed that the mutated proteins had an increased thermostability.

(B) *Hydrogen Bonds*:

Hydrogen bonds play a major role in the formation of the secondary and tertiary structures in the protein interior, e.g. α-helices and β-pleated sheets. Hydrogen bonds between uncharged donors and acceptors have been calculated to be between 0.5-1.8 Kcal/mol and those between charged groups about 6 Kcal/mol (Nosoh and Sekiguchi, 1993).

The energies of the hydrogen bonds in the folded and unfolded states are different due to differences in average geometry, entropy of formation and average number of interacting pairs of hydrogen bonds in the proteins (Alber, 1989). A survey of protein structures shows that the hydrogen bonding geometry is quite variable, and that unpaired hydrogen bond donors and acceptors are rare.

The stabilising effect of adding extra hydrogen bonds into proteases has been demonstrated with several enzymes. Subtilisin was stabilised by mutations that improved hydrogen bonding (Bryan *et al.*, 1986). A metallo protease from *Bacillus stearothermophilus* contains a buried Ala-170. Eijsink (1991) mutated the protein by replacing this residue with serine. Molecular dynamics simulations showed that Ser-170 stabilises the enzyme by the formation of an internal hydrogen bond. In addition, the -OH group could be increasing the stability by filling in an internal cavity.

In many proteins, buried water molecules may be present. Such water molecules usually participate in some form of hydrogen bonding (Rashin *et al.*, 1986). For example, in the mutant T_4 lysozyme, where isoleucine 3 was replaced by proline and tyrosine, the presence of a water molecule in the 3-dimensional structure could be clearly seen (Dixon *et al.*, 1992; Matsumura *et al.*, 1988). A water molecule was also bound in a crevice containing glycine 157, held there by 3 hydrogen bonds.

(C) *Electrostatic Interactions*:

Ionizable groups are not distributed randomly over protein surfaces. This reflects the structural and functional roles of these residues. On average, only about a third of ionizable groups are involved in ion pairs, with 70% of these pairs being buried (Alber, 1989). Overall, ion pairs tend to be poorly conserved in protein families.

Rice *et. al.* (1996) examined the structures of glutamate dehydrogenase from the hyperthermophile *Pyrococcus furiosus* and several mesophilic enzymes and found that a series of networks of ion pairs in regions of high charge density in the hyperthermostable enzyme. These networks were clustered at both the inter-domain and inter-subunit interfaces. It was suggested that these networks may be important in stabilising the enzyme.

Perutz and Raidt (1975) compared the 3-dimensional structures of mesophilic and thermophilic ferredoxins, based on the structure of the *Micrococcus aerogenes* ferredoxin. It was concluded that the increased thermostability of the thermophilic ferredoxins was due to an increased number of salt bridges (2-4).

Biesecker *et al.* (1977) compared the 3-dimensional structures of D-glyceraldehyde-3-phosphate dehydrogenases from lobster and *Bacillus stearothermophilus*. They concluded that 3 extra electrostatic interactions in each subunit in the thermophilic enzyme was a major contributor to its thermostability.

(D) *Helix Dipoles*:

Proteins can localise electrostatic charges by forming α-helix dipoles by showing preferences for negatively and positively charged residues at the first and last turn of the helix, respectively. The interaction of these dipoles with

charged residues in the local environment can be quite significant (Perutz *et al.*, 1985).

Richardson and Richardson (1988) showed that some amino acids tend to occur more frequently at certain positions within α-helices. In particular, glycine was often seen at the cap of the C-terminus of α-helices, asparagine at the N-terminus and proline at position N1, though there were exceptions. For example, Serrano and Fersht (1989) constructed a series of mutant barnase proteins where the N-cap residues threonine 6 and 26 were replaced with a variety of amino acids. The results were variable, but it was seen that in no case did the addition of asparagine to this position increase stability. It was concluded that this anomaly may have occurred because the side chains of the amino acids have different shapes, and they may require different backbone conformations in order to make acceptable hydrogen bonds to the amide group of the N3 position in the α-helix (Matthews, 1993). The overall conclusion is that the amino acid preferences at the ends of α-helices depends on the local stereochemistry of the existing members of the helix.

(E) *Disulphide Bonds*:

Disulphide bonds are covalent links between sulphur atoms in two different cysteine residues. They are considered to significantly contribute to the stability of a protein by forming covalent links between different parts of the protein, thereby aiding in holding the protein in the correct conformation. They are considered to stabilise by decreasing the entropy of the unfolded state.

The first type of protein stabilisation attempted using protein engineering was the introduction of disulphide bonds into T_4 lysozyme (Matsumura *et al.*, 1989). Other enzymes have also had disulphide bonds engineered into them, e.g. subtilisin, dihydrofolate reductase and λ repressor (see Nosoh & Sekiguchi, 1993). Only in some of these cases did the disulphide bond addition result in an enhancement of stability. The difficulty with the introduction of disulphide bonds is that the local environment must be correct for the bonds to form. Requirements such as bond distance, bond angle, orientation of the cysteine residues and the interactions in the surrounding environment may all be important in determining if a stabilising bond will form. Simply adding 2 cysteine residues to the protein in the same area may not be sufficient to ensure a bond will form. Thus, it is not suprising that not every attempt has been successful. Even where a bond has been formed, this does not ensure an increase in stability.

Native T_4 lysozyme has 2 free cysteine residues at positions 54 and 97 (Rossman and Argos, 1976). Perry and Wetzel (1984), using computer simulation of its known 3-dimensional structure, found positions in the protein that potentially had the correct requirements that would allow a disulphide bond to be formed. They used site-directed mutagenesis to introduce a cysteine residue at position 3, which could possibly form a bond with cysteine 97. The mutant protein was subjected to *in vitro* oxidation, and a disulphide bond was formed. This protein had a slower irreversible thermal inactivation and stabilisation towards reversible inactivation (Perry & Wetzel, 1984).

Proline 39 in *E. coli* dihydrofolate reductase was replaced by cysteine (Villafranca *et al.*, 1987). It formed a disulphide bond *in vivo* with native cysteine 85. The mutant protein was about 1.8 Kcal/mol more stable than either the wild-type or the reduced mutant proteins with respect to denaturation by guanidine hydrochloride.

Disulphide bonds are known to stabilise by decreasing the flexibility of the protein. Ribonuclease has a high thermostability, and it contains 4 disulphide bonds. (Spackman *et al.*, 1960). However, data does not show that disulphide bonds are more numerous in thermophilic proteins; in fact the opposite may be true (Ljungdahl and Sherod, 1976). For example, a thermostable enolase contains no half cysteine residues, while the less thermostable enolase does. Some thermophilic enzymes are actually activated by sulphydryl compounds, e.g. the aldolase from *Thermus aquaticus* (Freeze and Brock, 1970).

(F) *Dispersion Forces*:

During protein folding, residues that form the protein interior are forced together. Dispersion forces (Van der Waals forces) are attractive forces that cause residues to become densely packed (Nosoh and Sekiguchi, 1993). The packing of these molecules is not uniformly compact due to the differences in the side chains of the different amino acids found in the protein. These irregularities can form pockets or cavities in the protein interior, which tend to destabilise the protein. Therefore, substitutions that result in a 'filling in' of these cavities may increase the stability of the protein. Thus, dispersion forces are thought to play a significant role in stabilising the interior of proteins.

A comparison of the wild-type and mutant T_4 lysozyme proteins showed that dispersion forces and hydrophobic interactions of α-methyl group of

threonine 157 in the wild-type protein is stabilising (Alber *et al.*, 1987). Alanine 146 of this protein was then replaced by threonine, resulting in the 'filling-in' of the cavity at this site. This same cavity was expanded by replacing methionine 102 by threonine (Grutter *et al.*, 1983). In both cases, the protein was destabilised. Thus, cavities can have a significant effect on protein stability. This shows that not all cavity-filling mutations result in the stabilisation of a protein.

(G) *External Effects*:

Metal cations, such as Ca^{2+}, are known to stabilise some proteins. For example, proteases such as thermolysin (Matthews *et al.*, 1972b), thermitase (Frommel & Hohne, 1981), caldolysin (Khoo *et al.*, 1984) and Rt41A (Peek *et al.*, 1992a) are known to bind Ca^{2+} ions. These ions often significantly increase the thermostability of the proteases. For example, caldolysin is stabilised by binding 6 Ca^{2+} ions per enzyme molecule (Khoo *et al.*, 1984). Apocaldolysin (no Ca^{2+} ions) and Ca^{2+}-caldolysin have half lives of 40 minutes at *55°C* and 60 minutes at *95°C* respectively. These Ca^{2+} ions bind into 'pockets' by interacting with oxygen atoms in the enzyme, typically from aspartate residues and backbone carbonyl oxygen of amino acids.

Ca^{2+} can potentially stabilise proteases against both denaturation and autolysis. For example, in the case of Rt41A protease, 10 μM $CaCl_2$ was required to protect the protease against thermal denaturation, while 5 mM $CaCl_2$ was required to protect against autolysis (Peek *et al.*, 1992a).

Other metal ions can substitute for Ca^{2+} in increasing thermal stability. Caldolysin can bind lanthanide ions (e.g. Tb^{3+}, Ln^{3+}) in the Ca^{2+}-binding sites in a manner similar to Ca^{2+} (Khoo *et al.*, 1984). Other metals, such as Mn^{2+} and Co^{2+} have been known to slightly stabilise proteases. *Methanothermus fervidus* enzymes (e.g. malate dehydrogenase) show increased thermostability in the presence of high concentrations of K^+ (Hensel and Konig, 1988). This is not suprising as this organism contains high intracellular K^+.

The addition of sugars and other polyols to protein solutions has been demonstrated to strengthen hydrophobic interactions, and therefore make the proteins more resistant to denaturation (e.g. Piller *et al.*, 1996). For example, the half life of invertase at 60°C in 2.75M sucrose was ten times greater than in 1.5 M sucrose (Monsan and Combes, 1984). The increased stability was not due to substrate stabilisation as a similar stabilising effect was seen with non-substrate

polyols. It is thought that polyols and dextrans stabilise enzymes by binding twater molecules and thus reducing the free water availability of the solution.

Proteins tend to be more stable in concentrated solutions than dilute solutions. This has been noticed by studying intracellular proteins, which are usually more stable within the cell than in the diluted cell extracts (Ward and Moo-Young, 1988). However, the reverse tends to be true for proteases since these enzymes also undergo irreversible inactivation by autolysis. Thus increasing the protease concentration will effectively increase the 'substrate' concentration, and therefore increase the rate of autolysis.

1.1.2.3 Other Factors

i) Covalent Modification

Thermostable proteins were thought to be more likely to be susceptible to covalent modification reactions, e.g. deamidation of asparagine residues, than mesophilic proteins as the rate of these modifications increases with temperature (Daniel *et al.*, 1995). It was proposed that the temperatures where these reactions occurred significantly could determine the "upper temperature limit of life". However, studies by Daniel (1996) showed evidence that the rate of covalent modification reactions was higher for *mesophilic* proteins not thermophilic proteins at a given temperature. This suggests that these reactions are dependent on the flexibility of the protein as mesophilic proteins are more mobile than the equivalent thermophilic proteins at a given temperature. Thus, it appears that these reactions are slower in proteins in their native conformation, and the chemical modification reaction rates increase when the protein is denatured.

ii) Autolysis

Theoretically, as the temperature increases, the rate of autolysis of proteins increases also. This can have a large impact on the thermostability of only proteases. If a protease is heated at a pH where activity is insignificant (but the protease is stable at this pH), the rate of autolysis decreases significantly, and the thermostability increases (Toogood *et al.*, 1995).

Daniel *et al* (1982) demonstrated that the loss of activity due to proteolysis of purified L-asparaginase and β-galactosidase from mesophiles and thermophiles was dependent on the thermostability of the enzyme (not

protease). They found that the lower the thermostability, the more susceptible the enzyme was to proteolysis.

With proteases, as the temperature approaches the denaturation temperature, the rate of autolysis is expected to increase disproportunately as an unfolded protease is more susceptible to cleavage by a neighbouring protease in the native form.

1.2 THERMOSTABLE PROTEASES

While investigations of proteases have been carried out since the early days of enzymology, the study of thermostable proteases has a more recent history. An early report by Heinen and Heinen (1972) described the production of an extracellular protease by a *Bacillus* species growing at 72°C. Since then, thermostable proteases, as well as other thermostable enzymes, have been extensively studied. Perhaps the most studied of these enzymes is Thermolysin produced by *B. thermoproteolyticus*, which was first described by Endo (1962). Other early work focussed on the protease from *Thermonospora fusca* A20 (Desai and Dhala, 1969), Thermomycolase from *Malbranchea pulchella* var. *sulfurea* (Ong and Gaucher, 1976), Thermitase from *Thermoactinomyces vulgaris* (Hausdorf *et al.*, 1980) and Caldolysin from a *Thermus* sp (Cowan and Daniel, 1982a).

Since the mid 1980's, a large number of thermostable proteases have been characterised from eubacteria, and more recently from hyperthermophilic archaea. This section aims to give an overview of properties of some of the thermostable proteases characterised to date, with some emphasis on thermostability. Table 1.2 lists properties of some thermostable proteases. Proteases have been included if their half life is greater than 1 hour at 65°C or greater than 10 minutes at 70°C, or in a few cases if such a half life can be inferred from the 'temperature optima' data. The substrate specificity of some of these proteases is presented in Table 1.3, although the substrates listed are by no means exhaustive.

1.2.1 Serine Proteases

The serine proteases comprise the majority of the thermostable proteases characterised to date. They have a reactive serine residue at the active site, and their diagnostic inhibitors are disopropyl fluorophosphate (DFP) and phenylmethylsulphonyl fluoride(PMSF). There are two groups of serine proteases. Group I are chymotrypsin-like, while group II are subtilisin-like (Beynon and Bond, 1989). These groups differ from each other by their amino acid sequences and three-dimensional structures, but have very similar active site configurations. They typically have pH optima between pH 7-11, and often have broad substrate specificities.

1.2.1.1 *Bacillus* Serine Proteases

The classical *Bacillus* serine proteases are Subtilisin Carlsberg (DeLange and Smith, 1968) and Subtilisin BPN (Markland and Smith, 1967), isolated from *B. licheniformis* and *B. amyloliquefaciens* respectively. However, they are thermolabile by the criteria of this section, so they will not be discussed further.

The *Bacillus* serine proteases are principally alkalophilic, with pH optima typically >8.0. *Bacillus* sp. no. AH-101 produces an extremely alkalophilic protease with a pH optimum between 10-13 (Takami, *et al.*, 1989). It is moderately thermostable, and most readily hydrolyses insoluble fibrous proteins such as elastin and keratin (Takami, *et al.*, 1992). It has recently been cloned and expressed in *B. subtilis*, and shows high sequence homology to the alkaline subtilisin-like proteases (Takami *et al.*, 1990).

Another extremely alkalophilic protease is AprM produced by *Bacillus* st. B18' (Fujiwara *et al.*, 1993; Masui *et al.*, 1994). It has a half-life of 75 minutes at 70°C in the presence of 10 mM Ca^{2+}, and a high sequence similarity to *Bacillus* st. AH-101 protease (Fujiwara *et al.*, 1993). Recently it was cloned and sequenced, and its amino acid composition compared to similar proteases (Masui *et al.*, 1994).

The protease from *B. thermoruber* BT_2^T is moderately thermostable, with stability increased in the presence of Ca^{2+} (Manachini *et al.*, 1988). It showed the highest activity against Z-Ala-Ala-Leu-pNA, a typical substrate for subtilisins (Manachini *et al.*, 1988). *Bacillus thuringiensis* var. *kurstaki* HD-255 produces a thermostable protease with a pH optimum of 8.5-9.0 (Kunitate *et al.*, 1989). It retained 88% of its activity after more than 7 hours incubation at 60°C. It was inhibited by both PMSF and a cysteine protease inhibitor *p*-chloromercuribenzoate (PCMB), suggesting it is a member of the sub-family of SH-dependent serine proteases (Kunitate *et al.*, 1989).

B. subtilis NRRL B3411 produces an alkaline serine protease that is marginally thermostable (Keay *et al.*, 1970). It has a molecular weight of ~28-30 kDa, and a pH optimum of 10.0. After an incubation of 30 mins at 65°C, about 75% of its activity remained. The stability was found to be increased in the presence of Ca^{2+} (Keay *et al.*, 1970). This strain of *Bacillus* also produces a neutral metalloprotease (Keay and Wild, 1970; Pangburn *et al.*, 1976), but it has a much lower thermostability.

Source (Enzyme Name)		MW (kDa)	pI	pH_{opt}	Stability	References
SERINE						
Arthrobacter aureus		22	10	7.0	"T_{opt}"=70°C	Michotey and Blanco (1994)
Bacillus sp. no. AH-101		29-30	9.2	10-13[3]	60% left 10 min 80°C	Takami *et al* (1989, 1990), Fujiwara *et al.* (1993)
Bacillus st. AK.1		36.9	4.0	7.5	$t_{1/2}$ = 13 hours, 80°C	Peek *et al.* (1993)
Bacillus st. B18' (AprM)		28-30	-	12-13	$t_{1/2}$ = 75 min, 70°C	Fujiwara *et al.* (1993)
Bacillus st. K-295G-7	I	28	9.22	9.0	"T_{opt}"=65°C, 30 min assay	Park *et al.* (1987)
	II	29.5	9.45	9.0	"T_{opt}"=65°C, 30 min assay	Park *et al.* (1987)
Bacillus stearothermophilus F1		20-33.5	-	9.0	$t_{1/2}$ = 4 hours 85°C	Rahman *et al.* (1994)
B. stearothermophilus RM-67	II	19.95	-	8.0	20% left 30 min 65°C	Chopra and Mathur (1985)
B. subtilis NCIM No. 64		28	-	9.7	No act. loss 1 hour 70°C + Ca^{2+}	Kembhavi *et al.* (1993)
B. subtilis NRRL B3411	I	28.2-32.5	-	10.0	~75% left, 30 mins 65°C	Keay *et al.* (1970)
B. thermoruber BT_2^T		39	5.3	9.0	60% left 30 min 70°C	Manachini *et al.* (1988)
B. thuringiensis var. *kurstaki* HD-255		34	9.0	8.5-9	88% left >7 hours 60°C	Kunitate *et al.* (1989)
Desulfurococcus st. $Tok_{12}S_1$ (Archaelysin)		52	8.7	7.2	$t_{1/2}$ = 70-79 min, 95°C	Cowan *et al.* (1987c)
Malbranchea pulchella var.*sulfurea* (Thermomycolin)		32.7	6.0	8.5	$t_{1/2}$ = 110 min 73°C + Ca^{2+}	Gaucher and Stevenson (1976)
Pyrobaculum aerophilum (Aerolysin)		-	-	neu-alk	"T_{opt}"=100-130°C	Volkl *et al.* (1994)
Pyrococcus furiosus DSM 3638	(S66)	66	-	7.0	-	Blumentals *et al.* (1990), Connaris *et al.* (1991),
	(S102)[5]	102(Dimer?)	-	7.0	$t_{1/2}$ = 33h, 98°C(S66& S102)	
	(Pyrolysin)	65-140[5]	-	6.5-10.5	$t_{1/2}$ =20 min, 105°C	Eggen *et al.* (1990)
Staphylothermus marinus DSM 3639		30-300[2]	-	9.0(major)	$t_{1/2}$ = 5 h, 90°C	Klingeberg *et al.* (1991)
Streptomyces rectus var. *thermoproteolyticus*						Mizusawa and Yoshida (1972),
	Protease B	21.5	9.5	10.7[4]	65-70% left 10 min, 80°C	Mizusawa *et al.* (1964)
Sulfolobus solfataricus MT-4 (ATCC 49155)						
	Protease I	118	5.6	6.5-8[3]	$t_{1/2}$ = 342 min, 92°C	Burlini *et al.* (1992)
	Protease II	32	-	7.0[1]	Inactivated at 60°C, 15 min	Fusi *et al.* (1991)
Thermoactinomyces vulgaris (Thermitase)		28.4	9.1	8.5	"T_{opt}"=85°C	Gusek and Kinsella (1987), Frommel and Hohne (1981)
Thermobacteroides proteolyticus DSM 5265		30-300[2]	-	9.5	$t_{1/2}$ = 1.5h, 90°C	Klingeberg *et al.* (1991)
Thermococcus st. AN1		30-300[2]	-	7.0	$t_{1/2}$ = 30h, 90°C,	Klingeberg *et al.* (1991)
Thermococcus celer DSM 2476		30-300[2]	-	7.5	$t_{1/2}$ = 35-40h, 90°C	Klingeberg *et al.* (1991)
Thermococcus litoralis DSM 5473		30-300[2]	-	9.0	$t_{1/2}$ = >50h, 90°C	Klingeberg *et al.* (1991)
Thermococcus stetteri DSM 5262		30-300[2]	-	9.0(major)	$t_{1/2}$ = 30-35h, 90°C	Klingeberg *et al.* (1991)
Thermomonospora fusca YX		14.5	9.21	9.0	$t_{1/2}$ = 15 min, 85°C, pH 4.5	Gusek and Kinsella (1987)
Thermus st. Rt41A		32.5	~10.5	8.0	$t_{1/2}$ = 13.5 h, 80°C	Peek *et al.* (1992a)
Thermus st. Rt4A2		31.6	10.25	9.0	$t_{1/2}$ = 43 h, 80°C	Freeman *et al.* (1993)
Thermus st. Rt_6		27	8.5	7.7-8.8	$t_{1/2}$ = 360 min, 85°C	Cowan *et al.* (1987a)
Thermus st. Tok_3 (Caldolase)		25	8.9	9.5	$t_{1/2}$ = 840 min, 80°C	Saravani *et al.* (1989)
Thermus aquaticus st. T-351 (Caldolysin)		21	8.5	8.0[3]	$t_{1/2}$ = 30 h, 80°C	Cowan and Daniel (1982a)
Thermus aquaticus st. YT-1	(Aqualysin I)	28.5	>9-10	10.4	40% left 30 min, 90°C	Matsuzawa *et al.* (1983), Matsuzawa *et al.* (1988)
	(Aqualysin II)	-	-	7.0	"T_{max}"=95°C	
Thermus caldophilus GK24		31	-	7.8	$t_{1/2}$ = 120 min 80°C	Taguchi *et al.* (1983), Cowan *et al.* (1985)
METALLO						
Aeromonas proteolytica		29.5	3.0-3.5	8-8.5	No act. loss, few hours 70°C	Prescott and Wilkes (1976)
Aspergillus oryzae (NpII)		19.0	-	5.5-6.0	~70% left 10 min 90°C	Nakadai *et al.* (1973)
Bacillus st. EA.1		42	-	6.7	$t_{1/2}$ = 7 days, 75°C 10mM Ca^{2+}	Coolbear *et al.* (1991)
Bacillus st. OK3A.1		32	-	7.0	$t_{1/2}$ = 760 min 75°C 10 mM Ca^{2+}	Coolbear *et al.* (1991), Coolbear *et al.* (1992)
Bacillus brevis 7882		35	5.5-6.0	6.7	>50% left 1 hour, 80°C	Paberit *et al.* (1982)
B. caldolyticus YP-T (DSM 405)		-	-	7.0	No act. loss 8 hours, 80°C	Heinen and Heinen (1972), Van den Burg *et al.* (1991)

B. cereus DSM 3101	35	-	6.6-6.8	~60% left 20 min 70°C + Ca^{2+}	Sidler *et al.* (1986a)
B. stearothermophilus API (Aminopeptidase)	400(12sub)	-	7.5-9.4 [3]	No act. loss 15 hours, 80°C	Roncari *et al.* (1976)
B. stearothermophilus CU21 NprT	36	-	7.0	80% left, 30 min, 65°C	Fujii *et al.* (1983)
B. stearothermophilus IFO 12983 (Dipeptidase)	86 (2x43)	4.5	8.0	No act. loss 70°C, 10 min	Cho *et al.* (1988)
B. stearothermophilus KP 1236	33	7.5	7.0	No act. loss 80°C, 10 min	Takii *et al.* (1987)
B.stearothermophilus MK 232 NprM	34	-	7.5	No act. loss <70°C pH 5-10	Kubo *et al.* (1988)
B. stearothermophilus RM-67 I	67.61	-	8.0	63% left 30 mins 65°C	Chopra and Mathur (1985)
B. thermoproteolyticus (Thermolysin)	34.5	***	7.2	$t_{1/2}$ = 1 h, 80°C + Ca^{2+}	Edwards (1990), Aunstrup (19 Gusek and Kinsella (1988)
Chloroflexus aurantiacus I	66	6.2	8.0	60% left 1 hour 80°C	Watanabe *et al.* (1993)
Sulfolobus solfataricus DSM 1616	320(4x80)	4.4	6.5	76% left 15 min 75°C + Co^{2+}	Hanner *et al.* (1990)
Sulfolobus solfataricus MT-4 (ATCC 91?5) Carboxypeptidase	170(tetramer)	5.0	5.5-9[3]	No act. loss 85°C 15 min	Fusi *et al.* (1991), Colombo *et* (1992)
Talaromyces duponti AP-1 (Aminopeptidase)	400	-	6.9	No act. loss 8 h 55°C pH 7.2	Chaupis and Zuber (1970)
Thermomicrobium st. KN-22	35	-	8.5	"T_{opt}"=75°C 10 min assay	Murao *et al.* (1991)
Thermus aquaticus st. YT-1 (Aminopeptidase T)	108 (dimer)	-	8.5-9.0	60% left 20 h, 80°C	Minagawa *et al.* (1988)
(CPase *Taq*)	56-58	-	8.0	90% left 5 h, 80°C	Lee *et al.* (1992)
ASPARTIC					
Aspergillus niger F2078[5]	-	-	3-4	"T_{opt}"=60°C 10 min assay	Singh *et al.* (1994)
Bacillus st. MN-32 (Kumamolysin)	40-41	3.5	3.0	$t_{1/2}$ ~ 10 min, 80°C	Murao *et al.* (1993)
Bacillus st. Wai21a	45	4.0	3.0	$t_{1/2}$ = 2 min, 80°C, pH 3.0	Prescott *et al.* (1992), Prescott (1995)
Bacillus st. Wp22.A1	45	3.8	3.5	$t_{1/2}$ = 23 min, 80°C, pH 3.5	Toogood *et al.* (1994)
Sulfolobus acidocaldarius (Thermopsin)	46	4.0	2.7	$t_{1/2}$ = 48 h, 80°C, pH4.5	Lin and Tang (1990)
CYSTEINE					
Bacillus stearothermophilus 1503	-	-	6.9-7.2	9% left 10 min 65°C	O'Brien and Campbell (1957)
Pyrococcus st. KOD1	44-45	-	7.0	$t_{1/2}$ = 60 min, 100°C	Morikawa *et al.* (1994)
Sulfolobus solfataricus MT-4 (ATCC 49155) Protease III	27	-	7.0[1]	~90% left 15 min 90°C	Fusi *et al.* (1991)
OTHER					
Thermoplasma acidophilum (Proteosome)	53(27+25)	-	7.5[1]	-	Zwickl *et al.* (1992), Dahlmann (1989)
Bacillus st. P-OO1A	-	-	9.5	$t_{1/2}$ = 30 min 70°C	Atalo and Gashe (1993)
Thermoactinomyces sp. HS682	25	-	11.5-13.0	No act. loss ≤ 65°C, 60 min	Tsuchiya *et al.* (1991), Fujiwar *al.* (1993)
Thermoactinomyces vulgaris A60	23.8	-	9.0	$t_{1/2}$ = 1 hour ~ 85°C	Desai and Dhala (1969)
Thermomonospora fusca A29	21.5	-	8.5	$t_{1/2}$ = 1 hour 80°C	Desai and Dhala (1969)

Table 1.2 Properties of Some Thermostable Proteases

$t_{1/2}$ half life at temperature specified. [1] assay conditions. [2] possibly multiple forms of different subunit composition present. [3] depends on the substrate. [4] shoulder at pH 8-9 in pH profile curve. [5] from a crude cell extract which contains multiple activity bands of different molecular weights. - data not available. neu-alk = neutral to alkaline

Source (Enzyme Name)	Peptide Substrates	Insulin b Chain Cleavage	References
SERINE			
Bacillus st. AK.1	Suc-Ala-Ala-Pro-Phe-NH-Np	Leu^{15}-Tyr^{16}, Gln^{4}-His^{5}, Glu^{13}-Ala^{14}	Peek *et al.* (1993)
B. subtilis NRRL B3411	CBZ-Gly-pNO_2 phenyl ester	-	Keay *et al.* (1970)
B. thermoruber BT_2^T	Z-Ala-Ala-Leu-pNA, Z-Gly-Gly-Leu-pNA	-	Manachini *et al.* (1988)
Desulfurococcus st. $Tok_{12}S_1$ (Archaelysin)	A variety of Tripetide-pNA	Ala^{14}-Leu^{15}, Tyr^{16}-Leu^{17}	Cowan *et al.* (1987c)
Malbranchea pulchella var. *sulfurea* (Thermomycolin)	CBZ-Ala-ONp, CBZ-Tyr-ONp, CBZ-Phe-ONP	Glu^{13}-Ala^{14}, Leu^{15}-Tyr^{16}, Phe^{24}-Phe^{25}	Gaucher and Stevenson (1976), Ong and Gaucher (1976)
Pyrococcus furiosus DSM 3638 S66 & S102	Bz-Arg-ethyl ester, Bz-Arg-pNA	-	Blumentals *et al.* (1990)
Staphylothermus marinus DSM 3639	Succ-Ala-Ala-Pro-Phe-pNA, D-Val-L-Leu-Lys-pNA	-	Klingeberg *et al.* (1991)
Streptomyces rectus var. *thermoproteolyticus* Protease B	N-Acetyltyrosine ethyl ester	Phe^{24}-Phe^{25}, Leu^{11}-Val^{12}, Leu^{15}-Tyr^{16}, Gln^{4}-His^{5}	Mizusawa and Yoshida (1972), Matsue *et al.* (1982)
Sulfolobus solfataricus MT-4 (ATCC 49155)			
Protease I	CBZ-Tyr-pNA	Did not detect any cleavage	Burlini *et al.* (1992)
Protease II	Succ-Ala-Ala-Pro-Phe-pNA	ND	Fusi *et al.* (1991)
Thermoactinomyces vulgaris (Thermitase)	P-nitrophenyl acetate	-	Frommel and Hohne (1981)
Thermobacteroides proteolyticus DSM 5265	Succ-Ala-Ala-Pro-Phe-pNA, D-Val-L-Leu-Lys-pNA	-	Klingeberg *et al.* (1991)
Thermococcus st. AN1	Succ-Ala-Ala-Pro-Phe-pNA, D-Phe-Pip-Arg-pNA	-	Klingeberg *et al.* (1991)
Thermococcus celer DSM 2476	Succ-Ala-Ala-Pro-Phe-pNA, D-Phe-Pip-Arg-pNA	-	Klingeberg *et al.* (1991)
Thermococcus litoralis DSM 5473	Succ-Ala-Ala-Pro-Phe-pNA, Bz-Arg-pNA	-	Klingeberg *et al.* (1991)
Thermococcus stetteri DSM 5262	Succ-Ala-Ala-Pro-Phe-pNA, Bz-Lys-pNA	-	Klingeberg *et al.* (1991)
Thermomonospora fusca YX	SAAPF-pNA	-	Gusek and Kinsella (1987), Johnson *et al.* (1990)
Thermus st. Rt41A	Succ-Ala-Ala-Pro-Phe-NH-Np, Cbz-Gly-pNP ester	Leu^{15}-Tyr^{16}, Gln^{4}-His^{5}, His^{5}-Leu^{6}, Phe^{24}-Phe^{25}	Peek *et al.* (1992a)
Thermus st. Rt4A2	Succ-Ala-Ala-Pro-Phe-NH-Np, *N*-Bz-Phe-Arg-Nh-Np	-	Freeman *et al.* (1993)
Thermus st. Rt_6	Bz-Iso-Gly-Glu-Arg-pNA Succ-Ala-Ala-Ala-pNA	-	Cowan *et al.* (1987a)
Thermus st. Tok_3 (Caldolase)	Bz-Phe-Val-Arg-Nh-Np, Z-Ala-ONp, Z-Tyr-ONp	-	Saravani *et al.* (1989)
Thermus st. T-351 (Caldolysin)	Benzoyl-Phe-Val-Arg-pNA, CBZ-Gly-Pro-Leu-Gly-Pro	-	Cowan and Daniel (1982a)
Thermus aquaticus st. YT-1 Aqualysin I	Bz-Ala, Bz-Trp, Bz-Phe	Gln^{4}-His^{5}, Leu^{15}-Tyr^{16}, Phe^{24}-Phe^{25}, Phe^{25}-Tyr^{26}	Matsuzawa *et al.* (1983)
Thermus caldophilus GK24	CBZ-Leu-Tyr & esterase activity	-	Coolbear *et al.* (1992)
METALLO			
Aeromonas proteolytica	Leu-Gly, Leu-Val	-	Prescott and Wilkes (1976)
Aspergillus oryzae (NpII)	Cbz-Gly-NH_2, Cbz-Pro-Leu-NH_2, Cbz-Gly-Pro-NH_2	-	Nakadai *et al.* (1973)
B. brevis 7882	FAGLA	All bonds where Phe and Ala are at the N-terminus, plus others	Paberit *et al.* (1982)
B. cereus	FALGA	-	Sidler *et al.* (1986a)
B. stearothermophilus API (Aminopeptidase)	Leu-Gly, Leu-pNA, Gly-Leu-Tyr (broad specificity)	-	Roncari *et al.* (1976)
B. stearothermophilus CU21	Z-Gly-Leu-NH_2	Cleavage sites not listed	Takagi and Imanaka (1989)
B. stearothermophilus IFO 12983	Di, tri & tetra peptides esp. Val-Ala,Gly-Phe & Met-Gly	-	Cho *et al.* (1988)

B. stearothermophilus KP 1236	CBZ-Gly-Leu-amide, CBZ -Gly-Phe-amide	Cleavage sites not detected	Takii *et al.* (1987)
B. thermoproteolyticus (Thermolysin)		Tyr^{16}-Leu^{17}, His^{10}-Leu^{11}	Morihara (1974)
Chloroflexus aurantiacus J-10-fl	-	Ala^{14}-Leu^{15}, Gly^{23}-Phe^{24}	Watanabe *et al.* (1993)
Sulfolobus solfataricus DSM 1616	Ala-pNA, Leu-pNA, Arg-pNA, Phe-pNA	-	Hanner *et al.* (1990)
Sulfolobus solfataricus MT-4 (ATCC 49155) Carboxypeptidase	BZ-Gly-Lys, CBZ-Tyr-OEt	-	Fusi *et al.* (1991), Colomb *al.* (1992)
Talaromyces duponti AP-1 (Aminopeptidase)	Leu-pNA	-	Chaupis and Zuber (1970)
Thermomicrobium st. KN-22	-	Phe^{24}-Phe^{25}, Gly^{23}-Phe^{24}, His^{10}-Leu^{11}, Tyr^{16}-Leu^{17}	Murao *et al.* (1991)
Thermus aquaticus st. YT-1 (Aminopeptidase T)	Gly-Phe-Ala, Leu-2-NA, Leu-4-NA, Val-Tyr-Val	-	Minagawa *et al.* (1988)
(CPase *Taq*)	Cbz-Phe-Tyr-NH_2, Cbz-Gly-Phe-NH_2, Cbz-Gly-Leu-NH_2	-	Lee *et al.* (1992)
ASPARTIC			
Bacillus st. MN-32 (Kumamolysin)	-	Leu^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}	Murao *et al.* (1993)
Bacillus st. Wai21a	-	Leu^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}, Gln^{4}-His^{5}, His^{5}-Leu^{6}, Phe^{24}-Phe^{25}	Prescott *et al.* (1995)
Bacillus st. Wp22.A1	-	Val^{2}-Asn^{3}, His^{5}-Leu^{6}, Leu^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}	Toogood *et al.* (1995)
Sulfolobus acidocaldaris (Thermopsin)	Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu	Leu^{11}-Val^{12}, Leu^{15}-Tyr^{16}, Phe^{24}-Phe^{25}, Phe^{25}-Tyr^{26}, Tyr^{26}-Thr^{27}	Fusek *et al.* (1990), Lin ar Tang (1990)
CYSTEINE			
Bacillus stearothermophilus 1503	Leu-Gly-Gly, $(Gly)_3$, $(Gly)_4$	-	O'Brien and Campbell (19
Sulfolobus solfataricus MT-4 (ATCC49155) Protease III	BZ-Arg-pNA	-	Fusi *et al.* (1991)
OTHER			
Thermoplasma acidophilum (Proteosome)	Succ-Leu-Leu-Val-Tyr-NMec, Glu-Gly-Gly-Phe-NMec	-	Dahlmann *et al.* (1989)

Table 1.3 Substrate Specificity of Some Thermostable Proteases

- data not available. The list of substrates is not complete

B. subtilis NCIM No. 64 produces a thermostable, alkaline protease (Kembhavi *et al.*, 1993). Stability was increased in the presence of NaCl, with an optimal NaCl concentration of 15% when assayed at 72°C. Thermostability was furthur increased in the presence of Ca^{2+}, and the enzyme showed no loss of activity after 1 hour at 70°C with Ca^{2+} (Kembhavi *et al.*, 1993).

The protease from *Bacillus* st. AK.1 is the most thermostable of the *Bacillus* serine proteases (Peek *et al*, 1993). It has a half-life of 13 hours at 80°C and 19 minutes at 90°C in the presence of Ca^{2+}. The half life decreases to only 5 mins at 70°C in the absence of Ca^{2+}. The major cleavage sites of oxidised insulin B chain were Leu^{15}-Tyr^{16}, Gln^{4}-His^{5} and Glu^{13}-Ala^{14}, which is typical of other serine proteases such as Thermitase, Aqualysin 1 and Subtilisin BPN. It has been cloned and expressed in *Escherichia coli*, and showed high sequence homology to Thermitase, a serine protease from *Thermoactinomyces vulgaris* (Maciver *et al.*, 1994).

1.2.1.2 *Thermus* Serine Proteases

One of the earlier thermostable proteases characterised was Caldolysin, an extracellular serine protease from *Thermus aquaticus* st. T-351 (Cowan and Daniel, 1982a). The original data on caldolysin suggested that it was a metallo-protease (Cowan and Daniel, 1982a), however later studies showed that it is actually a serine protease (Cowan *et al.*, 1987b). It has a molecular mass of 21 kDa and a pH optimum of 8.0. The enzyme was specific for small aliphatic amino acids on either side of the sissile bond (Cowan and Daniel, 1982a).

Caldolysin is a very thermostable protease, with a half life of 228 minutes at 85°C (Cowan *et al.*, 1987a). It binds a total of 6 Ca^{2+} ions per molecule, but has two different types of Ca^{2+} binding sites with different binding affinities (Khoo *et al.*, 1984). These Ca^{2+} ions are thought to be responsible for the high thermostability of Caldolysin. An interesting finding is that lanthanide ions confer a greater thermostability on apo-caldolysin than Ca^{2+}, increasing its thermostability from 1 hour with Ca^{2+} to more than 4 hours at 95°C with La^{2+}. It was shown that the relative ability of various divalent cations to stabilise the enzyme was dependent on their ionic radius and directionality (Khoo *et al.*, 1984). When immobilised on Sepharose 4B and CM-cellulose supports, caldolysin had an increased thermostability, but a decreased activity, perhaps due to steric hinderance (Cowan and Daniel, 1982b).

Thermus aquaticus st. YT-1 produces two thermostable serine proteases named Aqualysin I and II (Matsuzawa *et al.*, 1983). Aqualysin I is an alkalophilic enzyme with a pH optimum of around 10. It contains four cysteine residues (possibly two disulphide bonds), and has an amino acid composition similar to those of other SH-dependent serine proteases (Matsuzawa *et al.*, 1988). Aqualysin I prefers to cleave ester substrates composed of amino acids with small hydrophobic and aromatic residues. The primary and secondary cleavage sites of oxidised insulin B chain were Leu^{15}-Tyr^{16} and Gln^{4}-His^{5} respectively. Thermostability was enhanced in the presence Ca^{2+} ions at temperatures >70°C (Matsuzawa, *et al.*, 1988).

Thermus st. Rt41A produces a thermostable extracellular serine protease (Peek *et al.*, 1992a) that has been recently been cloned and expressed in *E. coli* (Munro *et al.*, 1994). The protease has a much broader substrate specificity than AK.1 protease, as shown by the cleavage of a large proportion of the bonds of oxidised insulin B chain. It prefers substrates containing small aliphatic or aromatic amino acids at the P1 position. Like Aqualysin I, Rt41A protease contains 4 cysteine residues and has two disulphide bonds. It is stabilised against thermal denaturation at 10mM Ca^{2+}, and against autolysis at 5mM Ca^{2+} (Wilson *et al.*, 1994b). Immobilised onto controlled pore glass (CPG) beads (Wilson *et al.*, 1994b), the enzyme had a lowered pH optimum for the substrates azocasein and SucAAPFpNA, a similar effect to that found for caldolysin immobilised onto CPG beads (Cowan and Daniel, 1982a). The immobilised protease showed a dramatic increase in the thermostability at 70°C in the presence of 10mM Ca^{2+} compared to the free enzyme, with half lives of 110 and 5 hours respectively (Wilson, Peek and Daniel, 1994). The protease was capable of synthesising Bz-Ala-Tyr-NH2 with a yield of 26% in the presence of 90% dimethylformamide at 40°C (Wilson *et al.*, 1994a; Peek *et al* 1992b).

Not all *Thermus* serine proteases are dependent on Ca^{2+} for stability. Caldolase, from *Thermus* st. ToK_3, is an alkaline protease with a pH optimum of 9.5 (Saravani *et al.*, 1989). It contains approximately 10% carbohydrate and four disulphide bonds. Neither Ca^{2+} or Zn^{2+} were required for activity or stability. However, it was unstable in low ionic strength buffers and required 0.4M NaCl to maintain stability. It had a half life of 14 hours at 80°C (Saravani *et al.*, 1989). *Thermus* st. RT_6 also produces a protease that does not appear to be affected by Ca^{2+}, and that has a half life of 360 minutes at 80°C (Cowan *et al.*, 1987a). The protease from *Thermus* st. Rt4A2 showed less susceptibility to EDTA than other *Thermus* serine proteases such as Rt41A. The enzyme was more thermostable than caldolase, having a half life of 43 hours and 19.4

minutes at 80°C and 100°C respectively, although Ca^{2+} ions enhanced stability (Peek *et al.*, 1992a, Freeman *et al.*, 1993).. N-terminal sequencing has shown that it has a high homology with other *Thermus* serine proteases (Freeman *et al.*, 1993).

Table 1. shows that almost all of the thermostable proteases isolated from *Thermus* species are serine proteases. They are all quite thermostable, yet other properties such as the Ca^{2+} requirement, pH optimum and disulphide bond presence differ considerably.

1.2.1.3 Serine Proteases from Archaea

Hyperthermophilic organisms typically have a growth temperature optimum above 80°C (Stetter *et al.*, 1990). Almost all of these organisms are archaea. Some of these organisms have been shown to produce extremely thermostable proteases. Archaelysin, from *Desulfurococcus* st. $ToK_{12}S_1$ was the first archaeal protease to be described (Cowan *et al.*, 1987c, Coolbear *et al.*, 1988). It is an extracellular thermostable serine protease with a pH optimum of 7.2. It had a half life of 70-79 minutes at 95°C which appeared to be unaffected by Ca^{2+} or EDTA. These properties are similar to those of protease I from *Sulfolobus solfataricus* (Burlini *et al.*, 1992). However, it has a broader substrate specificity than protease I, preferring substrates containing hydrophobic residues, such as leucine, on the carboxyl side of the sissile bond (Cowan *et al.*, 1987c).

Pyrococcus furiosus DSM 3638 produces a number of intracellular proteases of different molecular weights (Blumentals *et al.*, 1990; Eggen *et al.*, 1990; Connaris *et al.*, 1991; Snowden *et al.*, 1992). Blumentals *et al.*, (1990) described two of these proteases (S66 and S102) which they found to be SDS-resistant. The proteases were extremely thermostable, as an extract containing both S66 and S102 had a half life of 33 hours at 98°C. S66 was slightly inhibited by EDTA at 37°C, but as inhibition at high temperature was not investigated, it is not known if Ca^{2+} is required for thermostability. Immunoblot analysis showed that S66 and S102 were unrelated (Blumentals *et al.*, 1990). Connaris *et al.*, (1991) identified 13 active proteases, including one metallo protease, produced by the same *Pyrococcus* sp., in contrast to the 5 identified by Blumentals *et al.* (1990). They saw no resistance to SDS of any of the proteases, though they did identify a 66 kDa protease which may correspond to S66. A few of these proteases were stabilised by Ca^{2+}. The 66 kDa protease had half lives of 117 and 15 minutes at 105°C in the presence and absence of

Ca^{2+} respectively (Connaris *et al.*, 1991). Eggen *et al.* (1990) described the properties of an enzyme named "Pyrolysin" from the same *Pyrococcus* sp. However on SDS/PAGE, pyrolysin had multiple activity bands, and the enzyme had a broad pH optimum of about 6.5-10.5. Eggen *et al.* (1990) contended that the multiple proteolytic bands of pyrolysin are processed to give a final 66kDa protease, but investigations by others do not support this conclusion (Blumentals *et al.*, 1990; Connaris *et al.*, 1991). Thus, it appears that *P. furiosus* produces a heterogeneity of highly thermostable intracellular proteases of molecular weights ranging from 66-135 kDa, with the exact relationships between them not certain.

The thermoacidophilic archaebacterium *Sulfolobus solfataricus* ATCC 49155 produces 6 intracellular proteases, two of which are serine proteases (Fusi *et al.*, 1991). One of these, protease I, is extremely thermostable, having a half life of 342 minutes at 92°C (Burlini *et al.*, 1992). Ca^{2+} did not significantly affect the enzyme, but Mn^{2+} appeared to activate it. It is unstable in the presence of high salt, but is stabilised by Triton X-100. This suggests that salt bridges and hydrophobic patches may play a significant role in stabilising the protease. Interestingly, it lacks cysteine and tyrosine residues. It has a narrow substrate specificity, and amino acid residues close to, but not directly involved in the peptide bond appeared to be crucial in substrate recognition (Burlini *et al.*, 1992).

Klingeberg *et al.* (1991) compared the properties of intracellular proteases from the hyperthermophilic archaebacterial species *Thermococcus celer, T. stetteri, Thermococcus* st. AN1, *T. litoralis, Staphylothermus marinus* and the hyperthermophilic eubacterial species *Thermobacteroides proteolyticus.* They are all serine proteases, and exhibited multiple activity bands on SDS/PAGE, similar to those seen with *P. furiosus.* They tend to have a preference for the presence of phenylalanine at the carboxylic side of the sissile bond. All of the proteases are extremely thermostable, with half lives between 5- >50 hours at 90°C. The most thermostable of them was the protease from *Thermococcus litoralis.* EDTA had little or no effect on the activities of the enzymes, though the temperature at which this experiment was carried out was not stated (Klingeberg *et al.*, 1991).

Recently, further work has been conducted on the protease from *Thermococcus stetteri* (Klingeberg *et al.*, 1995). This single polypeptide protease has a molecular weight of 68 kDa. It has a half life of 2.5 hours at 100°C and is SDS-resistant. Metal ions such as Ca^{2+}, Co^{2+} and Mg^{2+} did not

have any significant effect on activity, but activity was stimulated (170%) by 0.5M NaCl. This requirement for NaCl is not suprising as *T. stetteri* was grown in a medium containing 0.43M NaCl. The protease has a narrow substrate specificity, cleaving only n-protected basic or hydrophobic (phe or tyr) p-nitroanilides and p-nitrophenol esters. It also has transferase activity (Klingeberg *et al.*, 1995).

A subtilisin-type serine protease (aerolysin) from the hyperthermophilic archaeum *Pyrobaculum aerophilum* st. IM2 has been described (Volkl *et al.*, 1994). The gene encoding aerolysin has been obtained, and 3-dimensional structural models have been constructed based on its sequence similarity to thermostable proteases such as thermitase from *Thermoactinomyces vulgaris*. Based on these sequence and structural alignments, several sites were proposed to be important for its thermostability. Sites include positions where a charge addition or replacement may be affecting the stability of an a helix. Many of these changes were also present in thermitase (Volkl *et al.*, 1994). The role of Ca^{2+} ions in the stability of the protease has not been investigated. A comparison of the sequences of aerolysin and thermitase (Volkl *et al.*, 1994) shows that of the 10 amino acid residues observed to be involved in the binding of 2 Ca^{2+} ions in thermitase (Briedigkeit and Frommel, 1989), only one of them is conserved in aerolysin. This does not exclude the possibility that aerolysin binds Ca^{2+} ions, though it does appear that Ca^{2+}-binding is not important in the thermal stability of many hyperthermophilic proteases. However, it must be borne in mind that most hyperthermophilic proteases investigated so far are intracellular.

1.2.1.4 Other Thermostable Serine Proteases

Streptomyces rectus var. *proteolyticus* produces two serine proteases (A and B) that differ only in their amide content (Mizusawa and Yoshida, 1972). Protease B was found to retain 60-70% of its activity after a 10 minute incubation at 80°C (Mizusawa *et al.*, 1964). It cleaved oxidised insulin B chain most readily at Phe^{24}-Phe^{25}, Leu^{15}-Tyr^{16} and Leu^{11}-Val^{12}, and proangiotensin at Tyr^{4}-Ile^{5} (Matsue *et al.*, 1982).

Thermitase, from *Thermoactinomyces vulgaris*, is an thermostable alkaline serine protease (Hausdorf, Kruger and Hohne, 1980) which has a high sequence and structural similarity to the subtilisin-like serine proteases (Meloun *et al.*, 1985). It contains a cysteine residue near the active site that is apparently essential for activity. Thus, it is postulated to belong to the SH-dependent

subtilisin subgroup of proteases (Meloun *et al.*, 1985). Thermitase binds three Ca^{2+} ions per enzyme molecule. One of these ions binds weakly to the enzyme causing an increase in both thermostability and activity. The other two Ca^{2+} ions bind more strongly, and have only small effects on thermostability and activity (Frommel and Hohne, 1981).

The protease from *Thermomonospora fusca* YX is an alkaline serine protease with a low molecular weight of only 14.5-19 kDa (Gusek and Kinsella, 1987). When the pH was shifted from the activity optimum, to prevent autolysis, the enzyme exhibited substantial stability, having a half life of 15 minutes at 85°C at pH 4.5. ` Ca^{2+} does not appear to play a role in its thermostability (Kristjansson and Kinsella, 1990a,b). Lyotrophic salts affected the thermostability of the protease at 85°C, suggesting that hydrophobic interactions may play an important role in stabilising the enzyme above 80°C (Kristjansson and Kinsella, 1990a). It has a primary specificity for cleavage of peptides containing aromatic and hydrophobic amino acids (Kristjansson and Kinsella, 1990b). Immobilisation of the protease onto Sepharose-4B resulted in a 5-fold increase in half life at pH 8.5 at 85°C, though the specific activity to casein was reduced 3-fold (Johnson *et al.*, 1990).

Malbranchea pulchella var. *sulfurea* is a thermostable fungus which produces a thermostable serine protease called Thermomycolin (Ong and Gaucher, 1976). The protease preferentially hydrolyses substrates containing non-polar non-branched amino acids. Thermomycolin has a half life of 110 minutes at 73°C with Ca^{2+}, and is therefore relatively stable for a eukaryotic protease. Thermostability is lower in the absence of Ca^{2+} (Gaucher and Stevenson, 1976).

1.2.2 Metallo-Proteases

Metallo-proteases, as their name suggests, contain a metal ion at the active site that is essential for catalysis (see Beynon and Bond, 1989), as opposed to stability, although other metal ions may play a stabilising role. They tend to have pH optima in the range of 5-9, and are inhibited by metal-chelating reagents such as EDTA and o-phenanthroline. Most bacterial metallo-proteases contain one catalytic zinc atom per molecule.

There are basically two groups of metallo-proteases (see Beynon and Bond, 1989). The first group consists of thermolysin-like proteases, while the second

group of proteases are carboxypeptidase-A-like. These groups differ in their three-dimensional structure, but have very similar active site configurations.

1.2.2.1 Thermolysin

Thermolysin, from *Bacillus thermoproteolyticus* (Endo, 1962), is an endopeptidase that has been extensively investigated (eg. Titani *et al.*, 1972; Voordouw *et al.*, 1976; Dahlquist *et al.*, 1976; Holmes and Matthews, 1982; Sidler *et al.*, 1986). It has a molecular weight of 34.5 kDa and a pH optimum of 7.2 (Gusek and Kinsella, 1988). Its amino acid sequence has been determined (Titani *et al.*, 1972), and three dimensional structure refined to a high resolution (Matthews *et al.*, 1972a,b; Holmes, and Matthews, 1982). Sequence and structural studies have shown that metallo-proteases from *B. subtilis, B. stearothermophilus* and *B. cereus* are closely related to thermolysin (Yang *et al.*, 1984; Sidler *et al.*, 1986b; Pauptit *et al.*, 1988). The eukaryotic protease Carboxypeptidase A shows little structural and sequence similarity to thermolysin, but the active site structures and mode of binding of dipeptide inhibitors are quite similar (Kester and Matthews, 1977).

Thermolysin has a bilobal structure consisting of two domains of equal size, with the active site located at the interface between them (Matthews *et al.*, 1972b). It contains one zinc atom per enzyme molecule which is essential for activity. Thermolysin also binds four Ca^{2+} ions which are implicated in the thermostability of the protease (Matthews *et al.*, 1972b). Two of the four Ca^{2+} ions (Ca(1) and Ca(2)) form a double binding site by binding to a pocket of five acidic residues (Matthews *et al.*, 1972b). The two additional Ca^{2+} binding sites are located at exposed surface regions where Ca(3) and Ca(4) bind to the residues Asp^{57} and Asp^{59}, and Asp^{200} respectively (Matthews *et al.*, 1972b). Other residues have also been implicated in the binding of the four Ca^{2+} ions (Pangburn *et al.*, 1976). Ca^{2+} has been shown to be involved in the prevention of both thermal denaturation and autolysis of the enzyme (Roche and Voordouw, 1978).

An incubation of thermolysin in the presence of EDTA results in a rapid inactivation of the enzyme due to autolysis (Fontana, 1987). Early work suggested that the two weakest bound Ca^{2+} ions were those bound to the double Ca^{2+} binding site (Voordouw and Roche, 1974; Roche and Voordouw, 1978). However, Weaver *et al.* (1976) showed that the ranking of the binding affinities of the Ca^{2+} ions are $Ca(1) >> Ca(3) > Ca(4) \geq Ca(2)$. EDTA titration of the two weakest-bound Ca^{2+} ions (Ca(2) then Ca(4)) showed that Ca(2) had no

significant effect on thermostability, but the chelation of Ca(4) resulted in a fast and selective autolysis within the residues 190-205 (Weaver *et al.*, 1976; Fassina *et al.*, 1986). This polypeptide region corresponds to the binding site of Ca(4), which is within the most extended region of irregular conformation of thermolysin (Fontana, 1988).

The binding of terbium to either of the single Ca^{2+} binding sites, but not the double binding site, increased the thermostability of thermolysin considerably (Dahlquist *et al.*, 1976). This is comparable to caldolysin, which binds 6 Ca^{2+} ions, which was significantly stabilised by replacing Ca^{2+} with lanthanide ions (Khoo *et al.*, 1984). This suggests that the removal of Ca^{2+} ions from binding sites at the surface exposed regions of thermolysin (e.g. Ca(4)) results in structural changes in the polypeptide at these regions. These localised regions, or loops, are more flexible and therefore more susceptible to proteolysis (Fontana, 1988). The structure of thermolysin suggests that the function of Ca(3) may be to stabilise a loop on the surface of the molecule, while Ca(1) is more buried and serves to link together the two molecular lobes (Weaver *et al.*, 1976). Removal of Ca^{2+} ions (1) and (3) is therefore expected to lead to more extensive denaturation and therefore autolysis.

It is interesting to note that the metallo-protease from *B. subtilis* strain NRRLB3411 has a similar amino acid composition, as well as structural and functional similarities to thermolysin, but has a much lower thermostability (Pangburn *et al.*, 1976). A comparison of the amino acid sequences of these proteases showed that Asp^{200} in thermolysin was replaced by proline in *B. subtilis* protease, and three other residues in the region of Glu^{190}-Asp^{200} in thermolysin are absent in the *B. subtilis* protease (Pangburn *et al.*, 1976). This region corresponds to the Ca(4) binding site in thermolysin, which suggests that the lower thermostability could be due to a lack of a Ca^{2+} ion binding to a region homologous to the Ca(4) region in thermolysin. Also, Asp^{57}, Asp^{59} and Gln^{61} of the Ca(3) binding site of thermolysin have been replaced by Ser, Thr and Thr respectively in this protease (Sidler *et al.*, 1986b). This could account for the observation that *B. subtilis* protease binds fewer Ca^{2+} ions than thermolysin (Levy *et al.*, 1975).

1.2.2.2 Other *Bacillus* Metallo-Proteases

Early work on *Bacillus* neutral (metallo) proteases (not including thermolysin) focussed on proteases from organisms such as *B. caldolyticus* YP-T (Heinen and Heinen, 1972) now called *Bacillus* sp. IFO 15313, *B. cereus* (Melachouris and Tuckey, 1968), *B. subtilis* var. *amylosacchariticus* (Tsuru *et al.*, 1966) and *B. subtilis* NRRL B3411 (Keay and Wildi, 1970), though the latter two proteases are relatively thermolabile. The first of these, produced by *B. caldolyticus* YP-T, is a very thermostable protease (YP-T), with a half life of 45 minutes at 85°C with 10 mM Ca^{2+} (Saul *et al.*, 1995). Recently, the gene encoding the protease was cloned, sequenced and expressed first into *B. subtilis* (Van den Burg *et al.*, 1991), and then into *E. coli* (Saul *et al.*, 1995). This protease is nearly identical in sequence to, but is more stable than, the protease from *B. stearothermophilus* CU21 (Van der Burg *et al.*, 1991).

A thermophilic *Bacillus*, st. EA.1 (Coolbear *et al.*, 1991) produces an extracellular metallo protease with a high thermostability. It is a member of the thermolysin-type family of metallo-proteases, and has a half life of 2 hours at 85°C in the presence of 10 mM Ca^{2+}. There are two types of metal-ion binding sites . One is specific for ions that activate the enzyme, while the other is specific for ions that stabilise the enzyme. These sites are specific for metals with an ionic radius close to Zn^{2+} and Ca^{2+} respectively, the Zn^{2+}/Ca^{2+} couple being the most effective. Mercury (II) ions and thiol-reactive agents inhibited the enzyme, suggesting that thiol groups may play a role in maintaining enzyme integrity (Coolbear *et al.*, 1992).

Bacillus st. Ok3A.1 produces a metallo protease that is less thermostable than EA.1 (Coolbear *et al.*, 1991). It is sensitive to both EDTA and EGTA at high and low temperatures, suggesting that Ca^{2+} ions are important for both activity and stability (Eames, 1985). It has a half life of 40 minutes at 85°C which is reduced to 6 minutes in the absence of Ca^{2+} (Eames, 1985).

B. cereus DSM 3101 produces a metallo-protease which has a high sequence homology to thermolysin (63%), but is less thermostable (Sidler *et al.*, 1986a). It is a zinc-containing enzyme, and is stabilised by Ca^{2+} ions. It retained about 60% of its activity after an incubation of 20 minutes at 70°C in the presence of Ca^{2+} (Sidler *et al.*, 1986a). Its amino acid sequence is very similar to that of thermolysin, with fewer amino acid changes in the Ca^{2+} binding regions than in the *B. subtilis* protease. The crystal structure of the protease has been refined to 3.0Å resolution (Paupit *et al.*, 1988). When compared to the

crystal structure of thermolysin, it appears that the enhanced thermostability is not due to additional salt bridges, but is more likely to be due to extra hydrogen bonding interactions and the amino acid differences between them in the region of the double Ca^{2+} binding site (Paupit *et al.*, 1988). Sidler *et al.* (1986b) proposed that the difference in thermostability was due to effects such as lower hydrophobicity in the b-pleated sheets and lower bulkiness of the a-helical regions as compared to thermolysin.

B. stearothermophilus ATCC 12980 underwent spontaneous mutations to produce the strain CU21 (Imanaka *et al.*, 1982). It produces a thermostable metallo-protease (NprT) that has been cloned, sequenced and subjected to extensive site-directed mutagenic studies (eg. Fujii *et al.*, 1983; Takagi *et al.*, 1985; Takagi and Imanaka, 1989) It is moderately thermostable, retaining 80% of its activity after an incubation of 30 minutes at 65°C (Fujii *et al.*, 1983). It shows high homology to thermolysin (85%), and much lower homology to the more thermolabile *B. subtilis* metallo-protease (Takagi *et al.*, 1985).

Extensive site-directed mutagenic studies were conducted on the *B. stearothermophilus* CU21 protease to try to improve its thermostability (e.g. Van den Burg *et al.*, 1991; Eijsink, 1991). Three amino acid substitutions were made at positions corresponding to solvent-exposed regions of the protease (Ala^4 to Thr, Thr^{59} to Ala and Thr^{66} to Phe). These mutations resulted in the *B. stearothermophilus* protease having a thermostability identical to the *B. caldolyticus* YP-T protease, showing that these residues are important for thermostability (Van den Burg *et al.*, 1991). Also, Ala^{166} of NprT was changed to Ser (Vriend *et al.*, 1991) which resulted in an increased thermostability. Model building and molecular dynamics simulations of the mutant NprT showed that the serine hydroxyl group fits into a cavity that is occupied by a water molecule in the wild type NprT, and it was proposed that the increased stability of mutant NprT was due to a gain in entropy due to the release of a water molecule from the protein into the solvent. The hydrogen bonding around residue 166 was also improved in mutant NprT (Vriend *et al.*, 1991).

Eijsink (1991) changed Ala^{69} of NprT to proline, the residue present in thermolysin at this position. This resulted in an increase in thermostability of 5.5°C of the mutant NprT (shifting of the half life of 30 minutes at ~69°C to ~75°C), as opposed to the native NprT. This accounts for about 40% of the difference in thermostability between native NprT and thermolysin. It has been proposed that Pro^{69} has increased the thermostability of NprT by decreasing the

flexibility of a surface loop, possibly resulting in a decrease in autolysis at that site (Eijsink, 1991).

Other mutations have been conducted on NprT such as the substitution of Gly^{144} to Ala (Takagi and Imanaka, 1989), which resulted in an increased thermostability and catalytic rate with casein. Even though the changes in thermostability were quite small (~0.5 - 2°C), this illustrates the significance of single interactions in the overall stability of enzymes (e.g. Langridge, 1968).

B. stearothermophilus st. MK232 produces a metallo-protease (NprM) that has a higher thermostability than thermolysin (Kubo *et al.*, 1988). NprM has a half life of at least 25 minutes at 90°C, and a higher specific activity against casein than thermolysin (Kubo *et al.*, 1988). It has an identical amino acid sequence to thermolysin, except for the two amino acid changes Asp^{37} to Asn^{37} and Glu^{119} to Gln^{119} (Kubo and Imanaka, 1988). These two substitutions both resulted in the addition of an uncharged polar amino acid to NprM. It is postulated that these substitutions may have resulted in producing an additional hydrogen bond and/or decreasing electrostatic repulsion in their respective regions, thereby increasing the thermostability (Kubo and Imanaka, 1988). Thus, a single extra intermolecular interaction resulted in a significant increase in the thermostability of the enzyme NprM over thermolysin. This example shows that enzymes with increased stability can be sought in nature as an alternative to *in-vitro* manipulation. Kubo *et. al.* (1992) introduced a number of amino acid substitutions in this protease on the basis of the three-dimensional structures of thermolysin and NprM by site-directed mutagenesis using synthetic oligonucleotides. The amino acid substitutions were introduced at the catalytic site, the substrate binding site, at the junction of the two domains, at the tyrosine residues adjacent to the catalytic site and the substrate binding site on the surface of the enzyme and at a site involved in autolysis. Proteolytic activity was eliminated when Glu-143 at one of the proposed active sites was replaced by glutamine. Substitution of Phe-114 with alanine at the substrate binding site gave a mutant enzyme with higher activity than wild type. Substitutions involving Tyr-110 to Trp and Tyr-211 to Trp also gave enzymes with slightly higher activity than wild type.

NprM also has undergone site-directed mutagenesis to increase its lifetime at high temperature (Kubo *et al.*, 1992). Instead of substituting amino acids at sites that could possibly be important for conformational stability, they substituted an amino acid at an autolytic cleavage site (Tyr^{93} to Gly and Ser). The mutant enzymes had lower specific activities, but were less rapidly

inactivated at high temperatures because autolysis was reduced (Kubo *et al.*, 1992). Interestingly, the mutant enzymes were more effective catalysts in the peptide synthesis reaction producing more aspartame (50°C, 16 hours) than the native enzyme (Kubo *et al.*, 1992). The decrease in specific activity appears to have been offset by the increased stability. This suggests that for proteases significantly inactivated by autolysis, engineering autolytic resistance, as opposed to increasing conformational stability, may be the preferred engineering strategy.

B. stearothermophilus IFO 12983 produces an intracellular dimeric peptidase with a molecular weight of 86 kDa (Cho *et al.*, 1988). It is a zinc metallo-protease, but is also activated by trace levels of Mn^{2+} and Co^{2+}. A few other divalent cations such as Ca^{2+}, Ba^{2+} and Mg^{2+}, strongly inhibit the enzyme. The protease was relatively stable in the presence of denaturing agents such as ethanol and SDS. It specifically cleaves substrates such as Val-Ala and Gly-Phe, but is not a true dipeptidase as it also acts on some tri and tetra-peptides (Cho *et al.*, 1988).

Other strains of *B. stearothermophilus* produces three aminopeptidases, called API, APII and APIII (Roncari *et al.*, 1976). API was further characterised, and found to be composed of 12 subunits, with a total molecular weight of about 400kDa. It is very thermostable, showing no activity loss after a 15 hour incubation at 80°C (Roncari *et al.*, 1976). API binds two metal ions per subunit, one of which is catalytic while the other probably has a stabilising role (Zuber 1978). Both Zn^{2+} and Co^{2+} can bind to API. The Co^{2+}API is more flexible and more active than Zn^{2+}API, but is consequently less thermostable (Zuber, 1978). API has a broad substrate specificity as it can cleave next to neutral, acidic and basic amino acids. This is typical of other aminopeptidases (Roncari *et al.*, 1976).

Two other thermostable metallo proteases have been characterised from *Bacillus* sp. The first is a zinc-containing protease isolated from *Bacillus brevis* 7882 (Paberit *et al.*, 1982). It has a broad substrate specificity, hydrolysing many of the bonds of oxidised insulin B chain including Leu^{17}- Val^{18} and Phe^{24}-Phe^{25}. The protease was thermostable, with a half life of at least 1 hr at 80°C which is dependent on Ca^{2+} ions (Paberit *et al.*, 1984). *B. stearothermophilus* KP1236 produces a metallo-protease which shares some antigenic determinants with thermolysin (Takii *et al.*, 1987). Activity was inhibited by o-phenanthroline, which could be recovered by the addition of either Zn^{2+}, Co^{2+}

or Mn^{2+}. It was also thermostable, with no activity loss detected after an incubation of 10 minutes at 80°C (Takii *et al.*, 1987).

1.2.2.3 Other Metallo-Proteases

The archaebacterium *Sulfolobus solfataricus* strain MT-4 produced an intracellular tetrameric carboxypeptidase with a molecular weight of 170 kDa (Colombo *et al.*, 1992). It showed no sequence similarity or antibody crossreactivity to a number of other peptidases including thermolysin and swine carboxypeptidases. It exhibited a broad pH optimum between 5.5-9.0, which was dependent on the substrate. The enzyme had quite a broad substrate specificity as it could cleave basic, acidic and aromatic amino acids from benzoylglycylated and benzyloxycarbonylated amino acids (Colombo *et al.*, 1992).

The carboxypeptidase was thermostable, showing no loss of activity after about 15 minutes at 85°C (Villa *et al.*, 1993). However, thermostability was found to be dependent on Zn^{2+} ions rather than Ca^{2+} ions. Being a metallo-protease, Zn^{2+} was also required for activity. Co^{2+} could substitute for zinc, but was not as efficient an activator as Zn^{2+}. The activation free energies, enthalpies and entropies dropped when Zn^{2+} was removed. Thermal inactivation was found to be due to denaturation rather than autolysis in all of the conditions tested (e.g. with or without Zn^{2+}). Hydrophobic interactions and salt bridges were also proposed to be significant stabilising features due to the destabilising effects of chaotropic salts and sodium sulphate (Villa *et al.*, 1993). Another interesting feature of this enzyme was its relatively low activation energy of 31.0 kJ/mol (Colombo *et al.*, 1992) which resulted in significant activity being detectable at room temperature.

Sulfolobus solfataricus strain DSM 1616 produces a tetrameric intracellular aminopeptidase of molecular weight 320 kDa (Hanner, Redl and Stoffler, 1990). It prefers to cleave peptides containing Ala, Leu and Phe, which is similar to the substrate specificity of other known aminopeptidases (e.g. Roncari *et al.*, 1976). It was completely inhibited by EDTA, but activity was not significantly restored in the presence of Ca^{2+}, Mg^{2+} or Zn^{2+}. Instead, Co^{2+} or Mn^{2+} was required to restore the activity of the enzyme. Co^{2+} also significantly increased the thermostability of the enzyme. The aminopeptidase exhibited a "temperature optimum" of 75°C (six minute assay), yet the organism grows optimally around 85°C. This suggests that external factors other than Co^{2+} may be required for the thermostability of the protease *in vivo*. (Hanner *et al.*, 1990).

Thermus aquaticus YT-1 produces both an aminopeptidase (aminopeptidase T) and a carboxypeptidase (CPase *Taq*). Aminopeptidase T is an intracellular dimeric metallo-protease of molecular weight 108 kDa (Minagawa *et al.*, 1988). Unlike the aminopeptidase from *S. solfaraticus* DSM 1616, it is inhibited by o-phenanthroline suggesting that it contains a Zn^{2+} ion. The enzyme has a broad substrate specificity, though it preferred to cleave Gly and Leu from the N-terminal of substrates. Aminopeptidase T is quite thermostable as it retained about 60% of its activity after an incubation of 20 hours at 80°C (Minagawa *et al.*, 1988). However, it was readily destabilised by organic solvents at room temperature which is unusual for thermostable proteases (Daniel, 1986; Owusu and Cowan, 1989).

CPase *Taq* is a thermostable monomeric metallo-protease (Lee *et al.*, 1992). The native enzyme was found to be dependent on Co^{2+} for activity. When Zn^{2+} was substituted for Co^{2+}, only 4.8% of the activity remained. It belongs to the carboxypeptidase-A group of metallo-proteases, though these proteases tend to require Zn^{2+} for activity rather than Co^{2+}. CPase *Taq* has recently been cloned, sequenced and expressed in *E coli* (Lee *et al.*, 1994a). The protease showed no sequence homology with any other metallo-protease. Unlike the native protease, cloned CPase *Taq* was found to contain 1 Zn^{2+} per enzyme molecule, rather than Co^{2+} in spite of Co^{2+} being present in the culture medium. However, this enzyme was still activated by the addition of Co^{2+} (Lee *et al.*, 1994a).

CPase *Taq* was stable up to 80°C, which was independent of the presence of Co^{2+} (Lee *et al.*, 1992). It has a broad substrate specificity, cleaving all amino acids except proline from the C-terminus of substrates. It also sequentially cleaved amino acids from peptides making this enzyme a possible target for use in C-terminal sequencing (Lee *et al.*, 1992).

Chloroflexus aurantiacus J-10-fl produces two types of thermostable proteases, one of which (I) is a Ca^{2+}-stabilised, neutral metallo-protease (Watanabe *et al.*, 1993). The protease is stable to denaturing agents such as urea and SDS at room temperature. The primary and secondary cleavage sites of oxidised insulin B chain are Ala^{14}- Leu^{15} and Gly^{23}- Phe^{24} respectively. This indicates that the protease prefers to cleave peptide bonds on the N-terminal side of hydrophobic and aromatic residues, which is similar to other microbial neutral metallo-proteases (Watanabe *et al.*, 1993).

The fungus *Aspergillus oryzae* produces a thermostable metallo-protease (NpII) with a low molecular mass of 19.0 kDa (Nakadi, Nasuno and Iguchi, 1973; Tatsumi *et al.*, 1994). It requires Zn for activity, but shares no significant sequence similarity with any known protein. It has an unusual thermostability profile as it is most unstable after an incubation of 10 minutes at 75°C, but stability then increases with increasing temperature up to around 100°C. This drop in stability was not detected when similar experiments were conducted with the apoenzyme. It has been postulated (Tatsumi *et al.*, 1994) that upon heating, the protease undergoes reversible denaturation. At 75°C, its "optimum temperature", autolysis is occuring which irreversibly inactivates the enzyme. As the temperature increases above 75°C, the rate of autolysis decreases, hence the stability increases. At temperatures above 100°C, other irreversible reactions such as deamidation may occuring (Tatsumi *et al.*, 1994).

Three other thermostable metallo-proteases have been characterised. The most thermostable one was an extracellular aminopeptidase isolated from *Aeromonas proteolytica* (Prescott and Wilkes, 1976). It is stable for several hours at 70°C and is only partially inactivated by 8M urea. Activity is dependent on Zn^{2+} ions, but Co^{2+} and Mn^{2+} can partially activate the enzyme. Ca^{2+} and Mg^{2+} ions had no significant effect on the enzyme (Prescott and Wilkes, 1976). A less thermostable aminopeptidase from *Talaromyces duponti* has been characterised (Chapuis and Zuber, 1970). It has a high molecular weight of 400 kDa, so it may be composed of several subunits. It was activated by Co^{2+} at 55°C and 65°C (Chapuis and Zuber, 1970). The metallo-protease from *Thermomicrobium* sp. KN-22 has a "temperature optimum" of 75°C (10 minute assay) (Murao *et al.*, 1991). It is inhibited by o-phenanthroline, suggesting that it contains at least one essential Zn^{2+} ion (Murao *et al.*, 1991).

1.2.3 Aspartic Proteases

The aspartic proteases are characterised as having two aspartate residues at the active site and a pH optimum between about 1.5-5.0 (Fruton, 1987). They are typically inhibited by pepstatin, diazoacetyl norleucine methyl ester (DAN), and 1,2-epoxy,*p*-nitrophenoxy propane (EPNP), though many microbial aspartic proteases are known to be insensitive to one or more of these inhibitors (e.g. Prescott *et al.*, 1992; Murao *et al.*, 1993; Toogood *et al.*, 1995). They prefer to cleave substrates with bulky or aromatic amino acid residues on both sides of the sissile bond.

1.2.3.1 *Bacillus* Aspartic Proteases

Bacillus st. MN-32 produces a thermostable, pepstatin-insensitive aspartic protease called Kumamolysin (Murao *et al.*, 1993). It is also insensitive to the other diagnostic inhibitors DAN and EPNP, and inhibitors of the other classes of proteases. It has been classified as an aspartic protease by the demonstration that it contains catalytic carboxyl groups (Murao *et al.*, 1993). It has a molecular weight of 40-41 kDA, and a pH optimum of 3.0 (Murao *et al.*, 1993). Kumamolysin specifically hydrolysed Leu^{15}-Tyr^{16} and Phe^{25}-Tyr^{26} of oxidised insulin B chain, though the latter site was cleaved at a much lower rate (Murao *et al.*, 1993). It retained 60% of its activity after a 10 minute incubation at 80°C (Murao *et al.*, 1993).

The aspartic protease from *Bacillus* st. Wai21.A1 has a similar molecular weight and pH optimum to the MN-32 protease, but has a different inhibitor sensitivity (Prescott *et al.*, 1992). It is insensitive to pepstatin, partially sensitive to DAN, and sensitive to EPNP. It hydrolyses Leu^{15}-Tyr^{16} and Phe^{25}-Tyr^{16} of oxidised insulin B chain, as well as other sites such as Gln^{4}-His^{5} (Prescott *et al.*, 1995). It has a half life of 2 minutes at 80°C in the presence of Ca^{2+} (Prescott *et al.*, 1992).

Bacillus st. Wp22.A1 produces an aspartic protease with similar inhibitor sensitivities to Wai21.A1 protease, but with a longer half life of 21 minutes at 80°C (Toogood *et al.*, 1995). It is stabilised against thermal denaturation by Ca^{2+}, but this effect is only significant at temperatures less than 70°C. The stabilisation by Ca^{2+} is due to the prevention of thermal denaturation, rather than affecting autolysis. Its primary cleavage site of oxidised insulin B chain is Val^{2}-Asn^{3}, which is an unusual cleavage site among microbial proteases (Toogood *et al.*, 1995).

1.2.3.2 Other Thermostable Aspartic Proteases

Thermopsin, from *Sulfolobus acidocaldarius*, is an extracellular, extremely thermostable aspartic protease with unusual properties (Lin and Tang, 1990). It has a molecular weight of about 48 kDa, and a pH optimum of about 2. It is only non-specifically modified by DAN and EPNP, but is sensitive to pepstatin (Fusek *et al.*, 1990). It bears no sequence similarity to any known aspartic protease, and is missing the characteristic active site-specific Asp-Thr-Gly sequence found in virtually all aspartic proteases. Thus, it may represent a new sub-class of aspartic proteases. It has a half life of > 48 hours at 80°C, making it the most thermostable aspartic protease characterised (Lin and Tang, 1990). It

cleaves the oxidised insulin B chain between the characteristic Leu^{15}-Tyr^{16} and Phe^{25}-Tyr^{26} residues, as well as at a few other sites (Fusek *et al.*, 1990).

Aspergillus niger F2078 produces a mildly thermostable protease with a pH optimum of 4.0 (Singh *et al.*, 1994). It suffers only about a 10% loss of activity after an incubation of 1 hour at 60°C. It was not inhibited by EDTA, suggesting that Ca^{2+} may not be required for stability (Singh *et al.*, 1994).

1.2.4 Cysteine Proteases

Cysteine proteases are characterised as having a cysteine residue at the active site that is essential for activity (Beynon and Bond, 1989). They typically have a pH optimum between 5-8. These proteases are characteristically inhibited by low concentrations of sulphydryl reagents, such as *p*-chloromercuribenzoate and iodoacetamide, and tend to be activated by reducing agents such as cysteine and dithiothreitol.

The hyperthermophile *Pyrococcus furiosus* st. KOD1 produces a highly thermostable extracellular cysteine protease with a molecular weight of 44 kDA (Morikawa *et al.*, 1994). Its pH optimum is 7.0, and it retains about 90% of its activity at 120°C. The enzyme was insensitive to EDTA (Morikawa *et al.*, 1994), like most other hyperthermophilic proteases mentioned.

Sulfolobus solfataricus ATCC49155 produces an intracellular cysteine protease (protease III) as well as serine and metallo proteases (Fusi *et al.*, 1991). It is very thermostable, retaining ~ 90% of its activity after an incubation of 15 minutes at 90°C. It was not inhibited by EDTA, but this was determined only at room temperature, so it is not known whether Ca^{2+} plays a role in the stability of the enzyme (Fusi *et al.*, 1991).

1.2.5 Proteosome

Proteosomes, or high molecular mass proteolytic multi-subunit enzyme complexes, are ubiquitous in eukaryotes, but are also found in the archaeum *Thermoplasma acidophilum* (Zwickl *et al.*, 1992). Puhler *et al.* (1994) screened a large number of archaea from all the major lineages and some eubacteria, but found proteosomes only in *Thermoplasma* strains. These complexes are unusual as they can typically catalyse peptide bond cleavage on the carboxyl side of basic, hydrophobic and acidic amino acid residues, though the *Thermoplasma* proteosome has a more restricted substrate specificity (Rivett,

1993). The multicatalytic activities seen in the eukaryotic proteosome (Orlowski *et al.*, 1993) are believed to be located at independent sites within the proteosome, as activity towards a variety of substrates responded differently to a variety of activators and inhibitors. High levels of Ca^{2+} (500 mM) stimulate the activity of the *Thermoplasma* proteosome against Suc-Ala-Ala-Phe-NMec sixteen-fold, and metal chelators such as EDTA and EGTA, but not o-phenanthroline inhibit this activity strongly (Dahlmann *et al.*, 1989). This suggests that this activity was dependent on Ca^{2+}, not a heavy metal ion. Activity with the above peptide was also sensitive to diisopropylfluorophosphate, which led to the assumption that at least part of the *Thermoplasma* proteosome is a serine protease (Dahlmann *et al.*, 1989).

Recently, the 3-dimensional structure of the proteosome from *Thermoplasma acidophilum* has been determined (Lowe *et al.*, 1995). The 673 kDa complex consists of 14 copies of two different subunits a and b, which form a barrel-shaped structure of four stacked rings. The two inner rings consist of seven a subunits each (Lowe *et al.*, 1995). Proteolytic activity is confined to the b subunits only, though the presence of the a subunits increases the activity significantly.

Seemuller *et al* (1995a) performed extensive site-directed mutagenesis on the proteosome to determine the active site residues. They changed all of the serine, cysteine and histidine residues in the b subunits, and some serine residues in the a subunits. There was no change in the activity of the proteosome in any of the mutants. Mutations of aspartate residues in the a subunits that corresponded to the so-called 'universally-conserved' aspartates in acid proteases resulted in either no change, or a suprising increase in activity. These results, as well as the lack of inhibition of activity by chelators such as o-phenanthroline, suggests that this proteosome does not belong to any of the known classes of proteases.

Further site-directed mutagenic studies (Seemuller *et al.*, 1995b) showed that the active-site nucleophilic residue is the amino-terminal threonine residue of the b subunits. When mutated to serine, the activity was unchanged, but the proteosome's sensitivity to the serine protease inhibitor 3,4-dichloroisocoumarin was increased over 10-fold. It was also proposed that a neighbouring lysine residue (Lys^{33}) and the amino group of the active site threonine could possibly act as the proton acceptor-donors (Seemuller *et al.*, 1995b). Thus, the proteosome is a unique protease, belonging in a class of its own.

1.2.6 Other Thermostable Proteases

A number of thermostable proteases have been described that have not yet been classified into one of the classes of proteases. The protease from the deep sea thermophilic methanogen *Methanococcus jannaschii* is extremely thermophilic and barophilic,with activity being detectable up to at least 131°C (Michels and Clark, 1995). Activity was increased by the addition of 50MPa pressure. The protease was highly specific for leucine at the P_1 position, and showed some esterase activity at lower temperatures (Michels and Clark, 1995).

Sulfolobus shibatae, grown at pH 3.0 produces a 9kDa protease with a pH optimum of 7.2 (Vankley *et al.*, 1995). This activity is reported to change very little over the temperature range of 37°C-100°C.

Bacillus sp. (P-OO1A), produces a moderately thermostable protease (Atalo and Gashe, 1993). This protease was able to cleave a variety of fibrous proteins such as sheep skin, horn and feathers. It has a half life of 30 minutes at 70°C (Atalo and Gashe, 1993). *B. stearothermophilus* TP32 produces a thermostable protease with a molecular mass of 17kDa. It had an 'optimum temperature' between 75-80°C (Grey and Unger, 1995).

Thermoactinomyces sp. HS682 produces a thermostable alkaline protease of molecular weight 25 kDa (Tsuchiya *et al.*, 1991). The enzyme was stable for 60 minutes at 65°C at pH 11.0 (Tsuchiya *et al.*, 1991). *Thermoactinomyces vulgaris* (strain A 60) protease has a pH optimum of 9.0 and a half life of 1 hour at 85°C (Desai and Dhala, 1969). A protease with similar properties was produced by *Thermomonospora fusca* (A20) (Desai and Dhala, 1969). It had a pH optimum of 8.5 and a half life of 1 hour at 80°C. They both had the ability to cause the lysis of a number of Gram (+) bacteria and *E. coli* (Desai and Dhala, 1969).

Ladrat *et al.* (1995) isolated 77 thermophilic micro-organisms from hydrothermal units. Thermostable protease activity was detected in most of these isolates.

1.3 PURPOSE OF THIS STUDY

The purpose of this study was to investigate the properties of a variety of thermostable proteases. In particular, properties such as thermostability and substrate specificity were determined, and compared to the known or modelled three-dimensional structures of the proteases.

The initial proteases chosen for study were the thermostable metallo-proteases EA1 and YP-T from *Bacillus* st. EA1 and *B. caldolyticus* st. YP-T respectively. These proteases had previously been cloned, sequenced and expressed into *Escherichia coli* by Dr Dave Saul (Saul *et al.*, 1996). He discovered that these two proteases differed in sequence by only 1 amino acid. Yet these proteases differed in thermostability. Thus the proteases were investigated to determine how the amino acid difference affects the stability of the proteases.

The rest of the thesis is devoted to investigating some of the properties of a thermostable serine protease (subtilisin) called Ak.1 protease from *Bacillus* st. Ak.1. It was cloned, sequenced and expressed into *E. coli* by Dr Bryce Maciver (Maciver *et al.*, 1994). It has a 68% sequence similarity to thermitase, a thermostable subtilisin from *Thermoactinomyces vulgaris*. The protease was purified and properties such as thermostability and substrate specificity were studied in detail.

Dr Clyde Smith and Professor Ted Baker determined the three dimensional structure of Ak.1 protease during this project (Smith *et al.*, 1998). It was discovered that Ak.1 protease contains an unusual disulphide bond, located in the substrate binding cleft. Therefore the effect of the disulphide bond on properties of the enzyme such as substrate specificity and thermostability was investigated.

CHAPTER 2

GENERAL METHODS

2.1 MEDIA PREPARATION

2.1.1 *Bacillus* Species Liquid Medium

2.1.1.1 Nutrient Medium

	g/l
Nutrient Medium (dehydrated)	13

Dissolve the powder in ~800 ml of RO water (partially purified water by reverse osmosis). Adjust the pH to 7.0 with NaCl. Make up the volume to 1 l. Autoclave for 15 minutes at 121°C.

2.1.1.2 *Bacillus* st. EA.1 Liquid Medium (R Medium)

	g/l
KH_2PO_4	0.05
$MgSO_4.7H_2O$	0.25
$FeSO_4$	0.01
NaCl	0.002
Peptone	1.0
Yeast extract	1.0

Dissolve the components in ~800 ml of RO water. Adjust the pH to 7.6. Make up the volume to 1 l. Autoclave as above.

Note: This is the preferred medium for *Bacillus* st. EA.1 as it sporolates prolifically in the other media.

Variation: Add 1 g/l casein, or similar protein, to the medium to increase extracellular protease production.

2.1.1.3 *Bacillus. caldolyticus* Liquid Medium (Castenholz Medium D (CMD))

	g/l
Nitrilotriacetic acid	0.1
$CaSO_4.2H_2O$	0.06
$MgSO_4.7H_2O$	0.1
NaCl	0.008
KNO_3	0.103
$NaNO_3$	0.689
Na_2HPO_4	0.111
$FeCl_3$ (0.01M stock solution)	0.936 ml
Nitsch's trace elements	1 ml
Tryptone	3
Yeast extract	3

Dissolve the nitrilotriacetic acid first into ~900 ml of RO water in parts, adjusting the pH back to ~ 6.8 with KOH after each addition. Then add the other mineral salts, trace elements, tryptone and yeast extract. Adjust the pH to 7.8 at room temperature (equivalent to pH 7.4 at 70°C) with NaOH. Autoclave as above.

Variation: Add 1 g/l casein, or similar protein, to the medium to increase extracellular protease production.

Nitsch's Trace Elements

	g/l
$MnSO_4.H_2O$	2.2
$ZnSO_4.7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4$	0.016
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.046
H_2SO_4 (1N)	1 ml/l

Dissolve in water, make the volume up to 1 l. Dispense 1 ml/l.

2.1.1.4 Tryptic Soy Broth (TSB)

	g/l
Tryptic Soy Broth dehydrated media (Difco)	30

Dissolve the powder in ~800 ml of RO water. Adjust the pH to 7.0. Make up the volume to 1 l. Autoclave as above.

2.1.1.5 *Bacillus thermoglucosidacius* Liquid Medium

	g/l
Soluble starch	10
Peptone	5
Meat extract	3
Yeast extract	3
KH_2PO_4	3

Dissolve the components in ~800 ml of RO water. Adjust the pH to 7.0. Make up the volume to 1 l. Autoclave as above.

2.1.1.6 Basal Medium for *Bacillus* st. Ak.1

	g/l
MOPS buffer (25 mM final concentration)	5.233
Nitrilotriacetic acid	0.1
$CaSO_4.2H_2O$	0.06
$MgSO_4.7H_2O$	0.1
NaCl	0.008
KNO_3	0.103
$NaNO_3$	0.689
Na_2HPO_4	0.111
$FeCl_3$ (0.01M stock solution)	0.936 ml
Nitsch's trace elements	1 ml
Na glutamate (1% filter-sterilised stock)	10 ml
Carbon source (10% filter-sterilised stock)	10ml

Dissolve the nitrilotriacetic acid first into ~800 ml of RO water in parts, adjusting the pH back to ~6.8 with KOH after each addition. Then add the other mineral salts and trace elements. Adjust the pH to 7.0. Autoclave as above. Before innoculation, add the glutamate and carbon source.

2.1.1.7 Solid Medium

Dissolve the ingredients using any of the media recipies described above. Add 17.5 g/l agar to the medium and boil it until the agar has completely dissolved. Autoclave as above. Cool to about 50°C, then pour into sterile petri plates.

2.1.2 *Escherichia coli* Medium

2.1.2.1 Liquid Medium for Small Cultures (Luria Broth)

	g/l
NaCl	5.0
Tryptone	5.0
Yeast extract	5.0
Ampicillin (2.5 mg/ml stock, filter sterilised stock)	40 ml

Dissolve the ingredients, except the ampicillin, in ~800 ml RO water. Adjust the pH to 7.0. Make the volume up to 1 l. Autoclave as above. Add the ampicillin just before innoculation.

2.1.2.2 Liquid Medium for Fermentor Runs (Bulk Growth Medium 2)

This is the method of Peek *et al.*, 1993.

	g/l
Tryptone	10
Yeast extract	2
Glycerol	10 ml
NH_4Cl	3.22
KH_2PO_4	1.05
$MgSO_4.7H_2O$	0.135
K_2SO_4	0.0425
$CaCl_2.2H_2O$	0.002
$FeSO_4.7H_2O$	0.003
SL-10 Trace elements (DSM Medium 320)	1 ml
Ampicillin (2.5 mg/ml stock, filter sterilised)	40 ml

Dissolve the ingredients, except the ampicillin, in ~800 ml RO water. Adjust the pH to 7.5 with 1 M NaOH. Make the volume up to 1 l. Autoclave as above. Before innoculation, add the ampicillin.

2.1.2.3 SL-10 Trace Elements (DSM Medium 320)

	mg/l
HCl (25%; 7.7 M)	10.0 ml
$FeCl_2.4H_2O$	1.5 g
$ZnCl_2$	70.0
$MnCl_2.4H_2O$	100.0
H_3BO_3	6.0
$CoCl_2.6H_2O$	190.0
$CuCl_2.6H_2O$	2.0
$NiCl_2.6H_2O$	24.0
$Na_2MoO_4.2H_2O$	36.0

First dissolve the $FeCl_2$ in the HCl, then dilute in ~800 ml of RO water. Dissolve the other salts, then make up the volume to 1 l.

2.1.2.4. Solid Medium (Luria Agar)

Using the Luria broth recipe, dissolve the ingredients in 900 mls of RO water and adjust the pH to 7.0. Add 15 g/l agar and boil the solution to dissolve the agar. Autoclave as above, then cool down the agar to ~50°C. Add the ampicillin, swirl gently to mix, then pour ~20 ml into sterile petri dishes and allow to cool. Store at 4°C. Use the plates within 2 weeks.

2.2 PROTEASE ASSAYS

2.2.1 Azocasein Assay

This assay is based on the method of Peek *et al.* (1992).

SUBSTRATE

0.2% azocasein in 50 mM Hepes/NaOH buffer containing 5 mM $CaCl_2$. For Ak.1, the pH was 7.5. For EA1 and YP-T proteases, the pH was 6.5 and 7.0 respectively. The buffers were adjusted so the correct pH was at 75°C.

METHOD

Add 1 ml of substrate to 1.5 ml reaction vials. Use either duplicate or triplicate samples. Preincubate the tubes for 10-15 minutes at 75°C. Add 10µl of protease extract to the substrate (not the blanks), and incubate for 10 minutes at 75°C. Stop the reaction by adding 0.5 ml of 15% (w/v) trichloroacetic acid, then cool the tubes on ice for 10 minutes. At this stage, add the protease extract to the blanks. Centrifuge the tubes for 5 minutes at 15,000 rpm in a Beckman Microfuge E. Measure the OD_{420} nm of the samples and blanks. Average the replicate samples' absorbance, and subtract the absorbance of the blanks from it to determine activity.

1 U is expressed as the amount of protease required to produce a change in OD_{420} nm of 1.0 per minute at the given pH and temperature.

Variation: The substrate can be any soluble native (non-dyed) protein, e.g. haemoglobin, BSA, cytochrome c. In these cases, the wavelength used was 280 nm. Note: the background will be much higher than with azocasein. Other dyed proteins can also be used, and the wavelengths used in these cases will depend on the dye.

2.2.2 Peptide Substrates

2.2.2.1. *p*-Nitroanilide Substrates

Activity towards a variety of pNA-linked peptide substrates was determined at 5-75°C in a Perkin-Elmer Lambda #B Spectrophotometer fitted with a thermoelectric cell holder. The assay mixture consisted of 1 ml of 1.0 mM of the substrate (see Table 7.2) in 50 mM Hepes/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100 pre-equilibrated at the required temperature for 10 minutes. For some substrates, 10-50% acetonitrile was included to increase solubility. The reaction was started by adding 10 µl of the protease solution (diluted appropriately) and the change in absorbance at 400 nm was monitored continuously at the required temperature for 1-60 minutes, depending on the activity, using the PECSS program. 1 Unit (U) is defined as the amount of enzyme required to produce 1 µmol of pNA per minute The extinction coefficient for pNA under these conditions is 10,500, 10,400 and, 9,400 $M^{-1}cm^{-1}$ for assays with 0, 10%, and 50% acetonitrile respectively (see the standard curves in Appendix 1).

Later experiments were conducted, as above, with a total volume of 100 µl, with the calculations adjusted accordingly. 10 µl of protease solution was added to 90µl of substrate, and the activity was monitored as above using curvettes by Starna Pty, Ltd. A comparison of Ak.1 protease with 1ml and 0.1ml assays gave the same specific activities.

2.2.2.2. *p*-Nitrophenol Ester Substrates

Activity towards a variety of pNP-ester substrates was determined using the above equipment. The assay mixture consisted of 1 ml of 1.0 mM of the substrate (see Table 7.3) in 50 mM Hepes/NaOH pH_{40} 7.5 containing 0.01% (v/v) Triton X-100 and 50% acetonitrile, pre-equilibrated at 40°C for 10 minutes. The reaction was started by adding 10 µl of the protease solution (diluted appropriately) and the change in absorbance at 400 nm was monitored continuously at 40°C for 3-60 minutes. 1 Unit (U) is defined as the amount of enzyme required to produce 1 µmol of pNP per minute at 40°C. The extinction coefficient for pNP under these conditions is 7,400 $M^{-1}cm^{-1}$ (see the standard curve in the Appendix).

2.2.2.3. Methyl- and Ethyl-Ester Substrates

Activity towards a variety of methyl and ethyl-ester-linked substrates was determined at 20°C using the above equipment. This is an adaptation of the chromogenic pH shift method of Whittaker *et al.* (1994). The assay mixture consisted of 1 ml of 1.0 mM of the substrate (see Tables 6.1-6.2) in 5 mM Hepes/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$, 0.01% (v/v) Triton X-100 and 0.0025% (w/v) bromothymol blue, pre-equilibrated at 20°C for 10 minutes. The reaction was started by adding 10 µl of the protease solution (diluted appropriately) and the decrease in absorbance at 615 nm was monitored continuously at 20°C for 3 minutes. Activity calculations were determined from the *initial* slope of the curve. 1 Unit (U) is defined as the amount of enzyme required to cleave 1 µmol of substrate (equivalent to producing 1 µmol H^+) per minute at 20°C. Note: 1 µmol H^+ produced in 1 ml is equivalent to an absorbance change at 615 nm of 5.405.

2.2.2.4. α-Napthylester Substrates

Activity towards Ac-Ala-αnapthyl ester was determined at 40°C using the above equipment. The assay mixture consisted of 1 ml of 1.0 mM of the substrate (see Table 6.1) in 5O mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100, pre-equilibrated at 40°C for 10 minutes. The reaction was started by adding 10 µl of the protease solution (diluted appropriately) and the increase in absorbance at 340 nm was monitored continuously at 40°C for 3 minutes. 1 Unit (U) is defined as the amount of enzyme required to produce 1 µmol NAP of 1.0 per minute at 40°C (molar extinction coefficient = 1780) .

2.2.2.5. Furylacryl- Substrates

Activity towards furylacryl-linked substrates was determined at 40°C using the above equipment. The assay mixture consisted of 1 ml of 50 µM of the substrate (see Table 6.2) in 5O mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100, pre-equilibrated at 40°C for 10 minutes. The reaction was started by adding 10 µl of the protease solution (diluted appropriately) and the change in absorbance at 324 nm was monitored continuously at 40°C for 3-60 minutes. 1 Unit (U) is defined as the amount of enzyme required to produce 1 µmol of FA per minute at 40°C (molar extinction coefficient = 317).

2.2.3 Casein Agar Plates

This is the method of Eames, 1985.

	g/l
Casein (Sigma, technical grade)	10.0
Agar	17.5
NaN_3	0.2

Dissolve the casein and agar in 1 l of RO water by heating and mixing, readjusting the pH to 7.5 (or as appropriate) during heating with 1M NaOH. Autoclave as before, and allow to cool to 50°C. Add the NaN_3 and pour into sterile plates. Allow to set.

Cut wells (6 mm width) into the gels and add approximately 20 µl of enzyme solution (or culture) into the wells. Seal the plates with masking tape, and incubate them at the appropriate temperature (<70°C) for 16 hours (overnight). Proteolytic activity is observed as a ring of white *para*-casein precipitate around the sample wells. With high activity, the precipitate is only seen at the outer circumference of the rings. Activity is determined by measuring the diameter of the *para*-casein rings.

2.3 PROTEIN ASSAYS

2.3.1 Lowry Assay

2.3.1.1 Modified Lowry #1

This is the method of Peterson, 1977.
Reagent A: 0.5% $CuSO_4.5H_2O$ + 1% Na_3citrate
Reagent B: 2.0% Na_2CO_3 + 0.4% NaOH
Working Solution: 1 ml of reagent A + 50 ml of reagent B.
(Use within 1.5 hr)
BSA Standards: 0-100 mg/ml in Milli Q water

Add 200µl of the protein samples (diluted appropriately in RO water) and standards to 1 ml of the working solution. Vortex, then incubate at room temperature for 10 minutes. Add 100 µl of 2N Folins-Ciocalteu reagent (diluted 1:1 with RO water) and mix immediately. Allow to stand for at least 1 hour at room temperature. Read the absorbance at 750 nm.

2.3.1.2 Modified Lowry for Samples Containing Interferring Substances

This is the method of Peterson, 1983.

	g/100 ml	%	M
$CuSO_4.5H_2O$	0.1		
Sodium and/or potassium tatrate	0.2		
Na_2CO_3	10		
Sodium dodecyl sulphate (SDS)			5.0
NaOH			0.8
Folin-Ciocalteu phenol reagent			2.0 N
Sodium deoxycholate (DOC)		0.15	
Trichloroacetic acid (TCA)		72%, 6%	

CTC Reagent: Dissolve the Na_2CO_3 into 40 ml of RO water. Dissolve the $CuSO_4.5H_2O$ and tartrate into a second 40 ml of RO water. Add the first solution slowly, with stirring, to the second solution. It will keep indefinately at 10°C.

Reagent A: Mix 1 part of the CTC reagent with 2 parts of the SDS solution and 1 part of the NaOH solution. With time, the SDS will ppt out. This can be redissolved by warming. It will keep 2-3 weeks at room temperature. Discard if a dark ppt appears.

Reagent B: Mix 1 part of the 2N Folin reagent with 5 parts of RO water. It will keep for months at room temperature if covered from light.

BSA Standards: 0-100 mg/ml in Mili Q water.

i) *Sample Pretreatment*

To a 400 µl protein sample/standard in 1.5 ml reaction vials, add 40 µl of DOC, mix and allow to stand at room temperature for 10 minutes. Add 40 µl of 72% TCA, mix and centrifuge for 5 minutes in a Beckman microfuge E. Carefully remove the supernatant. If the original protein solution contained an interfering substance that the Lowry method is extremely sensitive to, e.g. Hepes buffer or histidine, wash the pellet by adding 1 ml of 6% TCA, mixing, and centrifuging as before and removing the supernatant. Repeat this washing process. Make 1 pin-hole through the top of the reaction vials, then dry the pellet *in vacuo* for about 1 hour. Resuspend the pellet in 400 µl of water. Any remaining precipitate will redissolve in the first step of the standard assay procedure (see below).

ii) Standard Assay Procedure

To 400 µl of sample/standard, add 400 µl of reagent A. Mix thoroughly and allow to stand for 10 minutes at room temperature. Add 200 µl of reagent B and mix immediately. Allow to stand for 30 minutes at room temperature, then read the absorbance at 750 nm.

2.3.2 Bradford Protein Assay

This is the method of Read & Northcote (1981).

Dye Reagent: Serva Coomassie Blue G in 1.6 M phosphoric acid/
0.8 M ethanol.

BSA Standards: 0-100 mg/ml in Milli Q water.

Add 40 µl of sample/standard to 1.2 ml of the dye reagent. Mix briefly and allow to stand for 5 minutes at room temperature. Read the absorbances at 595 nm.

2.3.3 280/205 Absorption Protein Assay

This is the method of Peterson (1983). This method is used to determine absolute quantities of a pure protein, and its extinction coefficient, E^{1}_{205} (mg/ml), at 205 nm. Dilute the protein solution into two concentrations; one between 5-

25 ug/ml and one between 50-100 ug/ml. Measure the absorbance of the solutions at 205 nm and 280 nm respectively. The absorbance values are then extrapolated to the original solution of the protein and its concentration is calculated from the following formula:

$$P \text{ (in mg/ml)} = A_{205}/[27.0 + 120(A_{280}/A_{205})]$$

The E^1_{205} (mg/ml) is equal to the value of the denominator with a <2% error.

2.4 ELECTROPHORESIS

2.4.1 Analysis of Proteins

2.4.1.1 SDS/PAGE Standards

This is a commercially prepared series of standards (Pharmacia).

Standards	mg/vial	MW (kDa)
Phosphorylase b	64	94
BSA	83	67
Ovalbumin	147	43
Carbonic anhydrase	83	29
Soybean trypsin inhibitor	88	20.1
α-Lactalbumin	121	14.4
Sucrose	27 mg	N/A

Add 100 µl of Milli Q water to the vial. Dilute as appropriate for silver staining (1/20-1/50).

2.4.1.2 Protease Pre-Treatment for SDS/PAGE

TCA-Acetone Treatment

This is the method of Peek *et al* (1993). Add 10 µl of 20% TCA to 10 µl of sample (final TCA concentration = 10%). Vortex, stand for 5 minutes at room temperature then centrifuge for 5 minutes in the Beckman microfuge E. Carefully withdraw the supernatant. Add 10 µl of 100% acetone to the pellet. Vortex, then centrifuge as before. Carefully remove the supernatant. Using either N_2 gas or a vacuum dessicator, evaporate the remaining solvent. Add 6 µl of Milli Q water and 2 µl of 2M NaOH.

2.4.1.3 SDS/PAGE Sample Preparation and Electrophoresis

This is the PhastGel method of Pharmacia.

Sample buffer(final concentration):10 mM Tris/HCl pH 8.0, 1 mM EDTA, 2.5% SDS, 5.0% β-mercaptoethanol and 0.01% bromphenol blue. Stock solution = x5 (requires heating to dissolve the SDS).

Add 2 µl of x5 SDS PAGE sample buffer to the above pretreated samples and standards. Mix well. If the solution is yellow, add 2M NaOH, 2 µl at a time, until the solution turns blue. Do not add too much NaOH or the protein will precipitate. Boil the samples for 10 minutes, then centrifuge them for 5 minutes as before. Load the supernatants onto the gel, then run according to the Pharmacia protocol.

2.4.1.4 Gel Staining

Silver Stain

This is the Bio-Rad method.

Step	Time	Solution	Comment
1.	30 min	40% methanol, 10% acetic acid	Fixative
2.	15 min	20% ethanol, 5% acetic acid	Fixative
3.	15 min	20% ethanol, 5% acetic acid	Fixative
4.	5 min	8.3% glutaraldehyde	
5.	-	Milli Q	Wash
6.	20 min	0.25% $AgNO_3$	Stain
7.	-	Milli Q	Wash
8.	3-10 min	0.04% formaldehyde, 2.5% Na_2CO_3	Developer
9.	10 min	5% acetic acid	Stop
10.	5 min	10% acetic acid, 5% glycerol	Preservative

2.4.2 Analysis of DNA

2.4.2.1 Reagents

5xTBE Buffer:	g/l
Tris buffer	54.0
Boric Acid	27.5
0.5M EDTA pH 8.0 (stock solution)	20 ml

Dissolve the ingredients in Milli Q water. Make up the volume to 1l. Adjust the pH to about 8.3.

Sample Preparation:

Add 5-7 µl of gel loading buffer (containing bromophenol blue, gelatin and glycerol) to 20 µl of the PCR product. Centrifuge for a few seconds, then mix the reagents.

2.4.2.2 Gel Preparation

Add 6.0g of SeaKem LE agarose to 400 ml of 1xTBE (final concentration = 1.5% agarose). Heat, with gentle swirling, to dissolve the agarose. Once dissolved, cool for at least 10-15 minutes till it is about 55°C, then pour into the electrophoresis apparatus. Allow to set with the combs in place. Add just over 2 l of 1xTBE buffer. Remove the combs and add 25 µl of the samples to each well. Run at 3.5 V/cm for 350 Vh.

2.4.2.3 Ethidium Bromide Staining of DNA

Stain:

Add 100 µl of a 10 mg/ml solution of ethidium bromide to 1l of water. Add to a container containing the gel. Swirl gently till the gel moves freely. Stain for 1 hour at room temperature.

Destain:

Tip off the ethidium bromide solution. Run tap water gently over the gel for 1 hour to destain.

Visualisation of the Bands:

Place the gel on a UV light box in a darkened room. Wearing safety glasses, turn on the UV light. The DNA bands will fluoresce pink.

2.5 BACTERIAL STAINING

2.5.1 Gram Stain

This is the method of Bartholomew (1962)

2.5.1.1 Reagents

Crystal Violet Stain:

Dissolve 2.0 g of crystal violet powder in 20 ml of 95% (v/v) ethanol. Dissolve 0.8 g of ammonium oxalate into 80 ml ofwater. Mix the two solutions together. Store for 24 hours at room temperature, then filter to remove any precipitate.

Iodine Mordant:

Grind 1.0 g of iodine and 2.0 g of potassium iodide in a mortar, then add water slowly (total volume = 300 ml) with continuous grinding until the iodine is dissolved. Store in amber bottles.

Decolourising Solvent:

95% (v/v) ethanol

Counterstain:

10 ml of a 2.5% (w/v) safranin O solution in 95% (v/v) ethanol is added to 100 ml of distilled water.

2.5.1.2 Procedure

Smear a small quantity of bacteria onto a slide. Air-dry and heat-fix the smear with a bunsen flame. Flood the slide with the crystal violet stain for 1 minute. Wash in a gentle stream of tap water for 2 seconds. Flood the smear with the iodine mordant for 1 minute. Wash in tap water as before, then blot dry. Flood the smear with 95% ethanol for 30 seconds, then blot dry. Flood the smear with the safranin counterstain for 10 seconds. Wash with tap water until no colour appears in the effluent. Examine under a light microscope. Pink cells are Gram (-), while blue cells are Gram (+).

2.5.2 Endospore Stain

This is the method of Shaeffer & Fulton (1933)

2.5.2.1 Reagents

Malachite Green Stain:

0.05% (w/v) malachite green in water.

Counterstain:

10 ml of a 2.5% (w/v) safranin O solution in 95% (v/v) ethanol is added to 100 ml of distilled water.

2.5.2.2 Procedure

Air dry a smear of bacteria on a glass slide, then heat-fix. Cover with blotting paper which is saturated with the malachite green stain. Place over a boiling water bath for 5 minutes. Remove the blotting paper and wash the slide in tap water. Counterstain the smear with safranin for 30 seconds as in the Gram stain. Wash the slide in tap water. Endospores appear bright green, while vegetative cells appear brownish-red.

2.6 REMOVAL OF SMALL MOLECULAR WEIGHT CHARGED COMPOUNDS FROM AK.1 PROTEASE

Sep-Pak C_{18} Cartridges

Sep-Pak C_{18} cartridges are obtained from Waters. They are used as a fast cleanup step for sample preparation prior to procedures which require salt-free preparations, or for the preparation of samples free from traces of inorganic salts that interfere with the procedure (e.g. sulphur-containing buffer salts and Ca^{2+} removal).

To prepare the dry Sep-Pak C_{18}, first wet it by passing 5 mls of 100% acetonitrile through the cartridge. This is followed by 10 mls of distilled water, to wash out the acetonitrile. Cartridges stored in 100% acetonitrile require only the water step.

Pass the enzyme solution through the Sep-Pak C_{18}. Wash the cartridge with a further 5 mls of distilled water. Elute the sample with 2 mls of 50% acetonitrile in distilled water. Alternatively, if several components have bound to the cartridge, pass 5 ml aliquots of 10-50% acetonitrile solutions onto the cartridge in a stepwise manner to achieve separation of the components. Finally, pass 10 mls of 100% acetonitrile through the cartridge to remove any remaining bound substances. The cartridge can be reused if stored in 100% acetonitrile.

Note: All additions to the Sep-Pak C_{18} cartridge are done with a syringe. Other water-miscible solvents can be used in place of acetonitrile such as methanol and isopropanol.

2.7 DISULPHIDE BOND REACTIONS

2.7.1 Ellman Assay

This is a modification of the method of Hermanson (1996).

2.7.1.1 Preparation of Reagents

The Ellman reagent consists of 4 mg/ml 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) in 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8.0 containing 10 mM ethylene-diaminetetra acetic acid (EDTA). The cysteine standards (0-100 µM) and enzyme samples were equilibrated in 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8.0 containing 10 mM (EDTA). The reagent used to break the disulphide bond and denature the protein was 10 mM dithiothreitol (DTT) in 10 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 containing 75 mM NaCl and 4M urea. All reagents were made up in RO water.

2.7.1.2 Assay Method

1. *Method for Proteins with Free Thiols*

Add 100 µl of the sample/standard to a curvette (Starna). Set the absorbance at 412 nm to zero. Add 20 µl of the Ellman reagent and mix well. Monitor the increase in absorbance at 412 nm for 15 minutes, or until the absorbance stops increasing. Note the final absorbance. The number of sulphydryl groups is computed from comparing the absorbance of the samples(minus 'no-enzyme' blanks) to the cysteine standard curve, or using the extinction coefficient of the 3-carboxylato-4-nitro-thiophenolate (CNT) product of 11,400 $\pm$ 600 cm^{-1} (13,600). The protein concentration is determined by the Lowry method (see section 2.3.1), and is used to determine the mole -SH per mole of enzyme.

2. *Method for Proteins Containing No Free Thiols*

The protein is first exposed to a reducing agent to open any disulphide bonds, and produce free thiols that will later be reacted with the Ellman reagent. Equilibrate the protein in the DTT reagent (see 2.7.1.1). Incubate for 1 hour at room temperature, followed by a 2 minute incubation in a boiling water bath to ensure the protein is fully denatured. Pass the solution through a Sephadex G25 column, pre-equilibrated with 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8.0 containing 10 mM EDTA. The EDTA is necessary to keep the protein in the reduced state. Once the solution has been loaded onto the column, pump the above buffer

through the column. The purpose of this column is to separate the reducing/denaturing agents from the protein sample. The protein will elute from the column before the DTT/NaCl/urea salts. Concentrate the protein eluent to by ultrafiltration (Amicon) using a YM-10 membrane. Take 100 µl of the solution and run the Ellman assay described in 1. above.

2.7.2 Reaction of Thiols with Disodium 2-nitro-5-thiosulphobenzoate (NTSB)

This is an adaptation of the method of Thannhauser *et al* (1984).

2.7.2.1 Preparation of NTSB from DTNB

Dissolve 0.1 g of DTNB (250 µmol) in 10 ml of 1M Na_2SO_3 at 38°C and adjust the pH to 7.5. A bright yellow-orange solution is produced. Bubble oxygen gas through the solution to decolourise it to a pale yellow. Add more Na_2SO_3 and bubble oxygen through the solution again. Repeat this step until no more yellow colour is produced when Na_2SO_3 is added (i.e. excess Na_2SO_3). These reactions should be complete after about 1 hour. No purification is required. The solution is diluted to 0.5 mM with 0.2 M Tris containing freshly prepared 0.1 M Na_2SO_3 and 3 mM EDTA and stored at -20°C until use.

2.7.2.2 Reaction of Thiols with NTSB

Add 0.1 ml of the enzyme to 1 ml of the NTSB solution (containing 2-3 M guanidine thiocyanate for buried disulphides). Incubate for 30 minutes at room temperature. Read the absorbance of the CNT product at 412 nm against a blank of the NTSB solution with 0.1 ml of water. The extinction coefficient of the product is the same as in the Ellman reaction.

CHAPTER 3

PRODUCTION AND COMPARISON OF THERMOSTABLE METALLO-PROTEASES FROM *BACILLUS* ST. EA.1 AND *BACILLUS CALDOLYTICUS*

3.1 INTRODUCTION

The metallo-proteases from *Bacillus* sp. have been extensively studied (see section 1.2). The most extensively studied member of this class is thermolysin, from *Bacillus thermoproteolyticus* (e.g. Titani *et al.*, 1972; Holmes and Matthews, 1982). Three metallo-proteases produced by strains of *B. stearothermophilus* have been characterised and their genes have been sequenced and expressed. They are *npr*M from strain MK232 (Kubo and Imanaka, 1988), *npr*T from strain CU21 (Takagi *et al.*, 1985) and *npr*S from strain TELNE (Nishiya and Imanaka, 1990). Each of these genes, and resultant peptide sequences, are closely related to each other, and show close homology to thermolysin.

Two other proteases have been characterised that belong to the thermolysin-like family of metallo-proteases. The first protease, produced by *B. caldolyticus* YP-T (DSM 405), was one of the earliest thermophilic proteases discovered. An early report by Heinen and Heinen (1972) described the production of this extracellular protease by a *Bacillus* species growing at 72°C. Recently, the gene encoding the protease was cloned, sequenced and expressed first into *B. subtilis* (Van den Burg *et al.*, 1991), and then into *Escherichia coli* (Saul *et al.*, 1996). Protease YP-T showed an approximate 87% identity with thermolysin, and was nearly identical to CU21 protease (Van der Burg *et al.*, 1991).

The second protease was produced by *Bacillus* st. EA1 (Coolbear *et al.*, 1991, 1992b). It is also thermostable, with a half-life of 2 hours at 85°C in the presence of 10 mM Ca^{2+}. It was cloned and sequenced (Saul *et al.*, 1996) and expressed into *Escherichia coli*. It was discovered that EA1 protease differs in its amino acid sequence from YP-T protease by only one amino acid (Val 61 of EA1 --> Gly 61 of YP-T; Saul *et al.*, 1996). Figure 4.1 shows an amino acid sequence comparison of the metallo-proteases EA1, YP-T, NprT and thermolysin.

The purpose of this study was to see if the single amino acid difference between EA1 and YP-T proteases resulted in a significant difference in any of the properties of the proteases, such as thermostability. It was not an attempt to quantify properties such as calcium binding, but rather to describe in a *qualitative* way the differences in the proposed Ca(3) binding site of the proteases.

To do this, the native and cloned organisms producing these proteases were grown, and their respective proteases were extracted and partially purified. The pH profiles and thermostability in the presence and absence of Ca^{2+} were investigated.

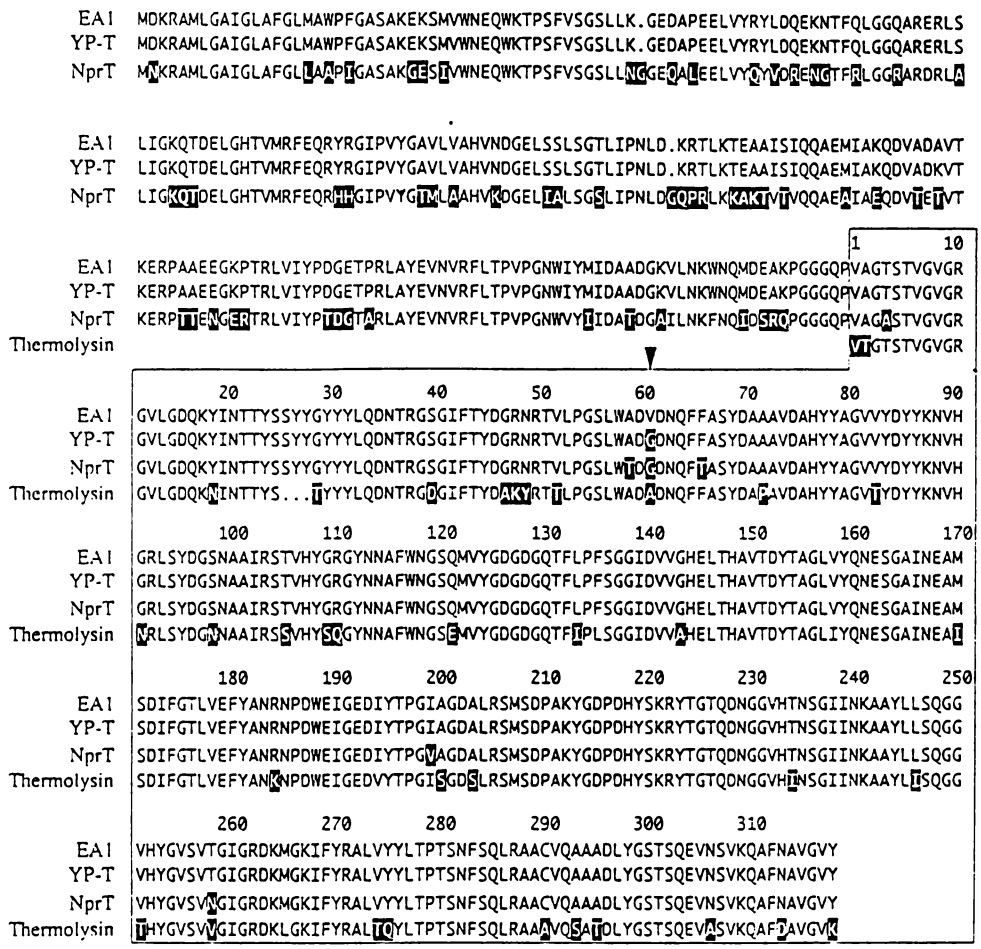


Figure 3.1 Amino Acid Sequences of Four Metallo Proteases.
From Saul *et al* (1997) with permission. EA1: from *Bacillus* st. EA1; YP-T: from *Bacillus caldolyticus* YP-T; Npr-T: from *Bacillus stearothermophilus* strain npr-T; Thermolysin: from *Bacillus thermoproteolyticus*.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

Bacillus caldolyticus YP-T (Heinen and Heinen, 1972; Van den Burg *et al.*, 1991) is also known as *Bacillus* sp. IFO 15313 (DSM 405). The other native organism is *Bacillus* st. EA1 (Coolbear *et al.*, 1991). The protease genes from these organisms were both introduced into *E. coli* strain DH5α (F- ◊80d*lac*ZαM15 α(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 hsdR17 ($r_k^-m_k^+$)*deo*R thi-1 *sup*E44α-*gyr*A96 relA1 (Saul *et al.*, 1996) The protease genes were introduced into the heat-inducable plasmid pJLA602. The *E. coli* clones were called PB6425 and PB6408 for YP-T and EA1 proteases respectively.

3.2.2 Growth of the Native and Cloned Organisms

The native organsims were grown aerobically in 2 l shake flasks overnight at 65°C with a final culture volume of 1.5 l. *B. caldolyticus* was grown on CMD medium (see 2.1.1.2) while *Bacillus* st. EA1 was grown on R medium (see 2.1.7.1). The two organisms were grown on different media as these were the media that maximised protease production for each organism. The medium was supplemented with 1 g/l casein to try to increase extracellular protease production.

The *E. coli* clones PB6425 and PB6408 were grown aerobically in 2 l shake flasks with a final culture volume of 0.75 l each. Each media was innoculated with an uninduced culture. They were both grown on Luria broth (see 2.1.2.1) containing 100 µg/ml ampicillin at 32°C until they reached an A_{600}nm of approximately 1.0. Production of the proteases was then induced by heating the cultures at 42°C for 20 minutes, followed by an incubation at 38°C for 2 hours.

3.2.3 Harvesting and Partial Purification of the Proteases

3.2.3.1 Harvesting of the Proteases

As the native proteases are extracellular, activity was detected in the supernatant of the cultures. The *Bacillus* cultures were centrifuged at 9000 rpm for 20 minutes to pellet the cells, and the supernatant was retained. The cell-free supernatants were concentrated on an Amicon YM10 ultrafiltration membrane

and equilibrated to 20 mM Bistris pH 6.5 containing 5 mM $CaCl_2$ in preparation for addition to phenyl Sepharose.

Protease activity was detected in the cell pellet of clone PB6408, and in both the cell pellet and the supernatant of clone PB6425. The activity in the supernatant of the latter culture is thought to be due to the significant degree of cell lysis that occurred after the induction. For the *E. coli* clones, the cultures were centrifuged, as before, and the supernatant discarded. The cells were resuspended in about 10 mls of 50 mM Hepes/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$, then sonicated on ice for 5 minutes. The suspension was then heat-treated for 20 minutes at 70°C to precipitate the majority of the *E. coli* proteins. This suspension was centrifuged at 10,000 rpm for 20 minutes, and the pellet discarded. Further centrifugation steps were required to clarify the PB6425 extract.

3.2.3.2 Partial Purification of the Proteases

Each of the 4 protease extracts was applied to a 10 ml phenyl Sepharose (Pharmacia) column, pre-equilibrated with 20 mM Bistris/NaOH pH 6.5 containing 5 mM $CaCl_2$ (buffer A) at a flow rate of 5 mls per minute. Following protease addition, the column was washed with buffer A. The protease was eluted by adding buffer A containing 1 M toluene-4-sulphonic acid. This was followed by a second wash with buffer A, then the remaining bound proteins were eluted by a step addition of 10 mM Hepes/NaOH pH 6.5 containing 5 mM $CaCl_2$ and 50% (v/v) ethanediol. The active fractions were pooled, concentrated on an Amicon YM-10 membrane, as before, and equilibrated to a final buffer composition of 20 mM Bistris/NaOH pH 6.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton-X-100.

3.2.4 Buffer Preparation for Thermostability Determination at 85°C

All buffers for the work with thermostability at 85°C were passed through a metal-ion chelating column (Chelex 100; Dow Chemical Company) before use to remove traces of Ca^{2+} and other metal ions. After passage through Chelex 100, samples of the buffers were analysed for Ca^{2+} content by Atomic Absorption Spectroscopy. Buffers containing less than 1μM Ca^{2+} were used in these experiments.

3.2.5 Pretreatment of the Native Proteases for Half Life Determination at 85°C

The native proteases were pretreated by incubating first with 1 mM *o*-phenanthroline, to prevent autolysis by removing Zn^{2+} ions, for 1 hour at room temperature. The proteases were then incubated with 20 mM EDTA, to remove Ca^{2+} ions. The metal ions-reagents were removed by extensive ultrafiltration (YM-1) against first Milli Q water with 0.01% Triton X-100, then 10 mM HEPES/NaOH pH_{75} 6.5 containing 0.01% Triton X-100 that had previously passed through Chelex-100 to remove Ca^{2+} ions.

3.2.6 Protease Assay

Protease activity was detected with azocasein by the method of Peek *et al.*, (1992). Activity was expressed as the amount of enzyme required to produce an A_{420}nm of 1.0 per minute (see section 2.2).

To determine the optimum pH of the enzymes, samples of enzyme were assayed with azocasein in the pH range of 5.5-8.0 for 30 minutes at 75°C. Activity is expressed as a percentage of the optimal activity.

3.2.7 Determination of Thermostability

The thermostability of the proteases at 85°C and 95°C were investigated by incubating samples of the proteases at the above temperatures at pH 6.5 in the presence of 5 mM and/or 10 mM $CaCl_2$. Samples were withdrawn at various time intervals, cooled in liquid N_2, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5, containing 5 mM $CaCl_2$ at 75°C (section 2.2). Activity is expressed as a percentage of the initial activity of the protease (non-incubated control).

For later thermostability studies at 85°C, the native proteases were pretreated as in section 3.2.5, then incubated with different concentrations (0-10 mM) of Ca^{2+} for the appropriate time lengths at 85°C, then cooled on ice. The remaining activity was was detected by assaying with 0.2% azocasein, as above.

3.2.8 Molecular Modelling

The molecular modelling was performed on a Silicon Graphics computer using the program Hyperchem. The 3-dimensional structural models of the third calcium binding site of EA1 and YP-T proteases were based on the known structure of thermolysin, a protease with a high sequence homology to the proteases. The structure of thermolysin was obtained from the Brookhaven Protein Databank (PDB). The calcium binding site of thermolysin was subjected to theoretical site-directed mutagenesis, to produce two structures with sequences identical to EA1 and YP-T proteases respectively. The structures were minimised, using the program Amber, to produce structures of minimum energy.

3.3 RESULTS AND DISCUSSION

3.3.1 Partial Purification and Yields of the Proteases

Phenyl Sepharose was chosen as the method of cleanup of the protease extracts due to the observation that this column appears to be acting as an affinity column for proteases (Prescott *et al.*, 1993; Toogood *et al.*, 1995). Toluene-4-sulphonic acid was used as the eluting substance as it is similar in structure to metallo-protease inhibitors, so it acts as an affinity ligand. Figures 3.2-3.5 show the elution profiles of the four protease extracts.

Bacillus st. EA1 produced about 130 U of protease per l of culture, as opposed to only about 40 U per l for *Bacillus caldolyticus* YP-T. PB6425(YP-T) produced about 544 U per l of culture, greater than the 8 U per l produced by PB6408 (EA1). Induction of PB6425 resulted in significant cell lysis within 1-2 hours, probably due to the high expression of the protease. No such lysis occurred with PB6408, consistent with the low protease yields.

After phenyl Sepharose, the yields of native and cloned EA1 proteases were 19.12 and 11.68 U respectively. The yields of the native and cloned YP-T proteases were 1.12 and 153.63 U respectively.

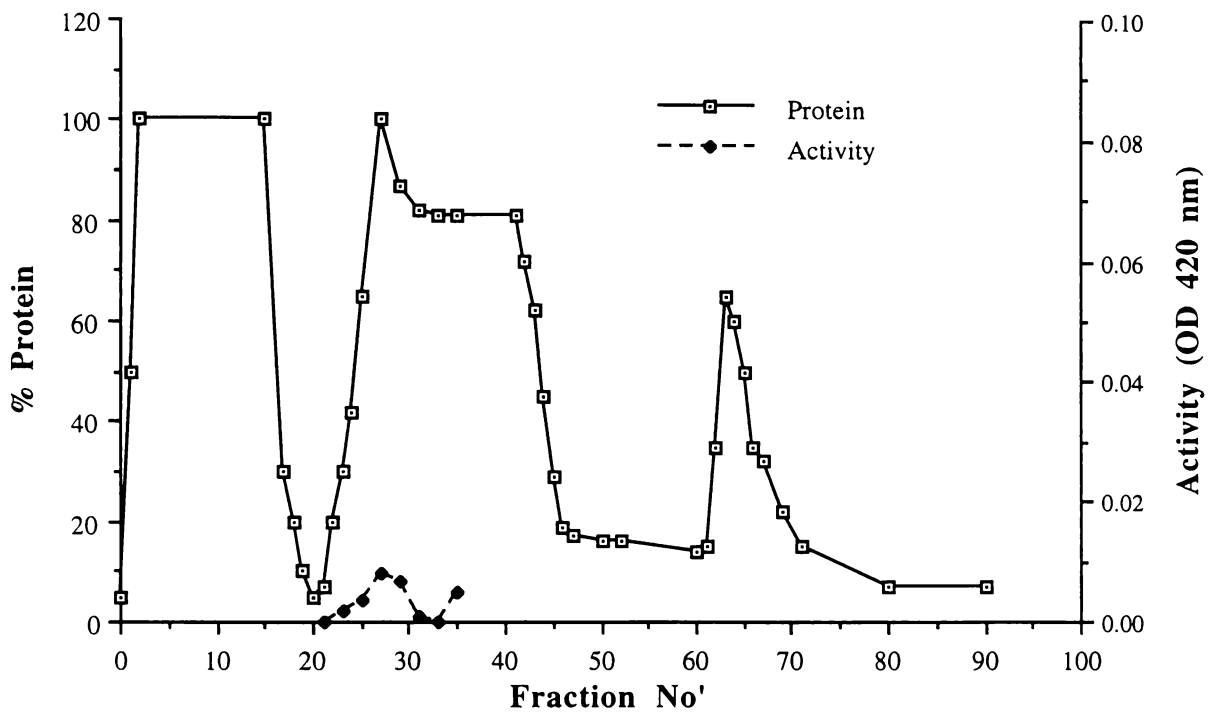


Figure 3.2 Phenyl Sepharose Elution Profile of Native YP-T Protease.
Fractions 1-20 = sample addition; fractions 21-35 = 20 mM Bistris/NaOH pH 6.5 + $CaCl_2$ + 1M toluene-4-sulphonic acid addition; fractions 36-54 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash; fractions 55-70 = 10 mM Hepes/NaOH pH 6.5 + 5 mM $CaCl_2$ + 50% (v/v) ethanediol wash.

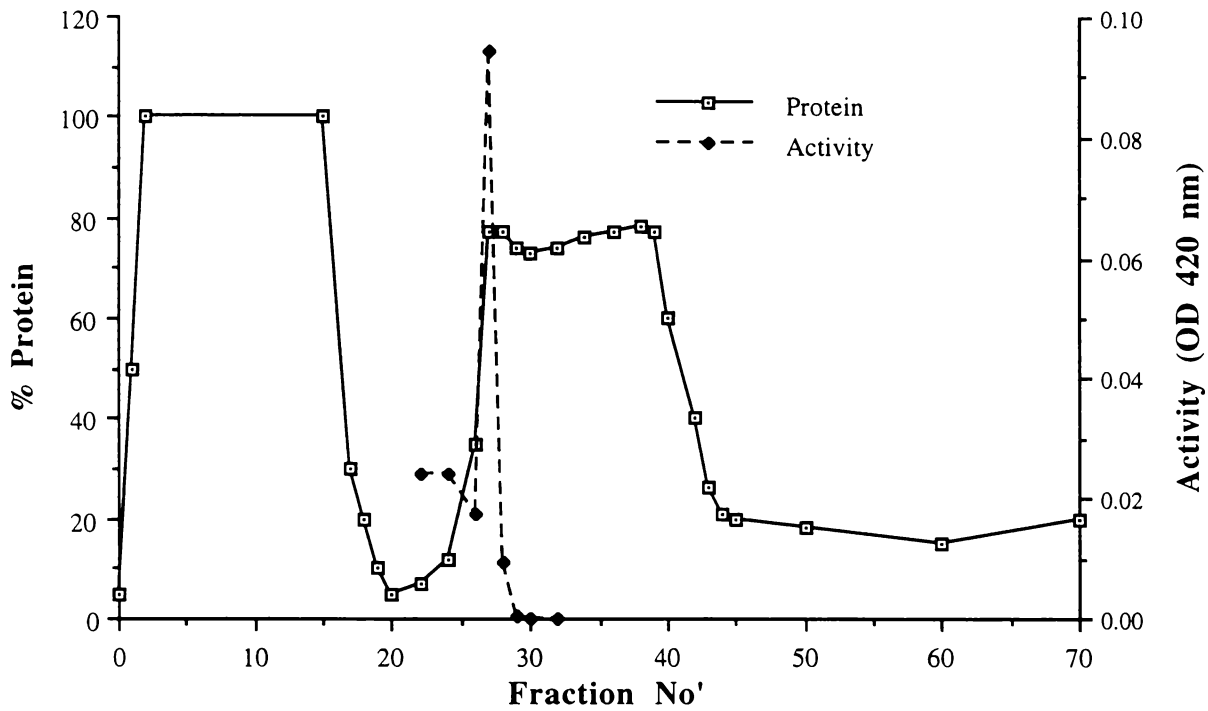


Figure 3.3 Phenyl Sepharose Elution Profile of Native EA1 Protease

Fractions 1-20 = sample addition; fractions 21-32 = 20 mM Bistris/NaOH pH 6.5 + $CaCl_2$ + 1M toluene-4-sulphonic acid addition; fractions 33-60 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash; fractions 60-70 = 10 mM Hepes/NaOH pH 6.5 + 5 mM $CaCl_2$ + 50% (v/v) ethanediol wash.

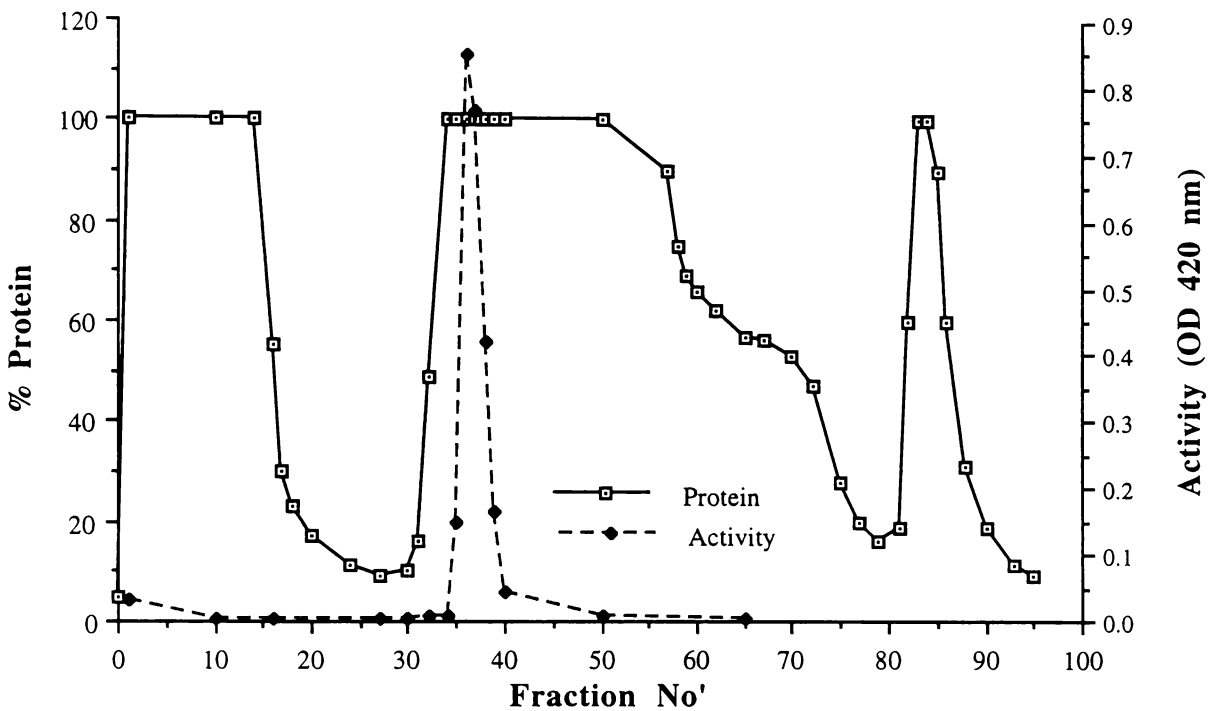


Figure 3.4 Phenyl Sepharose Elution Profile of Cloned YP-T Protease.
Fractions 1-24 = sample addition; fractions 25-42 = 20 mM Bistris/NaOH pH 6.5 + $CaCl_2$ + 1M toluene-4-sulphonic acid addition; fractions 43-74 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash; fractions 75-93 = 10 mM Hepes/NaOH pH 6.5 + 5 mM $CaCl_2$ + 50% (v/v) ethanediol wash; fractions 94-95 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash

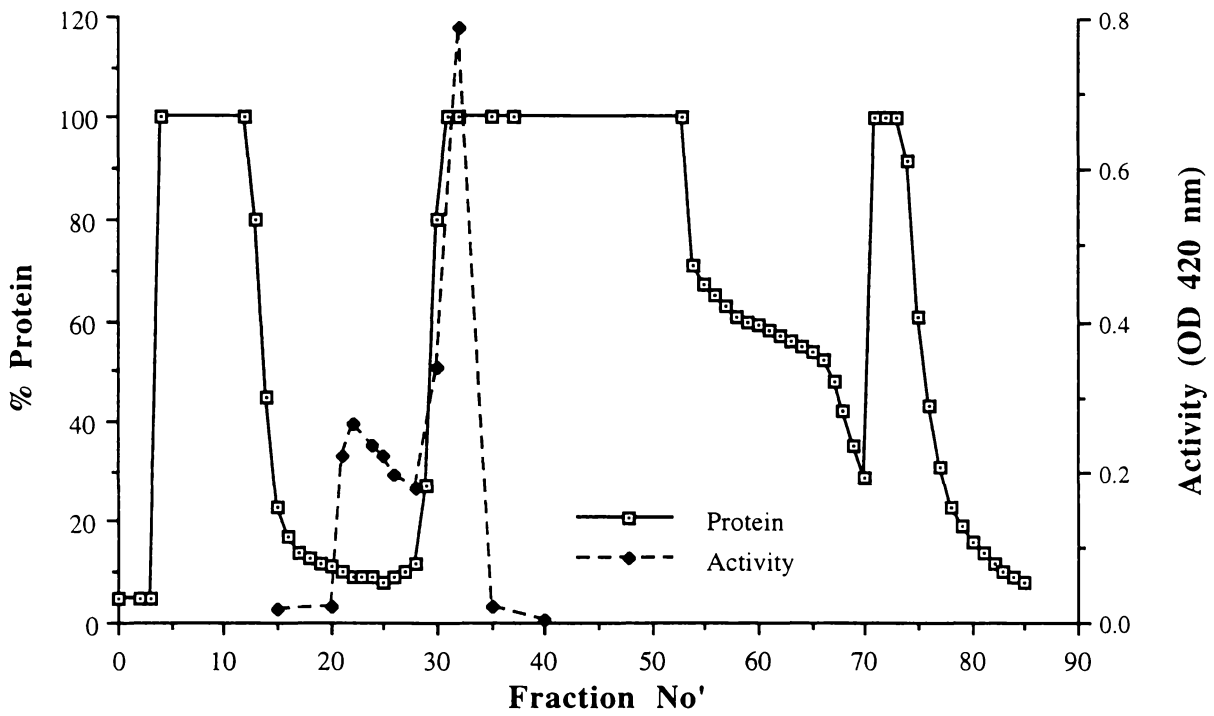


Figure 3.5 Phenyl Sepharose Elution Profile of Cloned EA1 Protease.
Fractions 1-20 = sample addition; fractions 21-35 = 20 mM Bistris/NaOH pH 6.5 + $CaCl_2$ + 1M toluene-4-sulphonic acid addition; fractions 36-62 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash; fractions 63-78 = 10 mM Hepes/NaOH pH 6.5 + 5 mM $CaCl_2$ + 50% (v/v) ethanediol wash; fractions 79-85 = 20 mM Bistris/NaOH pH 6.5 + 5 mM $CaCl_2$ wash

3.3.2 pH Optimum of the Proteases

Coolbear *et al* (1991) reported that the pH optimum for EA1 was 6.5. As Van den Burg *et al* (1991) used pH 7.0 for assaying YP-T protease, pH optimum studies were carried out to determine if the pH optimum was different between the proteases. Figure 3.6 shows that the pH optimum is 6.5 for both EA1 and YP-T proteases. Both the native and cloned proteases show a relatively broad pH optimum, with at least 80% of their activity present between pH 6-7.5. Thus the difference in amino acid sequence between EA1 and YP-T has no significant effect on the pH optimum of the proteases.

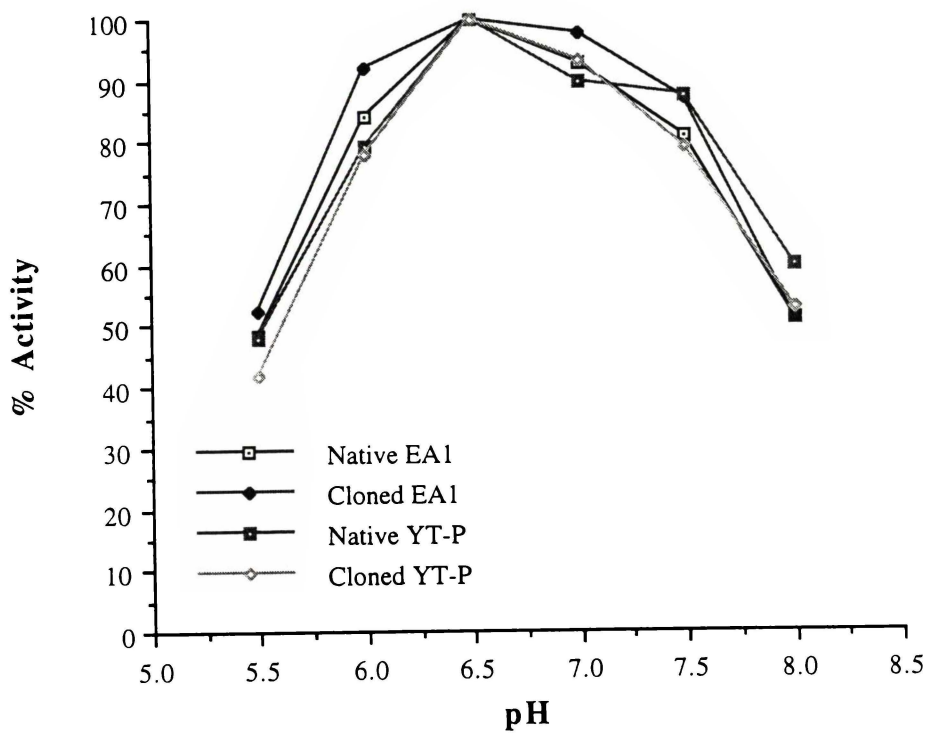


Figure 3.6 pH Optimum of the Proteases
The proteases were assayed with 0.2% azocasein in 50 mM Buffer (at the above pH values above) containing 5 mM $CaCl_2$ for 30 minutes at 75°C.

3.3.3 Thermostability of the Proteases

Table 3.1 shows the thermostability of the proteases at 85°C and 95°C. These results show that both the native and cloned forms of EA1 protease are significantly more thermostable than YP-T protease.

	Half Life at 85°C		Half Life at 95°C
Protease	5 mM Ca^{2+}	10 mM Ca^{2+}	5 mM Ca^{2+}
Native EA1	60 min	115 min	9.8 min
Cloned EA1	45 min	70 min	9.8 min
Native YP-T	21 min	45 min	6.5 min
Cloned YP-T	21 min	33 min	6.5 min

Table 3.1 Thermostability of the Native and Cloned Proteases at 85°C and 95°C.

The proteases were incubated at the above temperatures for the appropriate time lengths, cooled on ice, then assayed for remaining activity with 0.2% (w/v) azocasein in 50 mM Hepes/NaOH containing 5 mM $CaCl_2$ at pH 5.5-8.0 for 30 minutes at 75°C.

At 95°C, the half lives for EA1 and YP-T were 9.8 and 6.5 minutes respectively. The first order plots are close to linear, suggesting that thermal denaturation is the major cause of loss of activity (Figure 3.7). This is supported by the curved second order plots, indicating that autolysis does not play a significant role at 95°C.

At 85°C, in the presence of 5 mM $CaCl_2$, the half lives are 1 hour, 45 and 21 minutes for native EA1, cloned EA1 and the YP-T native and cloned proteases respectively (Table 3.1). As with thermostability at 95°C, the first order plots with 5 mM and 10 mM are essentially linear, although some autolysis is significant at 5 mM, suggesting that denaturation is most significant at 85°C. The thermostability of cloned EA1 is lower than the native EA1 at 85°C and this is confirmed by the results with 10 mM Ca^{2+}.

It is possible that the cloned enzymes have not been folded exactly the same in *E. coli* than in the native *Bacillus* species. This could explain the differences in thermostability between the cloned and native forms of the same enzyme.

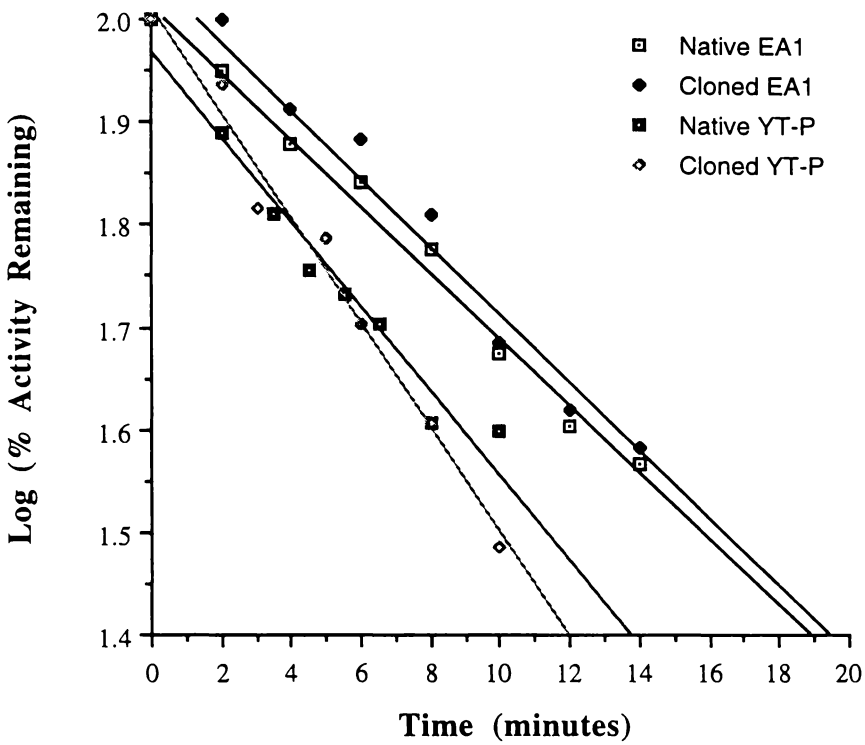


Figure 3.7 First-Order Plots of Native and Cloned YP-T and EA1 Proteases at 95°C.

The proteinases were incubated in 10 mM Hepes/NaOH pH 6.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100 at 95°C. Samples were withdrawn at various intervals, cooled, and assayed for remaining activity with 0.2% (w/v) azocasein at 75°C.

Figure 3.8 shows the first-order plots of cloned and native EA1 at 85°C in the presence of 5 and 10 mM $CaCl_2$. Increasing the $CaCl_2$ concentration from 5 to 10 mM has approximately doubled the thermostability of all the proteases, the highest being native EA1 protease with a thermostability of 115 minutes at 85°C. This is approximately the same half life reported by Coolbear *et al* (1991) under the same conditions with the purified protease (120 minutes). This shows that Ca^{2+} has a significant stabilising effect on the proteases, and suggests that K_S may be >5 mM Ca^{2+} for Ca^{2+} binding of EA1 protease implicated in thermal stability.

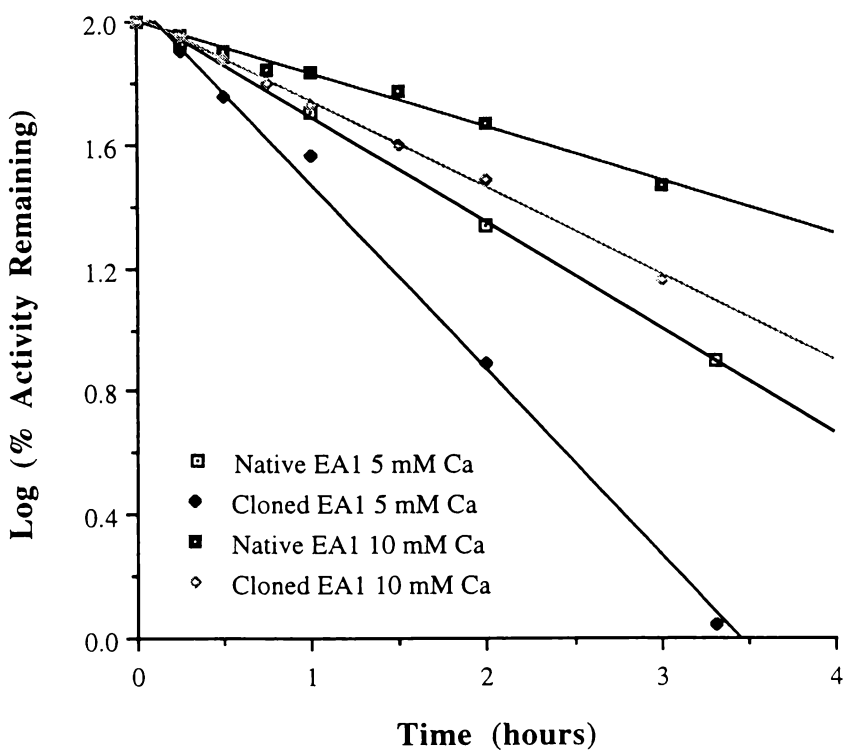


Figure 3.8 First-Order Plots of Native and Cloned EA1 Npr's at 85°C in the Presence of 5 and 10 mM $CaCl_2$.

The proteinases were incubated in 10 mM Hepes/NaOH pH 6.5 with 5-10 mM $CaCl_2$ and 0.01% (v/v) Triton X-100 at 95°C. Samples were withdrawn at various intervals, cooled, and assayed for remaining activity with 0.2% (w/v) azocasein at 75°C.

Looking more closely at the native EA1 and YP-T proteases, their half lives were determined at 85°C in the presence of 0-10 mM $CaCl_2$. Figure 3.9 shows the half lives of the proteases at 85°C as a function of the calcium concentration. This shows that the relationship between thermostability and calcium concentration is linear. This suggests that the proteases have a very low affinity for Ca^{2+} ions, as the half lives are still increasing at 10 mM Ca^{2+} ions.

Figure 3.10 shows the relationship between the log K_{obs} (log autolysis rate) with respect to the Ca^{2+} concentration (expressed as pCa). The dotted line for some of the YP-T protease results reflects the high errors in the autolysis rate determined due to the low thermostability of this protease under these conditions (<1 minute).

Table 3.2 shows the relationship between whether autolysis and/or denaturation are the most significant cause of loss of activity at 85°C with respect to the Ca^{2+} concentration. These results show that at low Ca^{2+} concentrations, with both proteases, autolysis is the most significant cause of loss of activity at 85°C. At 5 mM Ca^{2+} , significant denaturation is occuring, while at 10 mM Ca^{2+}, the major cause of loss of activity at 85°C is thermal denaturation. This shows that with both proteases, Ca^{2+} is stabilising by the prevention of autolysis. It also shows that the transition from autolysis to denaturation with both proteases occurs at *approximately* the same Ca^{2+} concentration, though this transition appears to be slightly earlier for YP-T protease as seen by the first and second order plots (see Appendix 3).

The first order plots (Appendix 3) show that at Ca^{2+} concentrations up to 1 mM, the denaturation rate is approximately the same. At higher concentrations, where denaturation is becoming the more significant cause of loss of activity (see Table 3.2), the denaturation rate decreases with increasing Ca^{2+} concentration. At lower Ca^{2+} concentrations (< 1 mM), Ca^{2+} appears to be stabilising the enzyme by the prevention of autolysis, and has little effect on the rate of denaturation. At higher Ca^{2+} concentrations, where denaturation is most significant, Ca^{2+} appears to be stabilising the enzyme by the prevention of thermal denaturation. This suggests that there is more than one calcium binding site.

Thus, Ca^{2+} appears to be having the same effect on the thermostability of the proteases, though the proteases have different thermostabilities. From these

results, it is proposed that the difference of 1 amino acid in the sequences of the two proteases could be in a region important for Ca^{2+}-induced thermal stability.

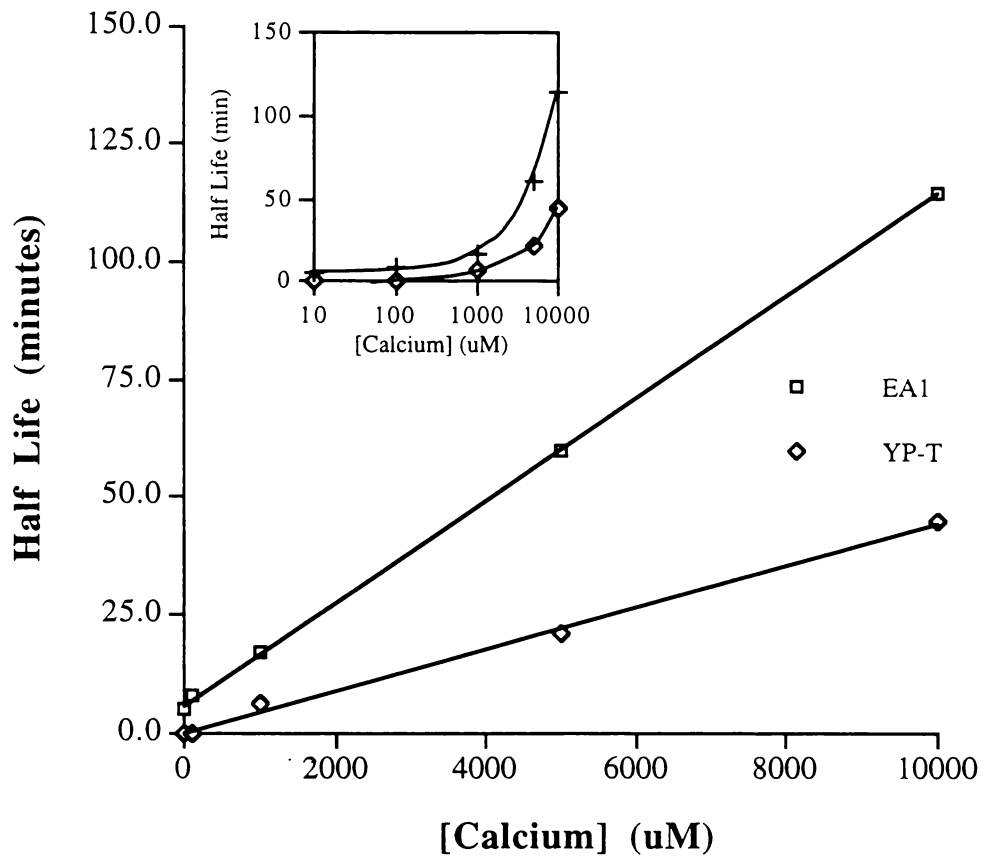


Figure 3.9 Half Life of Native EA1 and YP-T Proteases Vs Ca^{2+} Concentration at 85°C.

The native proteases were pretreated with o-phenanthroline and EDTA to remove metal ions, then extensively ultrafiltered (YM-1) to remove the metal-reagents. The proteases were then incubated with 0-10 mM Ca^{2+} ions for the appropriate time periods, cooled on ice, then assayed for remaining activity with 0.2% azocasein at 75°C (section 3.2.6). The inset is the same data, with the Ca^{2+} concentrations expressed in a log scale.

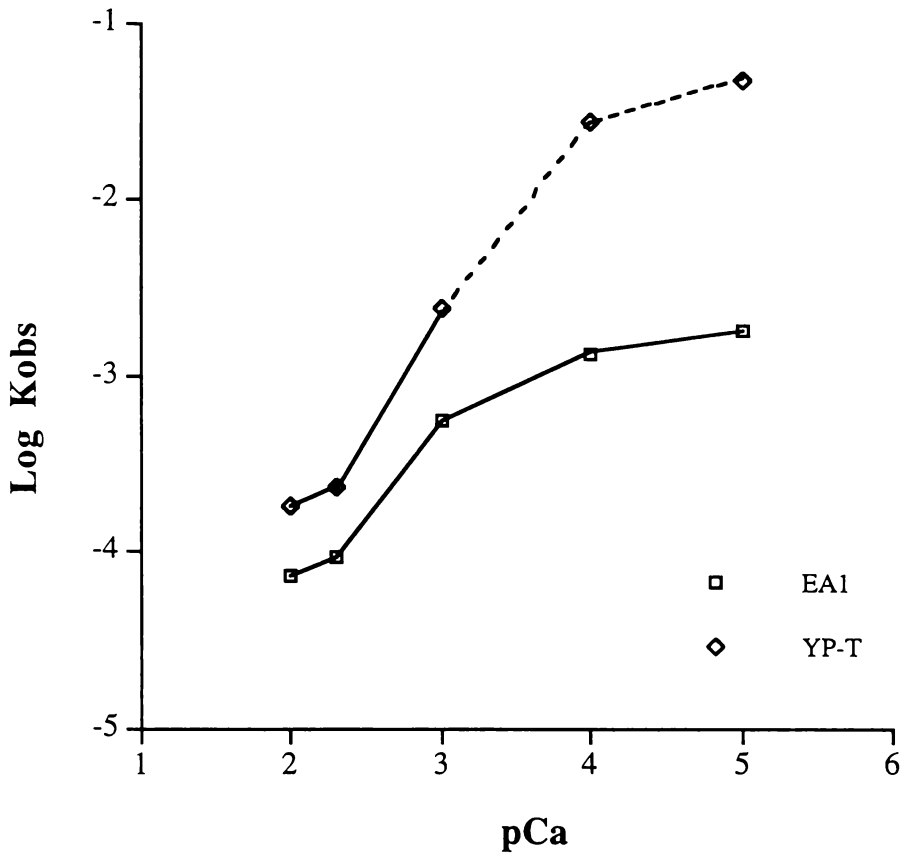


Figure 3.10 Log K_{obs} Vs pCa for Native EA1 and YP-T Proteases.

The native proteases were pretreated with o-phenanthroline and EDTA to remove metal ions, then extensively ultrafiltered (YM-1) to remove the metal-reagents. The proteases were then incubated with 0-10 mM Ca^{2+} ions for the appropriate time periods, cooled on ice, then assayed for remaining activity with 0.2% azocasein at 75°C (section 3.2.6).

$[Ca^{2+}]$ μM	Denaturation	Autolysis
EA1 Protease		
0		+
10		+
100		+
1000		+
5000	+	+
10000	+	
YP-T Protease		
0		+
10		+
100		+
1000		+
5000	+	+
10000	+	

Table 3.2 Autolysis or Denaturation as the Major Cause of Loss of Activity of EA1 and YP-T Proteases at 85°C in the Presence of 0-10 mM Ca^{2+} ions.

The native proteases were pretreated with o-phenanthroline and EDTA to remove metal ions, then extensively ultrafiltered (YM-1) to remove the metal-reagents. The proteases were then incubated with 0-10 mM Ca2+ ions for the appropriate time periods, cooled on ice, then assayed for remaining activity with 0.2% azocasein at 75°C (section 3.2.6). Significant autolysis and/or denaturation was determined by looking for linearity in the second and first order plots respectively (see Appendix 3 for the plots). In the cases when the plots were either both linear, or both non-linear, it was assumed that both causes were significant at that calcium concentration.

3.3.4 Molecular Modelling of the Proteases

Ca^{2+} stabilisation is a common feature among thermostable metallo-proteases. Thermolysin binds 4 Ca^{2+} ions which are essential for protection against both thermal denaturation and autolysis (e.g. Fontana, 1988). As both EA1 and YP-T proteases belong to the thermolysin-like family of proteases, stabilisation by Ca^{2+} is not suprising. Matthews *et al.* (1974) lists the residues involved in the binding of calcium. As EA1 (and YP-T) show a high sequence homology in the calcium binding regions of thermolysin, it is likely that they bind calcium in a similar fashion to thermolysin.

A sequence comparison between EA1, YP-T and thermolysin (Saul *et al.*, 1996) shows that the amino acid difference between EA1 and YP-T occurs within the region of the 3rd calcium binding site (Val/Gly 61 of EA1/YP-T corresponds to Ala 58 of thermolysin). Calcium 3 is important in the stability of thermolysin by minimizing the conformational change in the region around residue 57, thus preventing thermal denaturation and therefore autolysis (Dahlquist *et al.*, 1976). It has the second highest binding constant of the four calciums, and is chelated by a surface loop (e.g. Matthews *et al.*, 1974, Fontana, 1988). Therefore, the amino acid difference between EA1 and YP-T is within a region important for Ca^{2+} binding (and thermostability).

Matthews *et al.* (1974) showed that the residues involved in the binding of calcium 3 in thermolysin were Asp 57, Asp 59 and Glu 61. Ala 58 (Val/Gly 61 in EA1/YP-T) appears to play no direct role in the binding of calcium (Matthews *et al.*, 1974). Thus, the stabilisation caused by the substitution of Val for Gly at position 61 seems to be due to an interaction other than directly binding to the Ca^{2+} ion. Figure 3.11 shows a comparison of the structures of the amino acids valine, alanine and glycine. Val is a more hydrophobic amino acid than Gly as Val contains an isopropyl side chain, as opposed to the single H atom "side chain" of Gly. This suggests that perhaps the increased thermostability of EA1 over YP-T protease is due to extra hydrophobic interaction/s within the calcium 3 binding region.

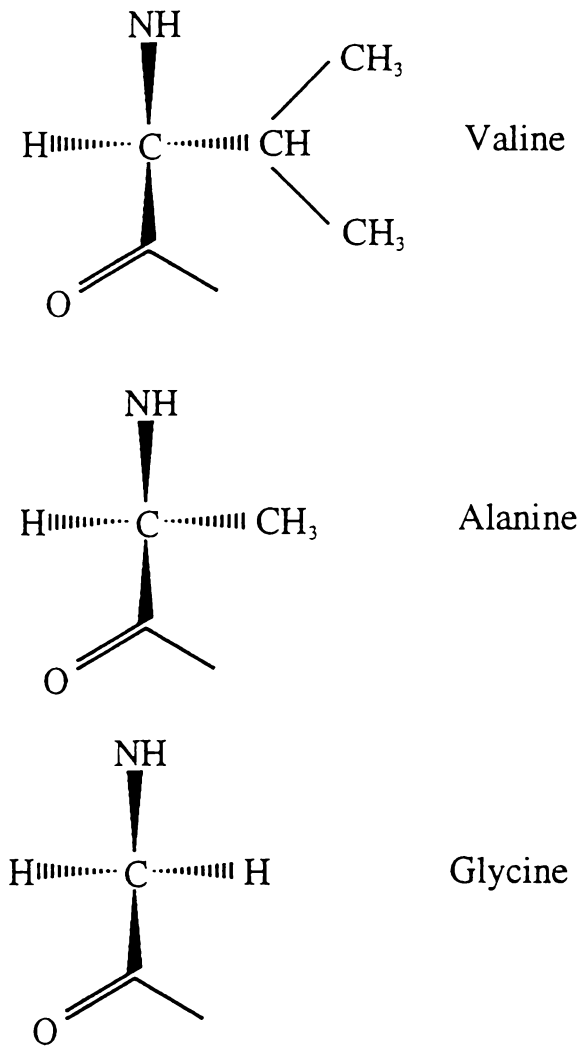


Figure 3.11 Structures of the Amino Acids Valine, Alanine and Glycine.

Figure 3.12 is the structure of the third calcium binding site of thermolysin. It shows that the methyl group of Ala 58 (analogous to Val61/Gly61 in EA1 and YP-T proteases) appears to be parallel to the benzene ring of Tyr 27, suggesting a possible interaction may be occuring. Figures 3.13-3.14 are the proposed analogous calcium binding sites of YP-T and EA1 proteases respectively, based on the structure of thermolysin (thermolysin numbering). The EA1 figure is in a slightly different orientation. YP-T protease contains glycine in the place of alanine in thermolysin. The 'side chain' of glycine is only hydrogen, so there is unlikely to be any interaction of it with Tyr 27.

EA1 protease contains valine in the place of alanine. Its side group is an isopropyl group. After minimization, the structure obtained suggests that the isopropyl group of Val 61 may be interacting with the aromatic side group of Tyr 27 (based on the structure of thermolysin). Such an interaction could increase the hydrophobic interactions within the calcium binding site. Therefore it is proposed that the amino acid difference between EA1 and YP-T proteases may be causing an extra hydrophobic interaction within a calcium binding region. This may increase the interactions between the amino acids in the 3rd calcium binding site, possibly changing the shape of the binding site and increasing the binding of Ca^{2+} this region. This may be the cause of the thermostability differences between the two proteases.

However, there are an extra 3 amino acids in EA1 and YP-T (Gly 29 - Tyr 30 - Tyr 31) which are situated close to residue 61. As no 3-dimensional studies have been conducted on these proteases, the structure of the calcium 3 binding region is difficult to predict. Thus while the extra stabilising interaction/s in EA1 appear to be due to increased hydrophobicity, the actual interaction/s are not known.

Therefore, it appears that the difference in the amino acid sequences appears to be important in the thermal stability of the proteases, by affecting the binding of Ca^{2+} at the proposed site 3 (thermolysin numbering). It is possible that either the binding constants are significantly different, or no Ca^{2+} ion binds to this site in YP-T protease. As *quantitative* data on the number of Ca^{2+} ions that bind to EA1 and YP-T proteases is not available, we can only propose that there is a binding site here in these proteases. This is not unreasonable due to the high sequence homology of the three proteases in these regions.

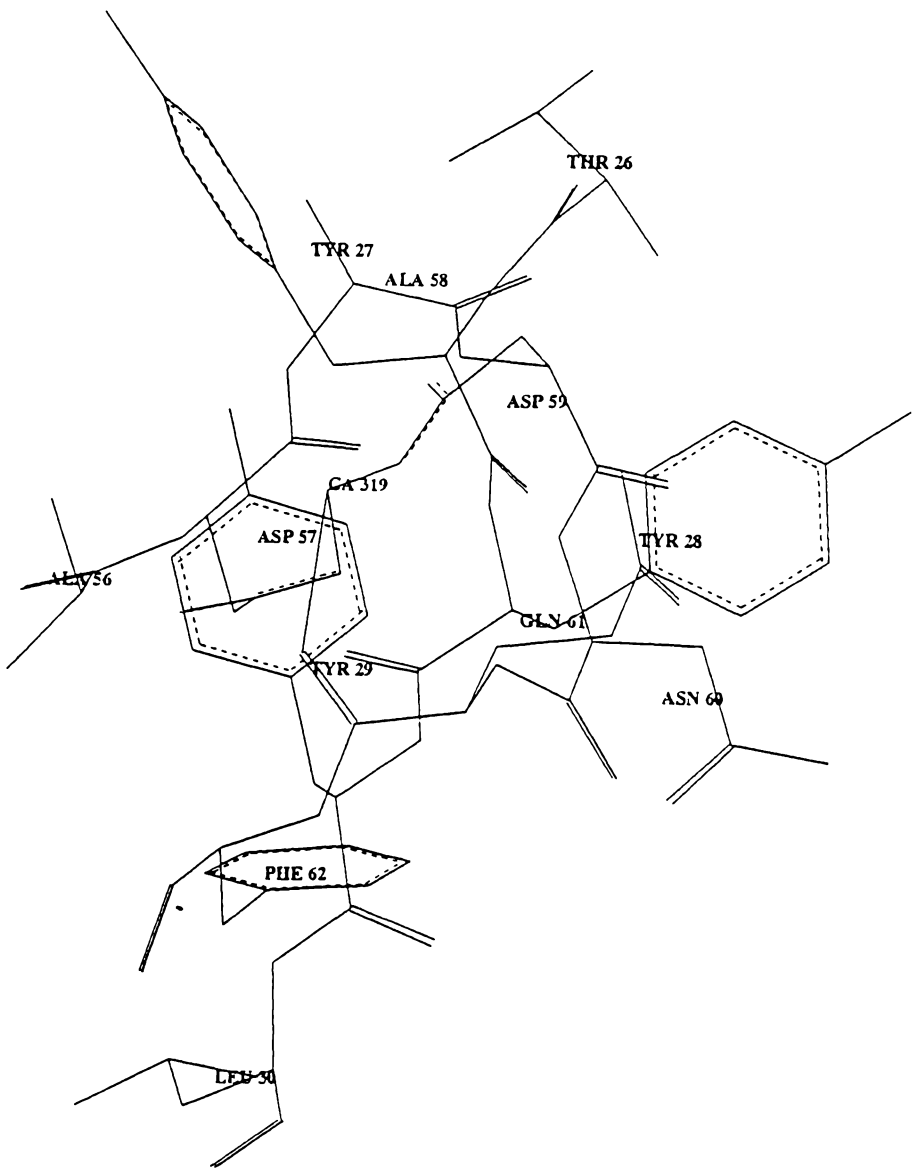


Figure 3.12 Structure of the Third Calcium Binding Site of Thermolysin.
The structure of thermolysin was obtained from the Brookhaven Protein Database.

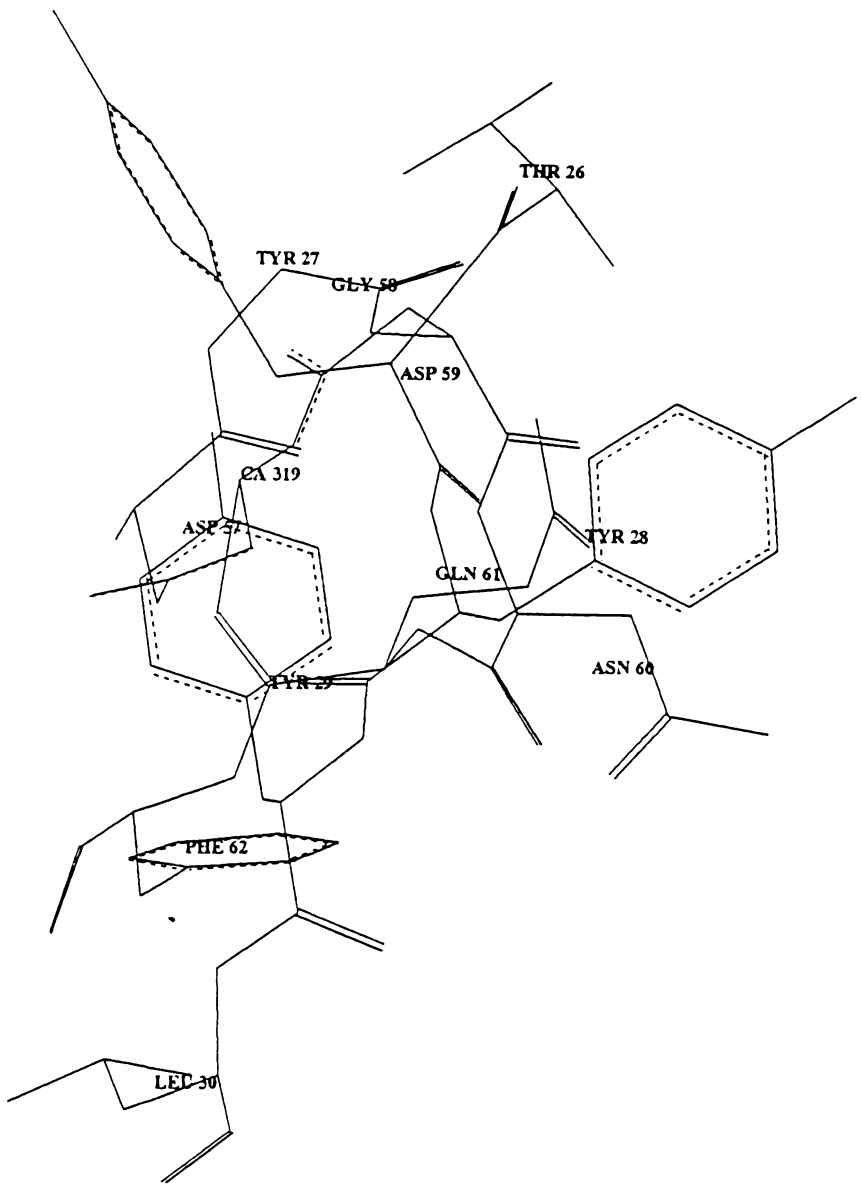


Figure 3.13 Proposed Structure of a Calcium Binding Site of YP-T Protease Based on Thermolysin.

The structure of thermolysin was obtained from the Brookhaven Protein Database. Molecular modelling studies of the EA1 and YP-T proteases were based on this structure.

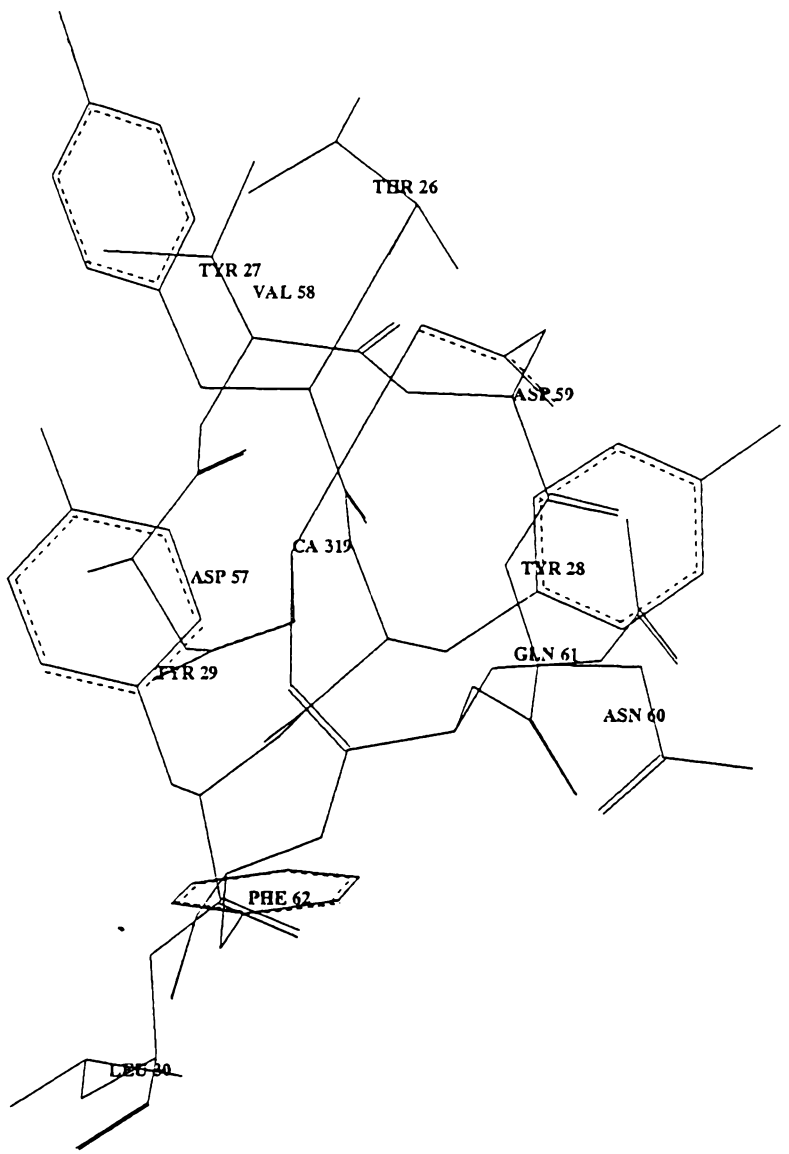


Figure 3.14 Proposed Structure of a Calcium Binding Site of EA1 Protease Based on Thermolysin.

The structure of thermolysin was obtained from the Brookhaven Protein Database. Molecular modelling studies of the EA1 and YP-T proteases were based on this structure.

3.4 CONCLUSIONS

A comparison of the pH optima of the native and cloned forms of EA1 and YP-T proteases shows that they all have a pH optimum of 6.5 at 75°C with azocasein. Thus the difference in amino acid sequences between the proteases has no effect on their pH optimum.

In contrast, there is a significant difference between the thermostability of the two proteases. EA1 protease has a higher thermostability than YP-T protease, and this is dependent on the Ca^{2+} concentration (Figure 3.9). An analysis of the half lives of the two proteases at 85°C at different Ca^{2+} concentrations shows that for both of them at low Ca^{2+} levels, autolysis is most significant, while at higher Ca^{2+} levels (> 5mM) denaturation is most significant.

It appears that the difference in the amino acid sequence of the two proteases appears to be in a region important for Ca^{2+} binding (and therefore thermostability). This is consistant with the observation that the amino acid difference between the two proteases is at the third Ca^{2+} binding site (based on the known structure of thermolysin). The residues are not directly involved in binding to the Ca^{2+}, but are located within this pocket.

Molecular modelling studies of EA1 and YP-T proteases, based on the structure of thermolysin, propose that the stabilisation of EA1 over YP-T protease could be due to extra interaction/s of the side chain of Val 61 of EA1 protease with the benzene ring of Tyr 27. YP-T protease has only Gly in this position. As the "side chain" of Gly is only a hydrogen, there is unlikely to be any such interaction with Tyr 27. Thermolysin contain Ala at this site (R-group = $-CH_3$). This may increase the interactions between the amino acids in the 3rd calcium binding site, possibly changing the shape of the binding site and increasing the binding of Ca^{2+} this region. This may be the cause of the thermostability differences between the two proteases.

CHAPTER 4

PROPERTIES OF *BACILLUS* ST. AK.1

4.1 INTRODUCTION

Saul *et al.*, (1996) compared the 16S rRNA sequences of several species of *Bacillus*. Figure 4.1 shows a consensus phylogenetic tree constructed from this comparison.

16S rRNA sequence of *Bacillus* st. Ak.1 shows a remarkable homology with that of *Bacillus thermoglucosidasicus* (Saul *et al.*, 1996). The two sequences are virtually identical. This comparison also put *Bacillus* st. EA1 and *B. caldolyticus* st. YP-T in the bacilli rRNA group 5, which also contains other thermophilic bacilli such as *B. stearothermophilus* (Saul *et al.*, 1996).

Consequently, in order to attempt to classify *Bacillus* st. Ak.1, some preliminary characterisation studies were done. Some comparisons to *B. thermoglucosidasicus* were conducted to compare the similarities and differences of the organisms. However, the characteristics of *Bacillus* st. Ak.1 was studied more extensively.

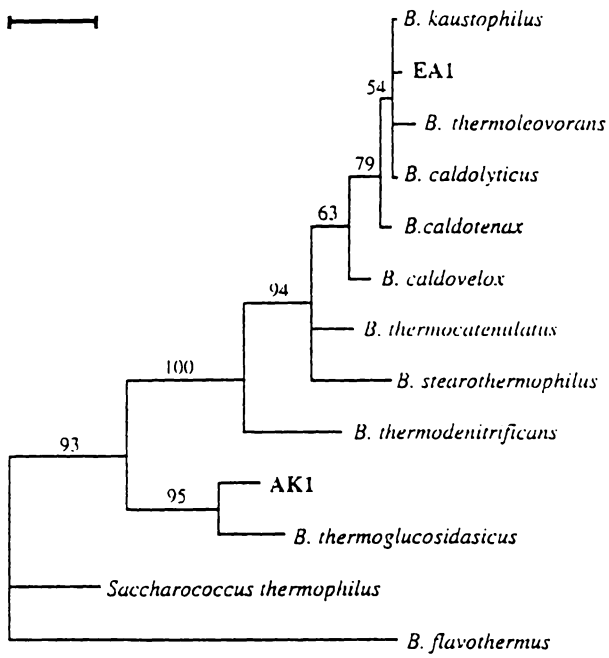


Figure 4.1 Consensus Phylogenetic Tree Constructed from 16S rRNA Sequences.

From Saul *et al* (1997)

4.2 MATERIALS AND METHODS

4.2.1 Organisms

Bacillus st. Ak.1 was isolated as a contaminant on a plate originally innoculated with *Bacillus* st. EA.1. It produces a thermostable serine protease (Peek *et al.*, 1993). It was cloned, sequenced and expressed into *Escherichia coli* by MacIver *et al.*, (1994).

Bacillus thermoglucosidasicus was described by Suzuki *et al.*, (1976). It was isolated from soil samples by enrichment at 65°C. It was designated strain KP 1006 (DSM 2542), the type strain. It was later officially named *Bacillus thermoglucosidasius* in the International Journal of Systematic Bacteriology **34** (2), 1984. It produces a thermostable extracellular α-glucosidase.

4.2.2 Growth on Rich Media

Innoculated side-arm flasks containing media at pH 7.0 with an active culture of *Bacillus* st. Ak.1. Incubated aerobically at 65°C overnight with shaking. To monitor growth, the absorbance at 650 nm was measured in the side-arm of the flask at various times during growth.

Media tested:
- Nutrient Broth (see 2.1.7.1)
- R Medium (see 2.1.7.2)
- CMD Broth (see 2.1.7.3)
- Tryptic Soy Broth/Agar (TSB/A) (see 2.1.7.4)
- *B. thermoglucosidasius*Medium (see 2.1.7.5)

To determine if anaerobic growth is possible, 100 µl of culture was added to belcho tubes containing 10 ml of liquid CMD agar supplemented with 0.001% methyl red (pH indicator). The suspension was sealed, mixed thoroughly, allowed to set, then incubated at 65°C overnight. Growth was detected by the presence of whitish colonies and/or a change in colour of the indicator. The colour was compared to an un-innoculated control. This experiment was repeated using anaerobic agar, with the headspace flushed with sterile oxygen after the suspension had set.

Universals containing media at pH 7.0 were innoculated with an active culture of *B. thermoglucosidasius*. The media tested were the same as for *Bacillus* st. Ak.1. They were incubated aerobically at 65°C overnight. Growth

was detected by visual inspection of the cultures and using a phase contrast microscope. Agar plates were also innoculated with a loopful of culture and incubated as before.

4.2.3 Growth on Minimal Media With a Single Carbon Source

Bacillus st. Ak.1 was grown on basal medium (see 2.1.1.6) containing 1 g/l of a variety of carbon sources. MOPS buffer was included in the medium to maintain the pH at pH 7.5 (the organism does not grow on MOPS buffer). 100 µl of an active culture was added to 10 ml universals containing basal medium and 1-10 g/l of a carbon source. The cultures were incubated overnight at 65°C. Growth was detected by measuring the absorbance at 650 nm for soluble carbon sources (e.g. glucose, trehalose), and by examination under a phase contrast microscope for insoluble carbon sources (e.g. starch, cellulose).

Initial innoculums were from CMD (rich) medium. Where growth was detected on a carbon source, transfers of that culture into fresh medium was incubated at 65°C as before to see if the growth was due to the carbon source tested or due to trace amounts of carbon sources in the initial innoculums. Where growth was not detected, or was very slight, growth was attempted again with a fresh innoculum from CMD medium.

The carbon sources found <u>not</u> to support growth were retested as before, except that all the media was supplemented with 0.1 g/l (sodium)glutamate. Growth was detected as before, and were compared to control universals that contained only 0.1 g/l glutamate as the carbon source.

B. thermoglucosidasius was grown on basal medium containing 1 g/l of a variety of carbon sources as above with *Bacillus* st. Ak.1. However, only a few of the carbon sources were tested.

4.2.4 Extracellular Enzyme Production

4.2.4.1 <u>Protease Activity</u>

Culture samples of the two organisms, grown on basal medium containing tryptone and yeast extract, were assayed for protease activity by the Casein agar plate method (see section 2.2.7). This method was chosen above the others as it is a non-specific protease assay, and activity can be detected by

direct observation. 100 µl samples of the cultures was added to wells of casein agar plates. The plates were then incubated at 65°C for at least 16 hours.

4.2.4.2 α-Glucosidase Activity

This assay is based on the method of Whitmore (1994). The organisms were grown on starch or maltose, with tryptone and yeast extract present.

Substrate: Dissolve 0.09 g of *p*-nitrophenol α-D-glucopyranoside in 10 mls of 50 mM HEPES/NaOH pH_{55} 7.0.
Stopping Reagent: 1M Na_2CO_3

Assay: Add 100µl of the substrate into a reaction vial. Add 800 µl of the above buffer and mix well. Preincubate at 55°C for 10 minutes. Add 100µl of the culture. Mix well. Incubate at 55°C until a yellow colour is visible. Stop the reaction by adding 500µl of the stopping reagent and mixing well. This also increases the degree of colour of the product (pNP). Read the absorbance at 400 nm. A blank is produced by adding the culture after the stopping reagent has been added.

4.2.4.3 β-Glucosidase Activity

This assay is based on the method of Whitmore (1994). The organisms were grown on cellulose or cellibiose.

Substrate: Dissolve 0.09 g of p-nitrophenol β-D-glucopyranoside in 10 mls of 50 mM HEPES/NaOH pH_{55} 7.0.
Stopping Reagent: 1M Na_2CO_3

Assay: Add 100µl of the substrate into a reaction vial. Add 800 µl of the above buffer and mix well. Preincubate at 55°C for 10 minutes. Add 100µl of the culture. Mix well. Incubate at 55°C until a yellow colour is visible. Stop the reaction by adding 500µl of the stopping reagent and mixing well. This also increases the colour of the product. Read the absorbance at 400 nm. A blank is produced by adding the culture after the stopping reagent has been added.

4.2.5 RAPD-PCR Method

4.2.5.1 Sources and Growth of the Cultures

Bacillus strains EA1 and Ak.1 were obtained from our culture collection. The other *Bacillus* species were obtained from the Deutsche Sammlung von Mikroorganismen (DSM) or the American Type Culture Collection (ATCC). *Saccharococcus thermophilus* was obtained from ATCC. The organisms were plated onto tryptic soy agar (TSA) plates and incubated for 16 hours at the appropriate temperatures. Table 5.1 lists the organisms used, and their optimal growth temperatures.

No.	Organism	$Temp_{opt}$ (°C)
1	*Bacillus* st. Ak.1	65
2	*Bacillus thermoglucosidasius*	65
3	*Bacillus* st. EA1	65
4	*Bacillus caldolyticus* YP-T	65
5	*Bacillus stearothermophilus* ATCC 8005 (formerly *B. kaustophilus*)	55
6	*Bacillus thermoloevorans*	60
7	*Bacillus caldotenax*	70
8	*Bacillus thermocatenulatus*	60
9	*Bacillus thermodenitrificans*	60
10	*Bacillus licheniformis*	37
11	*Bacillus* sp. DSM 411 (formerly *B. caldovelox*)	70
12	*Saccharococcus thermophilus*	55

Table 4.1 Organisms Tested and their Growth Temperatures.
The information was obtained from DSM and ATCC Culture Collections.

4.2.5.2 DNA Isolation and Partial Purification

The short method of DNA preparation by Ronimus *et al* (1997) was used in these experiments. 4 ml of sterile 50 mM Tris/HCl pH 8.0 containing 100 mM NaCl and 20 mM EDTA was added onto a well grown agar plate. The buffer was allowed it to soak for 5 minutes. The growth was removed and placed into sterile 10 ml reaction vials. Centrifuged the suspension at 4000 X *g* (Runne Hettich Centrifuge) for 8 minutes. Discarded the supernatant. The cell pellet was stored at -20°C until further use. Add between 0.5-2.0 ml of sterile purified water., depending on the pellet size. Resuspend the cells with a vortex mixer. Punch a hole in the top of the tubes with a sterile, forceps. Boil for 10 minutes. Centrifuge (as before) for 10 minutes to pellet the cellular debree. Carefully remove the supernatant into sterile 1.5 ml reaction vials. Discard the pellet. Measure the quantity of DNA by reading the absorbance at 260 nm. Adjust the concentration of the DNA to an OD_{260nm} of 0.3 by adding the appropriate amount of sterile purified water. The DNA is stored at -20°C.

4.2.5.3 PCR Conditions

RAPD-PCR assays were performed in 25µl volumes. The 'master mix' of all the components was prepared by mixing together 75µl of 10x PCR buffer (100 mM Tris/HCl containing 500 mM KCl, 0.01% gelatin and 1.5 mM $MgCl_2$), 75µl of dNTP (Boehringer Mannheim), 75µl of 25 mM $MgCl_2$, 30 µl of the primer OPR-13 (GGACGACAAG; Operon Technologies), 412.6 µl of milli Q water and 7.5 µl of *Ampli*Taq (1.25 U; Perkin Elmer) in this order.

Dispense 22.5 µl of the master mix into each PCR tube using aerosol-resistant tips. Add 2 drops of mineral oil into each tube. Add 2.5 µl of the DNA samples to each tube, then centrifuge briefly. The PCR machine utilised was a water-cooled EriComp thermocycler. The conditions used were: an initial denaturation step at 94°C for 3 minutes and 45 seconds was followed by 35 cycles of 94°C for 15 seconds, 36°C for 15 seconds and 72°C for 2 minutes. This was followed by a final step at 72°C for 4 minutes. Each DNA sample was amplified in duplicate, to check for reproducibility, and included was a negative control (no template).

4.2.5.4 Agarose Gel Electrophoresis

The components of each sample after PCR (20 µl) were separated by electrophoresis on 1.5% agarose gels (SeaKem LE agarose) at 3.5 V/cm for a total of 350 Vh (see section 2.4.5). The gels were stained with 0.5 µg/ml ethidium bromide for 1 hour and destained for 1 hour as described in section 2.4.6.

4.3 RESULTS AND DISCUSSION

4.3.1 Growth of the Two Organisms on Rich Media

4.3.1.1 *Bacillus* st. Ak.1

1. *Morphology*

Non-motile rods, with swollen terminal spores (Figure 4.2).

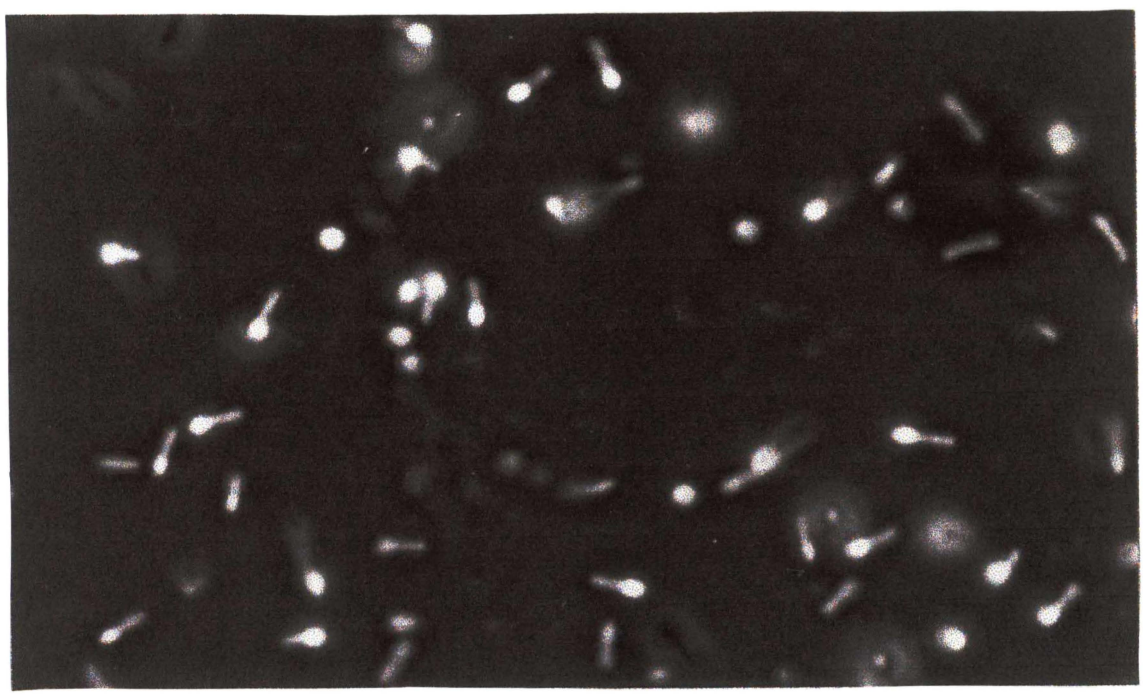


Figure 4.2 Morphology of *Bacillus* st. Ak.1 Under a Phase Contrast Microscope.
Bacillus st. Ak.1 was grown overnight in CMD medium (section 2.1.7.3) at 75°C

2. *Growth of Bacillus st. Ak.1*

The organism grew well on all the media tested. Best growth was observed on CMD medium. The organism also grew on nutrient agar plates. Figure 4.3 shows the growth profile of *Bacillus* st. Ak.1 on CMD medium.

Growth on tryptone and yeast extract resulted in an *increase* in the pH of the medium of about 1 Unit. This was seen as a change in the colour of the indicator dye from orange to red, and checked with a pH meter. This could be due to the production of ammonia via deamidation of amino acids.

Growth (colonies) were detected on the top of the agar, where oxygen was present. A slight pH change was detected within the depths of the agar as compared to the uninnoculated control indicating a slight growth, though no colonies or cloudiness were visible. No such colour change was detected in the anaerobic agar suggesting that the slight growth in the previous experiment was due to the presence of trace amounts of oxygen. Thus, the organism appears to have a requirement for oxygen.

4.3.1.2 *Bacillus thermoglucosidasius*

Morphology: Non-motile rods (like *Bacillus* st. Ak.1)

This organism proved to be more difficult to grow than *Bacillus* st. Ak.1. It often would not grow even in the rich media. It grew in the CMD broth, TSB and *B. thermoglucosidasius* media. The later medium contains starch. It prefers the later medium above the others, with CMD medium a close second. It did not grow well on TSB medium. It is difficult to get it to grow well on any of the agar plates.

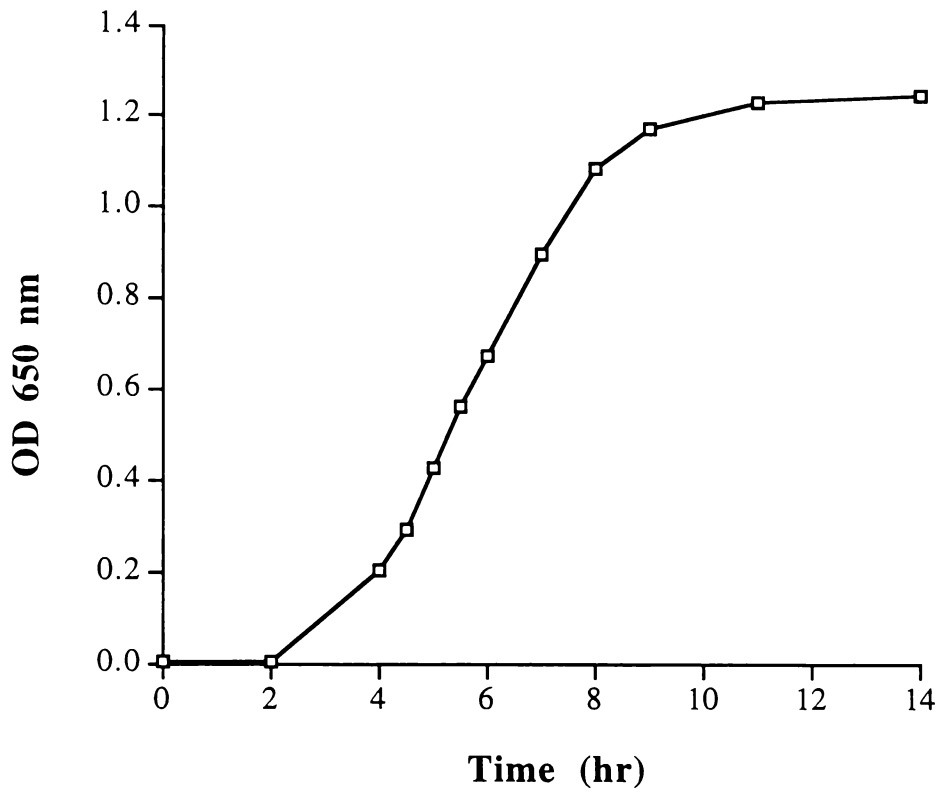


Figure 4.3 Growth Profile of *Bacillus* st. Ak.1 at 65°C in CMD Medium.
Bacillus st. Ak.1 was incubated in CMD medium at overnight at 65°C. Growth was monitored by measuring the OD 650 nm.

4.3.2 Carbon Sources Supporting Growth of the Two Organisms

4.3.2.1 *Bacillus* st. Ak.1

In the initial experiments, growth was detected only in the media containing either tryptone, yeast extract, casamino acids or glutamate as the carbon source/s. As glutamate is present in all of these carbon sources, this suggests that the organism has a requirement for glutamate. Thus, the carbon source experiments were retested in the presence of 0.1 g/l glutamate, the lowest quantity of glutamate required to support good growth. At this concentration, the 0.1g/l glutamate controls had either no growth or a slight growth (OD 650 nm < 0.05).

Table 4.2 shows the carbon sources tested, and whether they support growth or not. The degree of growth is indicated by the number of '+' in the column. Carbon sources that supported only slight growth, or grew only occasionally after repeated transfers were classified as +/-.

This table shows that in the presence of a minimal amount of glutamate, the organism will grow on a variety of carbon sources. It grows very well on monosaccharides such as glucose and sucrose. It grows less well on other compounds such as glycogen, arabinose and fumerate. Growth was variable on substances such as acetate and lactose. No growth was detected with compounds such as urea, methionine, ribitol and rhamnose. Suprisingly, while the organism had a requirement for the amino acid glutamate, it would not grow on glutamine, a similar amino acid. Figure 4.4 shows a comparison of the structures of glutamate (glutamic acid, sodium salt) and glutamine. The only difference between the structures is the presence of an additional amine group in the place of the carboxylic acid group in glutamate.

Growth		+/- Growth	No Growth
Yeast Extract	+++	Acetate	Methyl-D-galactopyranoside
Tryptone	+++	Isoleucine	Sorbose
Casamino acids	+++	Pullulan	Glutaric Acid
Glutamate	+++	Lactose	Citrate
Methyl-D-glucopyranoside	+++		Glyceraldehyde*
Trehalose	+++		Dihydroxyacetone*
Sucrose	+++		Glutamine
Maltitol	+++		Melezitose
Mannitol	+++		Urea
Salicin	+++		Asparagine
Fructose	+++		Methionine
Cellulose^	+++		D-Valine
Starch^	+++		Amygdalin
Glucose	++		Ribitol
Glycerol	++		Raffinose
Succinate	++		Rhamnose
Inositol	++		Glucuronic Acid
Maltose	++		
Ribose	++		
Melibiose	++		
Glycogen	++		
Erythritol	++		
Arabinose	++		
Fumerate	+		
Lactate	+		
Pyruvate	+		

Table 4.2 Growth of *Bacillus* st. Ak.1 on Different Carbon Sources.

Bacillus st. Ak.1 was grown on minimal medium containing 0.1 g/l glutamate and 1 g/l of the carbon sources listed overnight at 65°C. * Broke down during incubation. ^ 10 g/l carbon source

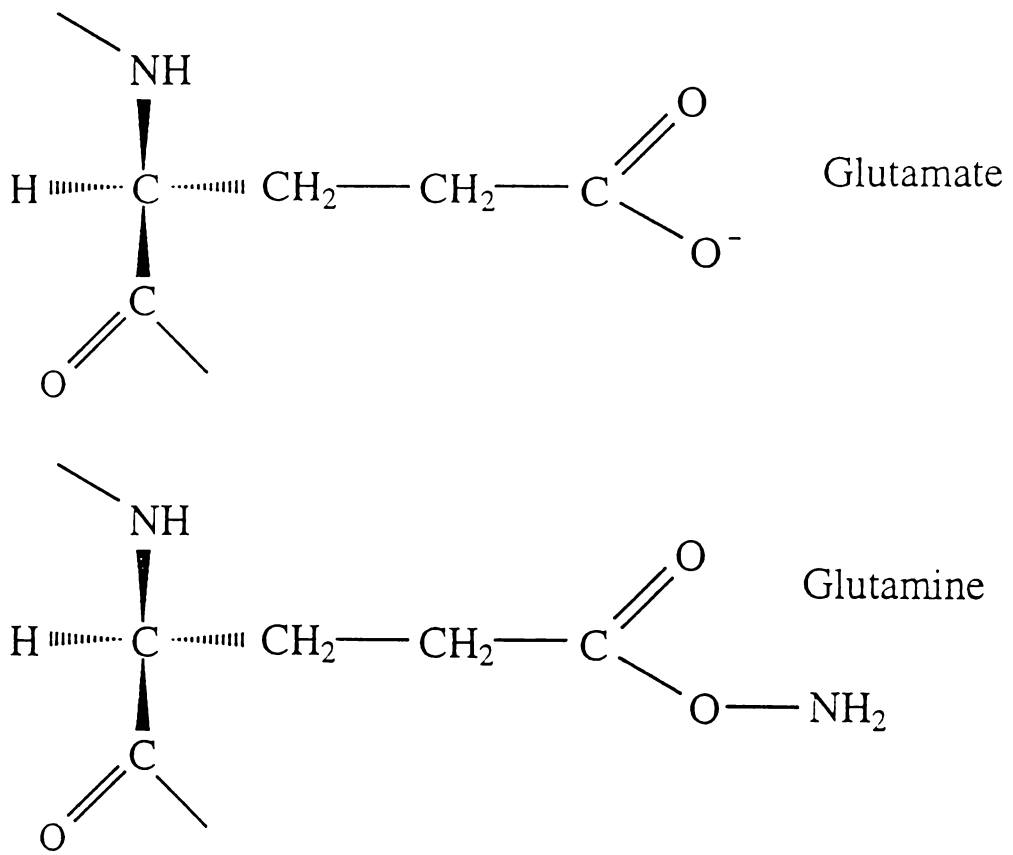


Figure 4.4 Structure of the Amino Acids Glutamate and Glutamine.

4.3.2.2 *Bacillus thermoglucosidasius*

Table 4.3 shows the results of the growth experiments. From this you can see that *B. thermoglucosidasius* also has a growth requirement found in tryptone and yeast extract, but unlike *Bacillus* st. Ak.1 this requirement is not met by glutamate. It grows well on starch and maltose, consistant with the knowledge that it has α-glucosidase activity. It was able to grow slightly on maltose as a sole carbon source.

Carbon Sources	Growth
None	-
Starch	-
Glutamate	-
Tryptone + Yeast Extract	++
Starch + Glutamate	-
Starch + Tryptone + Yeast Extract	+++
Maltose	+

Table 4.3 Growth of *B. thermoglucosidasius* on Various Carbon Sources.
Bacillus thermoglucosidasius was grown on minimal medium containing 1 g/l of the carbon sources listed overnight at 65°C.

4.3.3 Extracellular Enzyme Production

4.3.3.1 Protease Activity

Bacillus st. Ak.1 produced a large *p*-casein precipitate ring on casein agar after 16 hours of incubation, indicating the presence of a thermostable protease. *B. thermoglucosidasius* cultures, however, did not produce any *p*-casein precipitate, even after several days incubation at 55°C or 65°C. Thus, no extracellular protease activity has been detected.

4.3.3.2 α-Glucosidase Activity

Both of the organisms exhibited significant α-glucosidase activity. This is not suprising as both organisms grow on starch and maltose as carbon sources.

4.3.3.3 β-Glucosidase Activity

Bacillus st. Ak.1 exhibited no β-glucosidase activity, in spite of it growing very well on cellulose and cellibiose. In contrast, *B. thermoglucosidasius* exhibited very high β-glucosidase activity.

4.3.3.4 Conclusions

Table 4.4 is a summary of the extracellular enzyme activities. It shows that apart from both organisms containing α-glucosidase activity, the organisms have some differences in their extracellular enzyme production. However, it is possible that where no activity was seen, the conditions may not have been optimal for activity to be detected. However, these differences in activity do show an interesting difference between the two organisms.

Organism	Protease	α-Glucosidase	β-Glucosidase
Bacillus st. Ak.1	+++	++	-
Bacillus thermoglucosidasius	-	+++	+++

Table 4.4 Extracellular Enzyme Activities.
Culture samples were assayed for extracellular enzyme activity at 65°C according to the methods in section 4.2.4.

4.3.4 Comparison of Phenotypic Characteristics of *Bacillus* st. Ak.1 and *Bacillus thermoglucosidasius*

Table 4.5 shows a comparison of the carbon sources that support growth of *Bacillus* st. Ak.1 and *Bacillus thermoglucosidasius*. This shows that these organisms have different growth requirements. Both organisms have a requirement for a carbon source contained in yeast extract and/or tryptone. Glutamate is required for *Bacillus* st. Ak.1, but not for *B. thermoglucosidasius*. *Bacillus* st. Ak.1 can grow on a wider range of carbon sources.

	Growth	
Carbon Source/s	*B.* st. Ak.1	*B. thermogluc.*
None	-	-
Starch	+++	-
Glutamate	+++	-
Starch + Glutamate	+++	-
Tryptone + Yeast Extract	+++	++
Starch + Tryptone + Yeast Extract	+++	+++
Maltose	++	+

Table 4.5 Comparison of the Growth of *Bacillus* st. Ak.1 and *B. thermoglucosidasius* on Different Carbon Sources.

Bacillus st. Ak.1 and *Bacillus thermoglucosidasius* were grown on minimal medium containing 0.1 g/l glutamate and 1 g/l of the carbon sources listed overnight at 65°C.

4.3.5 Analysis of 12 Organisms by RAPD-PCR

RAPD-PCR (Random Amplified Polymorphic DNA - Polymerase Chain Reaction) is a method of DNA fingerprinting. It was first used in 1990 by Williams *et al* (1990). It has been used to identify pathogenic bacterial strains, and for the generation of fingerprints of nonclinical prokaryotic, fungal and plant species (Welsh & McClelland (1990); Williams *et al* (1993)). Recently, Ronimus *et al* (1997) optimised the conditions of RAPD-PCR for the identification of a variety of *Bacillus* species. Due to the close grouping of *Bacillus* st. Ak.1, *B. thermoglucosidasius* and some other organisms by 16S rRNA sequence analysis seen in Figure 4.1, most of these organisms were compared by RAPD-PCR.

Figure 4.5 is a photograph of the agarose gel containing the DNA bands of the twelve organisms after PCR. These results show that all the 12 organisms have unique banding patterns. They all contain a large number of bands, with many of them in common. *Bacillus* st. Ak.1 is suprisingly very different from all the other banding patterns, even that of *B. thermoglucosidasius*. Thus, RAPD-PCR is a useful technique for the identification of closely related organisms, as organisms that are closely grouped by 16SrRNA analysis exhibit different banding patterns with RAPD-PCR.

Bacillus st. EA1 and *B. caldolyticus* YP-T have a very similar banding pattern. This agrees with the 16s rRNA analysis of the two organisms (Saul *et al.*, 1996). Also, as mentioned in Chapter 3, the two organisms each produce an extracellular thermostable metallo-protease which differ in the mature enzyme sequence by only one amino acid.

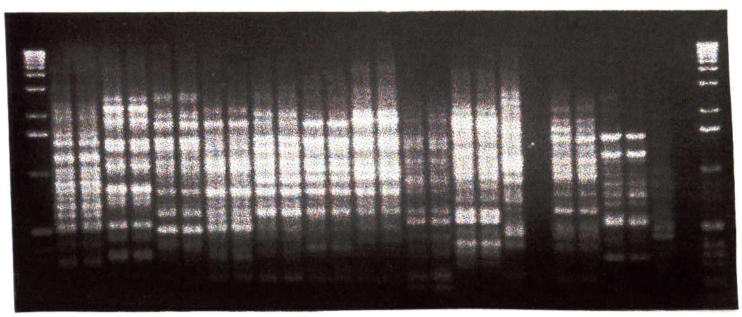


Figure 4.5 Agarose Gel Electrophoresis of PCR Products of 12 Organisms.
PCR products of the 12 organisms were separated by agarose gel electrophoresis, and the bands were visualised by UV light. Lane 1 = molecular weight ladder; lanes 2-3 = *Bacillus* st. Ak.1; lanes 4-5 = *B. thermoglucosidasius*; lanes 6-7 = *Bacillus* st. EA1; lanes 8-9 = *B..caldolyticus*; lanes 10-11 = *B. kaustophilus*; lanes 12-13 = *B. thermoloevorans*; lanes 14-15 = *B. caldotenax*; lanes 16-17 = *B. thermocatenulatus*; lanes 18-19 = *B. thermodenitrificans*; lanes 20-21 = *B. licheniformis*; lanes 22-23 = *B. caldovelox*; lanes 24-25 = *Saccharococcus thermophilus*; lane 26 = negative control; lane 27 = blank; lane 28 = molecular weight ladder.

4.4 CONCLUSIONS

The 16S rRNA analysis (Saul *et al.*, 1996) closely groups *Bacillus* st. Ak.1 and *B. thermoglucosidasius*. Their sequences are virtually identical. However, the results presented here show that the two organisms exhibit significant phenotypic and genotypic differences.

The two organisms have different growth requirements. *Bacillus* st. Ak.1 has a requirement for glutamate, while *B. thermoglucosidasius* has a requirement for a compound found in yeast extract and tryptone, but not glutamate (possibly maltose). RAPD-PCR analysis of twelve organisms show that the banding patterns of the organisms are all unique, though the closest two were between *Bacillus* st. EA1 and *B. caldolyticus*.

CHAPTER 5

PRODUCTION AND PURIFICATION OF AK.1 PROTEASE FROM *ESCHERICHIA COLI* CLONE PB5517

5.1 INTRODUCTION

Ak.1 protease is a thermostable serine protease originally isolated from *Bacillus* st. Ak.1 and partially characterised by Peek *et al.* (1993). It was cloned, sequenced and expressed into *Escherichia coli* by Macivor *et al.* (1994). The protease belongs to the subtilisin-like family of serine proteases. It has a 68% sequence similarity to thermitase, a thermostable subtilisin from *Thermoactinomyces vulgaris*.

5.2 MATERIALS AND METHODS

5.2.1 Expression Method for *Escherichia coli* clone PB5517

5.2.1.1 Clone Information

E. coli strain DH5α (see 4.2.1) was used as the host strain. The protease gene was inserted into the plasmid pGEMSZf(+). This strain was then named clone PB5517. This work was carried out by Macivor *et al.*, 1994.

5.2.1.2 Growth of *Escherichia coli* Clone PB5517

To determine the growth profile of *E. coli* clone PB5517, a 750 ml volume of Bulk Growth Medium 2 (see 2.1.2.2) was innoculated with an uninduced overnight culture of the clone and was incubated at 37°C until the A_{600}nm was approximately 0.2 (about 3 hours). The culture was then induced with 1 mM isopropyl β-thiogalactopyranoside (IPTG) (final concentration), and the culture was incubated for a further 10 hours at 37°C. Samples were withdrawn periodically and the A_{600}nm and activity (after heat-treating for 2 hours at 70°C

to activate the protease) of each was determined. Morphology was determined by an inspection of several drops of culture under a phase contrast microscope, and by carrying out Gram (see 2.5.1) and Spore (see section 2.5.2) stains.

To determine if IPTG or lactose is required to induce expression of the protease, three sets of cultures were set up. All three media samples were innoculated with an uninduced culture, and incubated for several hours at 37°C until the culture was in the log phase. Culture 1 is the non-induced control. Cultures 2 and 3 were then given 1 mM final concentration of IPTG and lactose respectively and incubated for a further 2 hours at 37°C. Morphology of each culture was determined by an inspection of several drops of culture under a phase contrast microscope. Following this, 10 µl samples of each culture was added to a casein agar plate and the plates were incubated at 70°C overnight to determine which samples contained thermophilic protease activity (see section 2.2.2).

5.2.2 Production and Purification of Ak.1 Protease

5.2.2.1 Fermentor Run of *E. coli* Clone PB5517

E. coli clone PB5517 was grown aerobically in a 10 l LH Series Fermentor on Bulk Growth Medium 2 (see section 2.1.2.2) in a fed-batch manner at 35°C. Extra nutrients were added during a later stage when the OD 600 nm started to decrease to increase growth and, therefore, protease production. Growth was continued until cell density began to drop again (approximately 17 hours).

5.2.2.2 Extraction and Heat-Treatment of Ak.1 Protease

The culture was harvested and concentrated on an Amicon Hollow Fibre Cartridge (S1OY10). The resultant cell slurry was centrifuged at 10,000 rpm (Beckman JA-10) for 20 minutes and the pellet was discarded. The supernatant was retained (3 l) and was incubated with 0.01g lysozyme and 0.001g DNAase at room temperature overnight to clarify it.

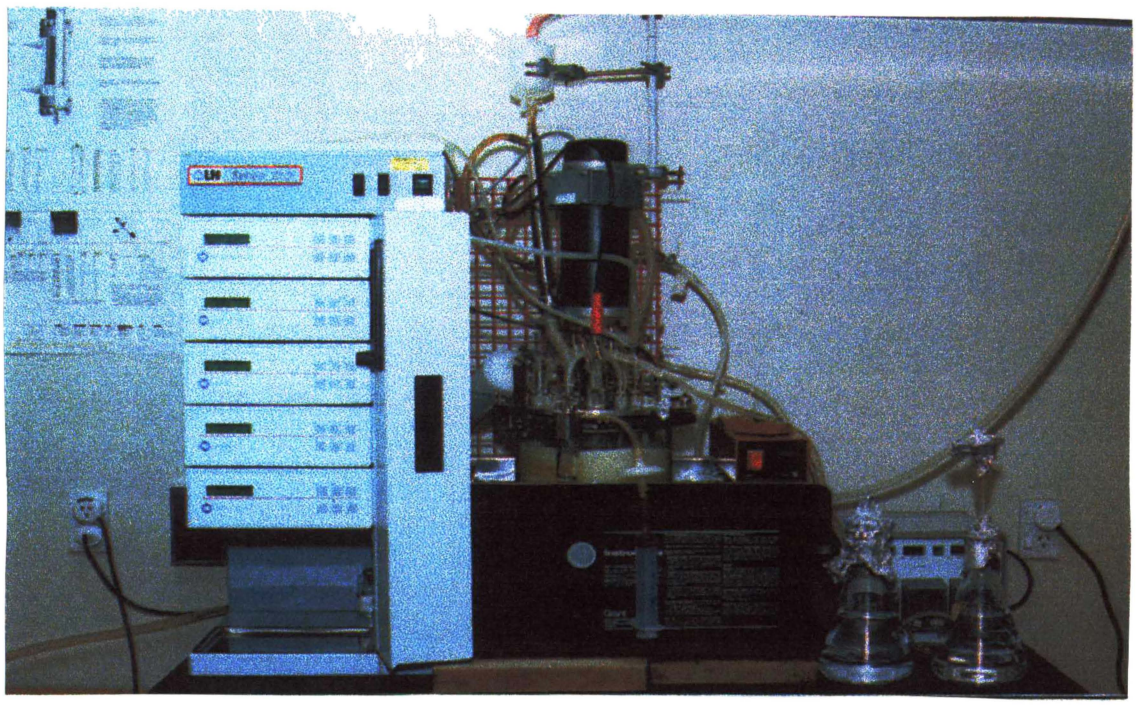


Figure 5.1 LH Series Fermentor Setup

The supernatant was heat-treated at 70°C for 15 minutes to precipitate the majority of the *E. coli* proteins. After heating, the mixture was cooled to 4°C, centrifuged as before and the pellet discarded. The supernatant (2.9 l) was heated-treated for a further 2 hours at 70°C to activate the protease. This solution was concentrated on an Amicon YM-10 Ultrafiltration membrane (1.6 l), and equilibrated to 10 mM Tes/NaOH pH 7.0 containing 5 mM $CaCl_2$ and 1 M NaCl.

5.2.2.3 Purification of Ak.1 Protease

The concentrated extract (1600ml) was applied to a 510 ml phenyl Sepharose (Pharmacia) column pre-equilibrated in the same buffer. The column was washed with equilibration buffer, followed by NaCl-free equilibration buffer, distilled water then a gradient of 0-50% (v/v) ethanediol to elute the protease. The active fractions were pooled (452 ml) and diafiltered to a volume of 27 ml and a final buffer composition of 10 mM diethanolamine/H_2SO_4 pH 8.5 containing 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100.

The solution was applied to a Mono Q 5/5 (Pharmacia) column, pre-equilibrated in the above diethanolamine buffer at a flow rate of 1.5 ml/min. The protease was eluted with a 120 ml linear gradient of 0-0.5 M NaCl. The active fractions were pooled and diafiltered to a volume of 42 ml and a final buffer composition of 20 mM Tes/NaOH pH 7.5 containing 5 mM $CaCl_2$ and 1 M NaCl.

The solution was applied to a 10 ml phenyl Sepharose (Pharmacia) column, pre-equilibrated in the above Tes buffer at a flow rate of 3 ml/min. The column was washed with NaCl-free Tes buffer, followed by a 240 ml linear gradient of 0-0.15% (w/v) of each of phenylalanine, histidine and alanine in the above buffer. The column was washed with 60 ml of the amino acid-containing buffer at the top of the gradient, then with the NaCl-free Tes buffer, and finally with the above buffer containing 50% (v/v) ethanediol. The active fractions were pooled (1124 ml) and diafiltered to a final volume of 94 ml and a buffer composition of 10 mM Tes/NaOH pH 7.5 containing 1 mM $CaCl_2$ and 0.01% (v/v) Triton X-100. The fractions were frozen and stored at -70°C until required for further use.

5.2.2.4 Protease Assays

Protease activity with azocasein was carried out by the method of Peek *et al.* (1993) at 75°C as described in section 2.2.1. As the assay was linear with respect to enzyme concentration over an A_{420nm} range of only 0.05, a standard curve was constructed and the assay values were adjusted accordingly (see Appendix 1.1). Activity was also determined by the Casein Agar Plate method of Eames (1985; Section 2.2.3).

5.2.2.5 Protein Concentration Determination

The protein concentration was estimated by four different methods to ensure accuracy. The methods used were two modifications of the Lowry method described by Peterson (1977) and Peterson (1983); followed by the Bradford method of Read and Northcote (1981) and the $A_{280/205}$ method of Peterson (1983) (see section 2.3).

5.2.2.6 Electrophoresis

The purity of the protease solutions was determined by running samples on several SDS/PAGE 10-15% gradient PhastGels according to the method of the Pharmacia Phast System. To prevent autolysis during SDS/PAGE pretreatment, the samples were pretreated according to the TCA/Acetone method of Peek *et al.* (1993) (see section 2.4.2). The molecular weight standards were from the low molecular mass calibration kit (Pharmacia; section 2.4.1) and silver staining of the gels was performed by the Bio Rad method (see section 2.4.4).

5.3 RESULTS AND DISCUSSION

5.3.1 Expression Method of Ak.1 Protease

5.3.1.1 Morphology

E. coli clone PB5517 is present in two forms. Figure 5.2 is a photograph of these morphologies taken from a phase contrast microscope. The most common form is the short, fat rods typical of *E. coli*. Another form is elongated cells, some 20 times their normal length, containing shiny bands irregularly throughout their length. Both of these types were Gram (-) and negative on a spore stain. This unusual form has been seen previously in other *E. coli* clones by Saul (personal communication) during overexpression of the recombinant gene. The shiny bands could possibly be stores of the recombinant protease.

This unusual morphology was not treated as a contaminant as extensive attempts to eliminate this morphology from the cultures was unsuccessful. Also, thermophilic protease activity was approximately proportional to the concentration of these elongated cells.

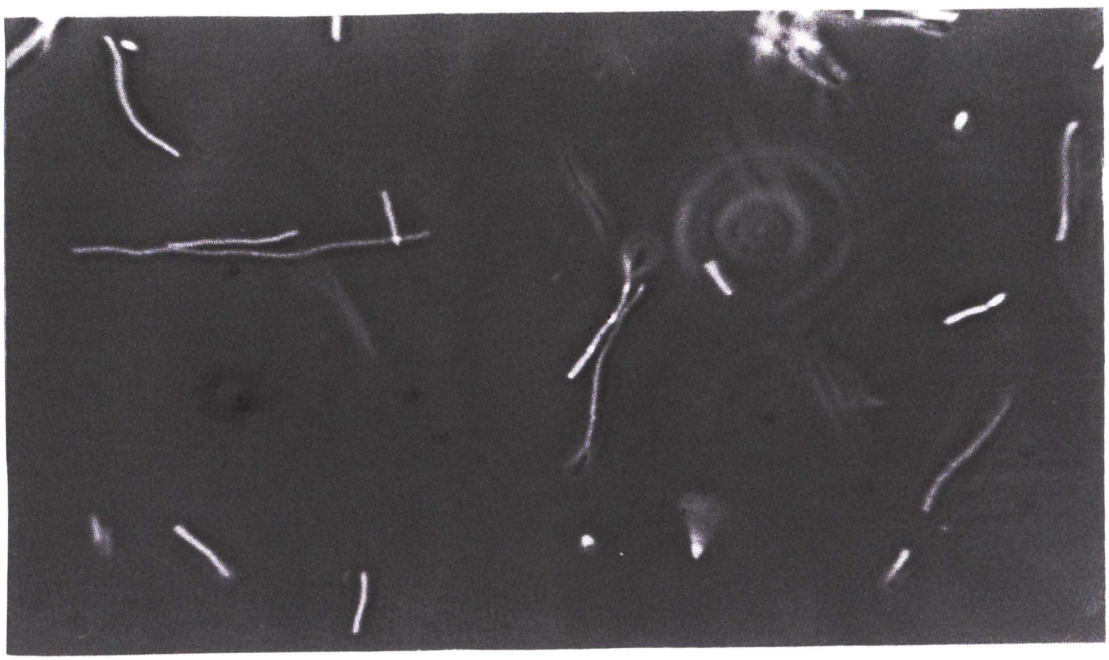


Figure 5.2 Photograph of the *Escherichia coli* Clone PB5517 Exhibiting Two Different Morphologies as Seen Under a Phase Contrast Microscope.
E. coli was grown in Luria broth containing 100 µg/ml ampicillin overnight at 35°C.

5.3.1.2 Growth Profile

Figure 5.3 shows the growth and activity profile of *E. coli* clone PB5517 in a batch culture.

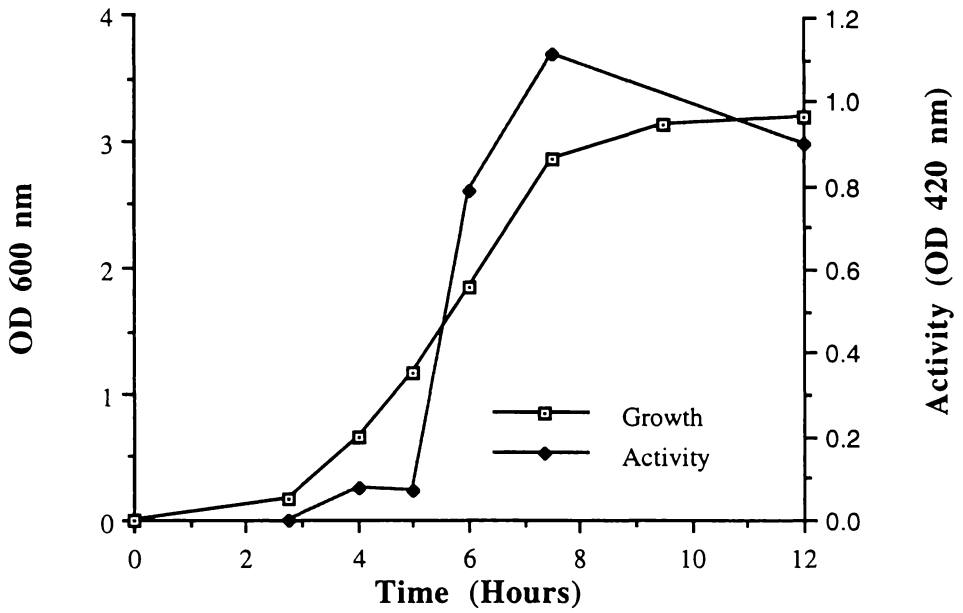


Figure 5.3 Growth and Activity Profile of a Batch Culture of *Escherichia coli* Clone PB5517. The cultures were 'induced' with 1 mM IPTG after 3 hours of incubation.

When extrapolating the maximum detected activity in a batch culture, the max yields of protease that can be obtained from this clone is approximately 2275 U/l, corresponding to 5.3 mg protease/l culture

5.3.1.3 Optimal Expression System of *Escherichia coli* Clone PB5517

E. coli clone PB5517 was initially 'induced' with IPTG to stimulate the production of Ak.1 as this gene had been inserted next to a *lac* operon promotor (Macivor *et al.*, 1994). Peek *et al.* (1993) induced the clone when producing a small quantity of the protease. However, it was noticed that the unusual morphology of the clone, suggesting that recombinant protein expression was occuring, was present in *uninduced* cells. Thus a check was performed to determine if either IPTG or lactose was required for Ak.1 protease expression.

Table 5.1 shows the results of the casein agar plate assays. This shows that approximately the same activity was detected in all 3 cultures. Thus, the non-induced cultures had produced the same quantity of protease as the induced ones. This suggests that the original starter culture was already expressing the protease without any induction having occurred.

Culture Induction	Protease Activity
1. No Induction	+++
2. 1 mM IPTG	+++
3. 1 mM Lactose	+++

Table 5.1 Protease Activity of the Non-Induced and Induced Cultures of *Escherichia coli* Clone PB5517.
Activity was detected by the Casein Agar Plate method of Eames (1985).

Closer inspection of the clone PB5517 revealed that at some point, the *lac* promoter had become mutated, resulting in a *constitutive* recombinant protease production (MacIvor *et al.*, 1994). Thus, no induction is required to induce Ak.1 protease production in the clone. This also means that there is no control over the expression, therefore maximum production is dependent only on the cell mass of the culture and the proportion of the cell mass containing the gene.

5.3.2 Large-Scale Production and Purification of Ak.1 Protease

5.3.2.1 Growth Profile of the Fermentor Run

Figure 5.4 shows the growth profile of *E. coli* clone PB5517 during the fermentor run. It shows that protease production closely followed cell growth, as expected from a clone with constitutive protease production. The total cell mass obtained was 344 g (wet weight) from a 10 l run.

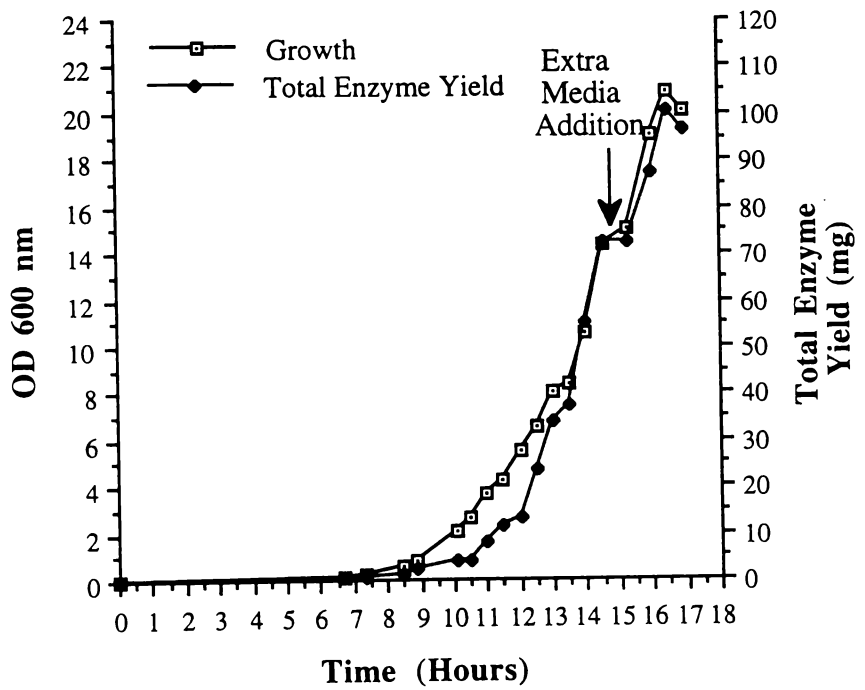


Figure 5.4 Growth and Activity Profile of the Fermentor Run of *E. coli* Clone PB5517

Growth was detected by measuring the OD 600 nm. Activity was determined with the substrate 0.2% azocasein pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

Figure 5.5 shows the relationship between protease yield and both the volume of the culture, and the cell mass as a function of the time of incubation. This data shows that there is not a linear relationship between the yields of protease and the mass of cells produced. Where significant growth was occuring (after 10 hours), the enzyme production was proportunitely increasing. This levelled off to about 0.3 U protease per gram of cells (wet weight) after about 14 hours. Thus, revertant bacteria (missing the plasmid containing the protease gene) were not significately affecting the yields of protease.

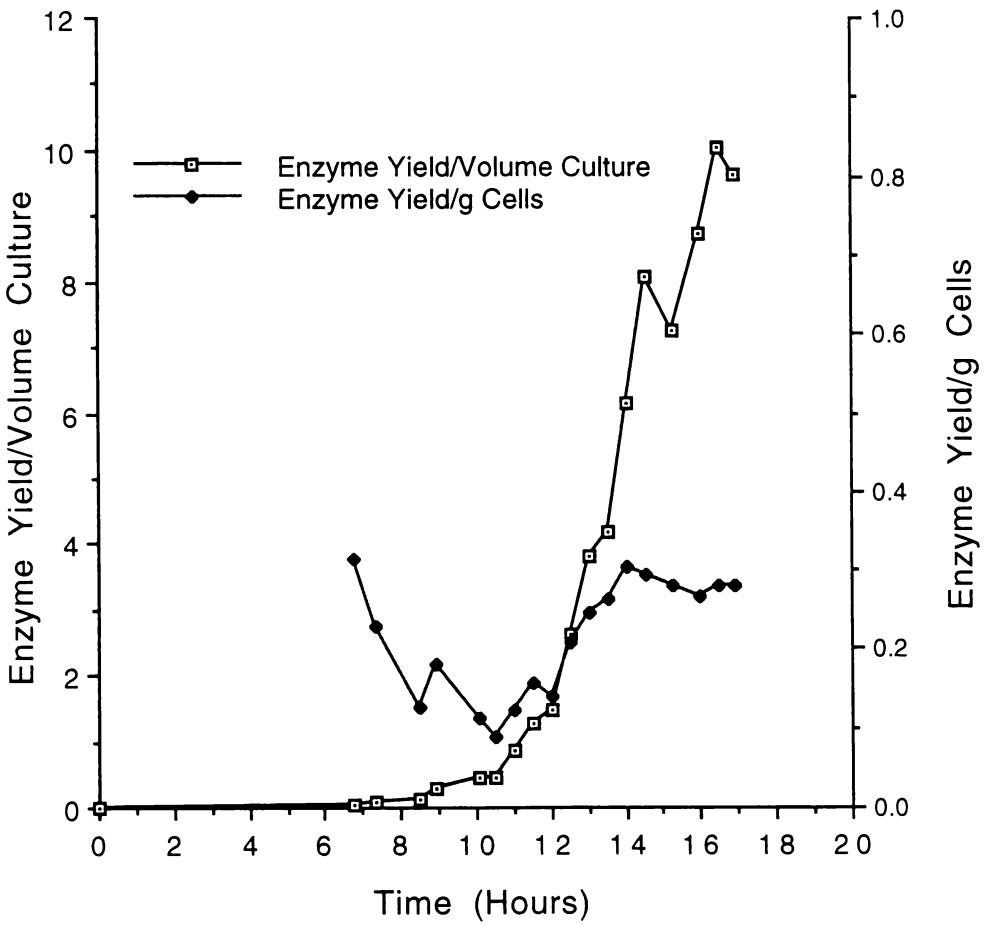


Figure 5.5 Protease Yield/Volume Culture and Protease Yield/g Cells Vs Time of Incubation.

Growth was detected by measuring the OD 600 nm. Activity was determined with the substrate 0.2% azocasein pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

5.3.2.2 Purification of Ak.1 Protease

Table 5.2 shows the purification profile of the Ak.1 protease fermentor run. The first phenyl Sepharose step was very effective, achieving about a 90-fold purification with only about a 20% loss of activity. The Mono Q step was less effective, achieving little purification, while retaining most of the activity. The second phenyl Sepharose step achieved about a 4-fold purification, with about a 50% loss in activity. Figures 5.6-5.8 show the elution profiles of the three purification steps, while Figure 5.9 shows the SDS/PAGE gel of the purified Ak.1 protease.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Heat-Treated Extracts	19200	44800	0.43	1.0	100
Phenyl Sepharose #1	15800	414	38.2	88.7	82
Mono Q FPLC	15300	356	44.0	100	80
Phenyl Sepharose #2	8820	51	174.0	404	46

Table 5.2 Purification Table of Ak.1 Protease
Activity was determined with the substrate 0.2% azocasein pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C (section 2.2.1). The protein concentration was determined by the Lowry method (section 2.3.1).

The major loss of protease occurred during the last purification step. The protease began to elute at the highest concentration of amino acids (1.5%) in the gradient. It appears that the amino acids used (or their concentrations) were not optimal for the elution of this protease. This worked well for WP22.A1 protease (Toogood *et al.*, 1995), but this is a different class of protease with different substrate specificities.

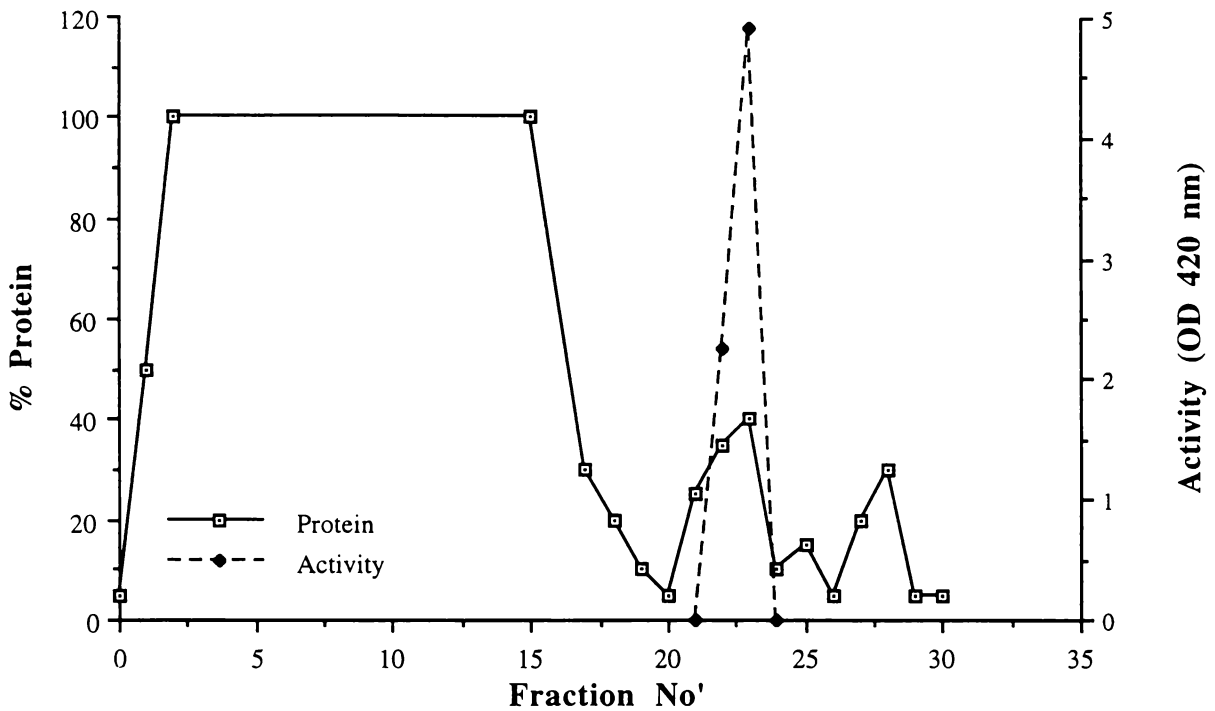


Figure 5.6 Elution Profile of Ak.1 Protease with Phenyl Sepharose #1.
Fractions 1-20 = sample addition; fractions 21-23 = 10 mM Tes/NaOH pH 7.0 + 5 mM $CaCl_2$ wash; fraction 24 = RO water wash; fractions 25-30 = gradient of 0-30% ethanediol in RO water.

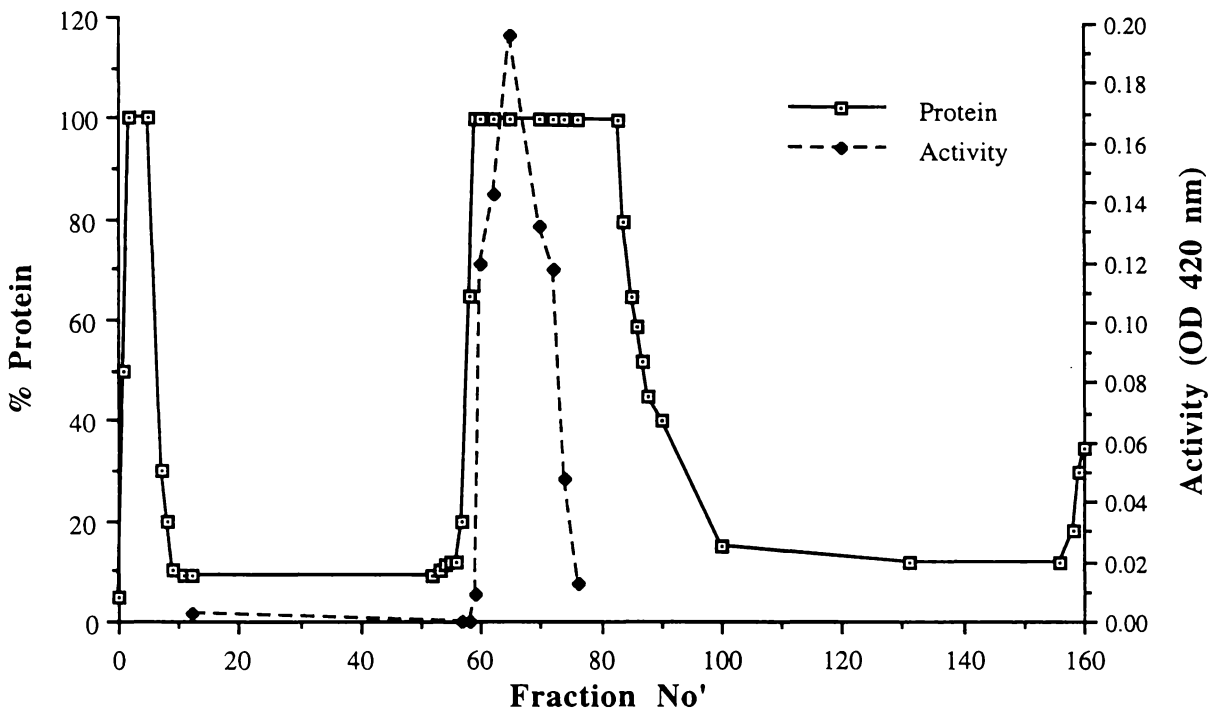


Figure 5.7 Elution Profile of Ak.1 Protease with Mono Q 10/10.

Fractions 1-11 = sample addition; fractions 12-131 = gradient of 0-0.5M NaCl in 10 mM diethanolamine/H_2SO_4 pH 8.5 + $CaCl_2$ + 0.01% (v/v) Triton X-100; fractions 132-156 = 10 mM diethanolamine/H_2SO_4 pH 8.5 + $CaCl_2$ + 0.01% (v/v) Triton X-100 + 0.5M NaCl wash; fractions 157-160 = 10 mM diethanolamine/H_2SO_4 pH 8.5 + $CaCl_2$ + 0.01% (v/v) Triton X-100.

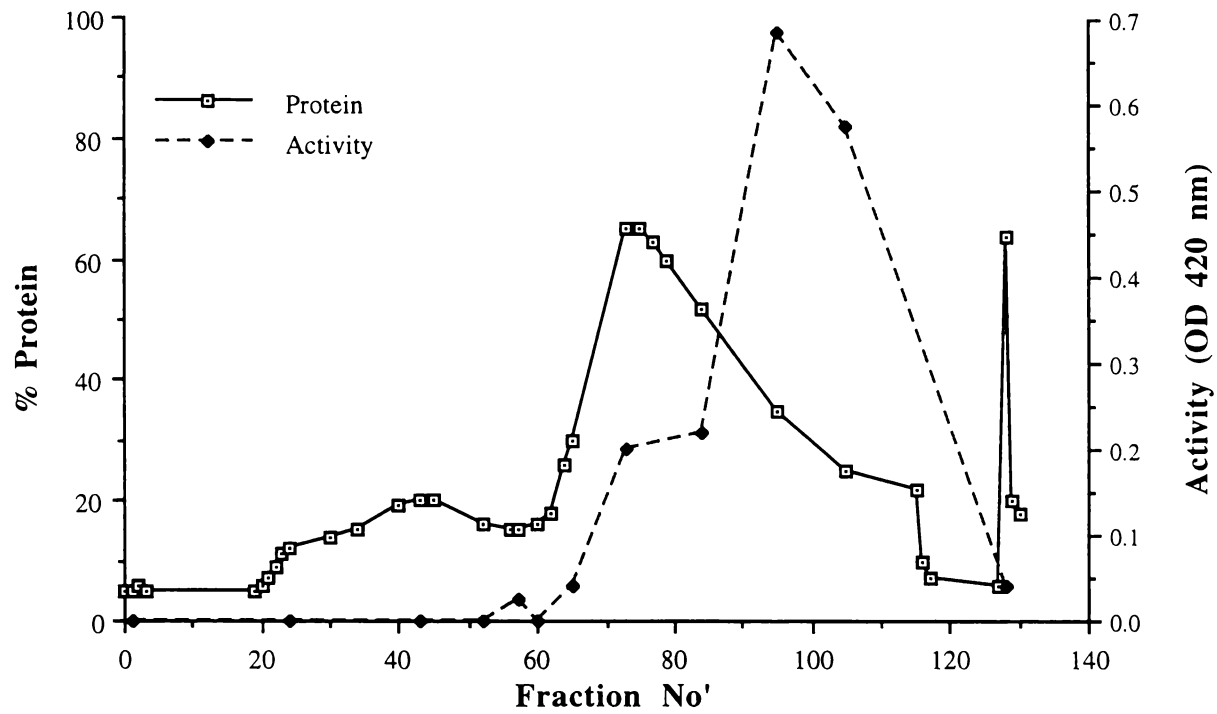


Figure 5.8 Elution Profile of Ak.1 Profile with Phenyl Sepharose #2.

Fraction 1 = sample addition; fraction 2 = 20 mM Tes/NaOH pH 7.5 + 5 mM $CaCl_2$; fractions 3-105 = gradient of 0-0.15 of each of the amino acids alanine, histidine and phenylalanine in 20 mM Tes/NaOH pH 7.5 + 5 mM $CaCl_2$; fractions 106-117 = 0.15 of each of the amino acids alanine, histidine and phenylalanine in 20 mM Tes/NaOH pH 7.5 + 5 mM $CaCl_2$ wash; fractions 118-130 = 20 mM Tes/NaOH pH 7.5 + 5 mM $CaCl_2$ wash + 50% ethanediol wash.

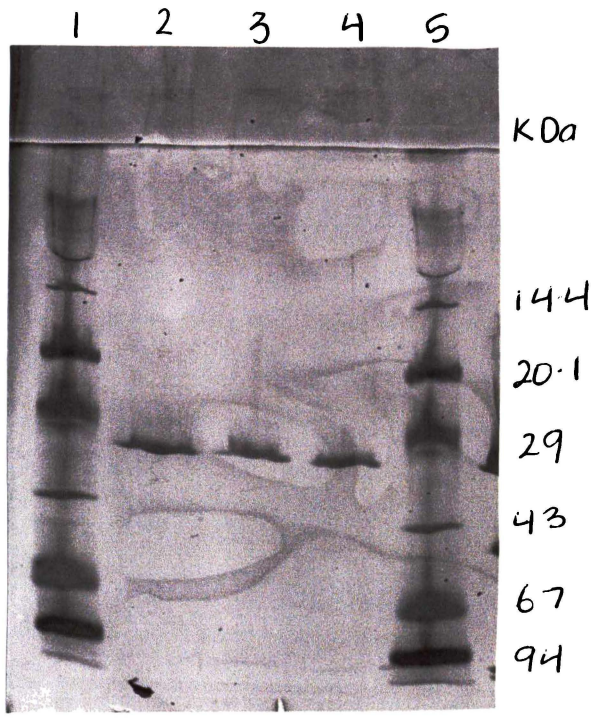


Figure 5.9 SDS/PAGE Gel of Purified Ak.1 Protease.

Ak.1 protease was inactivated by the TCA-Acetone method (section 2.4.1.2) then pretreated with SDS PAGE sample buffer and run on Gradient 10-15% polyacrylamide gels according to the method of Pharmacia (section 2.4). The gel was stained by the BioRad Silver Stain method (section 2.4.1.4). 0.54 µg (1 µl of a solution of 0.54 mg/ml) of purified Ak.1 protease was analysed by this method. Lane 1 & 5 = Molecular mass markers; Lanes 2-4 = Purified Ak.1 protease.

The protease was purified to homogeneity, as determined by the presence of 1 band after SDS PAGE. This was done 2 times to confirm its purity. The determined molecular mass from SDS/PAGE was 36 kDa, while the predicted molecular mass (according to the sequence) was 27 kDa. Kaufmann *et al.* (1984) found that acidic proteins tend to migrate more slowly on SDS/PAGE, resulting in a higher molecular mass determination. Ak.1 protease has an isoelectric point of 4.

5.4 CONCLUSIONS

Escherichia coli clone PB5517 produces Ak.1 protease constitutively at 35°C. A 10 l fermentor run was conducted, and 51 mg of the protease was purified to homogeneity by the criterion of a single band on SDS PAGE.

CHAPTER 6

STABILITY OF AK.1 PROTEASE

6.1 INTRODUCTION

The interactions between metal ions and proteins have been the subject of considerable research. In particular, the interactions between cations and proteases has been extensively studied (e.g. Frommel & Hohne, 1981; Roche & Voordouw, 1978; Khoo *et al.*, 1984). These interactions can influence the activity and/or stability of the protease.

It is known that divalent cations, such as Ca^{2+}, increase the thermostability of many proteases e.g. trypsin, thermolysin and subtilisins (Briedigkeit & Frommel, 1989). Detailed studies of the interactions of Ca^{2+} with thermolysin have shown that these metal ion-protein interactions rely on precise conformations of specific amino acids in the protein relative to the metal ion (Roche & Voordouw, 1978).

Other chemicals, such as polyols and proteins are known to stabilise proteins. The effects of a variety of chemicals on the thermostability of Ak.1 protease were also investigated.

6.2 MATERIALS AND METHODS

6.2.1 Metal Salts and Buffers

Laboratory reagent grade metal salts were used in all cases. All buffers for the work with cations were passed through a metal-ion chelating column (Chelex 100; Dow Chemical Company) before use to remove traces of Ca^{2+} and other metal ions. After passage through Chelex 100, samples of the buffers were analysed for Ca^{2+} content by Atomic Absorption Spectroscopy. Buffers used in these experiments all contained less than 1μM Ca^{2+}.

Lanthanide chlorides were prepared from their respective oxides by dissolving them in hot, anaerobic, concentrated HCl (aristar grade), sealed under nitrogen. The HCl was evaporated off under a constant stream of nitrogen, and the salts were redissolved in anaerobic 50 mM Hepes/NaOH pH_{75} 7.5 in an anaerobic chamber. Europium (III) chloride was prepared by dissolving the salt in water. The oxovanadium chloride ion (VO^{2+}) was prepared by dissolving $VOSO_4.H_2O$ in the above anaerobic Hepes buffer under nitrogen.

6.2.2 Cleaning of Glass and Plasticware

All equipment which came in contact with the enzyme prior to the assays (e.g. reaction vials, capillary tubes, pipettor tips) had to be treated to remove trace amounts of metal ions. This was done by soaking them in a solution of 0.1M HCl for 30 minutes, followed by extensive rinsing with Milli Q water. To ensure the equipment was no longer acidic, it was rinsed with a solution of Ca^{2+}-free 50 mM HEPES/NaOH pH_{75} 7.5 which had previously passed through Chelex 100.

6.2.3 Preparation of Calcium-Free Ak.1 Protease

Ca^{2+}-free Ak.1 protease (apo-Ak.1) was prepared by incubating the protease with 10 mM EDTA in 10 mM Hepes/NaOH pH_{75} 7.5 containing 0.01% (v/v) Triton X-100 for 1.5 hours at room temperature. This was followed by exhaustive dialysis against 10 mM Hepes/NaOH pH_{75} 7.5 containing 0.01% (v/v) Triton X-100 in a 1 ml dialysing chamber with continuous buffer exchange for 2 days at 4°C.

6.2.4 Determination of Thermostability

Apo-Ak.1 (0.11 μg per assay), in 50 mM Hepes/NaOH pH 7.5 (at the required temperature) containing 0.01% (v/v) Triton X-100 and the appropriate concentration of metal chloride salt/additive, was incubated at the specified temperature in reaction vials. Samples were withdrawn at various time intervals, fast-frozen in liquid N_2 then stored on ice until assayed. For high temperature incubations (>90°C) or for very long incubations (>1 day), samples were sealed in acid-cleaned capillary tubes (including the zero time control) and heated in a thermostated oil bath. These tubes were cooled quickly in liquid nitrogen, and stored on ice until assayed.

The samples were assayed for remaining activity with 0.2% azocasein at 75°C (see section 2.2.1) and the half lives determined.

6.3 RESULTS AND DISCUSSION

6.3.1 Crystal Structure of the Calcium Binding Sites of Ak.1 Protease

6.3.1.1 Introduction

Ca^{2+} ions strongly prefer to bind to oxygen donors rather than to nitrogen or sulphur donors (Martin, 1984). This is supported by the 0.26Å increase in the Ca-N bond length as compared to the Ca-O bond length found in crystal structures of Ca^{2+} with nitrilotriacetate and EDTA. Ca^{2+} is known as a "hard" acid, meaning it is of small size, has a high positive charge density and is usually without unpaired electrons in its valence shell. Therefore, coordination about Ca^{2+} is essentially ionic and spherical without strong directionality (a requirement for ligands corresponding to the set configurations of the protein-metal ion molecular orbitals; Khoo *et al.*, 1984; Martin, 1984).

To provide a good Ca^{2+} binding site, a nearly spherical pocket of the size appropriate to the coordination number of Ca^{2+} (see Table 6.2) is required, with at least two negatively charged donor groups, preferably carbonyl or carboxyl groups (Martin, 1984). Thus, Ca^{2+}-binding pockets frequently contain aspartate residues. Ca^{2+} prefers to have a coordination number of 6 or 7 in these interactions. Figure 6.1 shows the structures of typical interactions between Ca^{2+} and aspartate residues.

6.3.1.2 Structure of the Calcium Binding Sites

Recently, the three dimensional structure of Ak.1 protease was determined to 1.75 Å resolution by Smith *et al* (1998). The structure revealed the presence of **4** Ca^{2+} binding sites in the protease. Three of these sites correspond to the Ca^{2+} binding sites of thermitase, while the fourth site is unique among subtilisins. The numbering of these sites corresponds to the numbering of thermitase, the binding strengths being Ca(1) > Ca(2) >>Ca(3). Ca(4) is the site unique to Ak.1 protease.

Figure 6.2 shows a representation of the structure of Ak.1 protease (Smith *et al.*, 1998). The structure was resolved based on the starting model of thermitase, resolved to 1.4Å. The four yellow balls are the positions of the four Ca^{2+} ions.

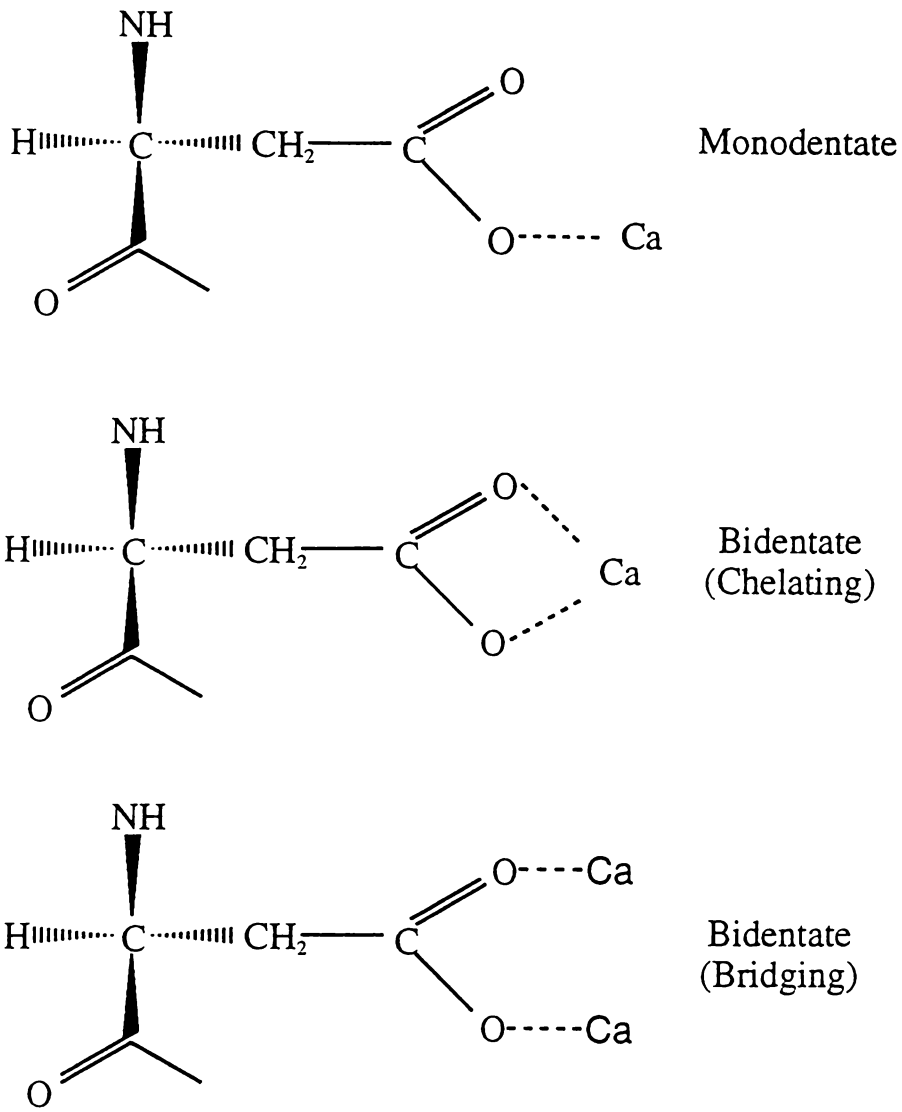


Figure 6.1 Structures of Typical Interactions between Ca^{2+} and Aspartic Acid.

Note: The metal can also bind to the backbone carbonyl group of amino acids.

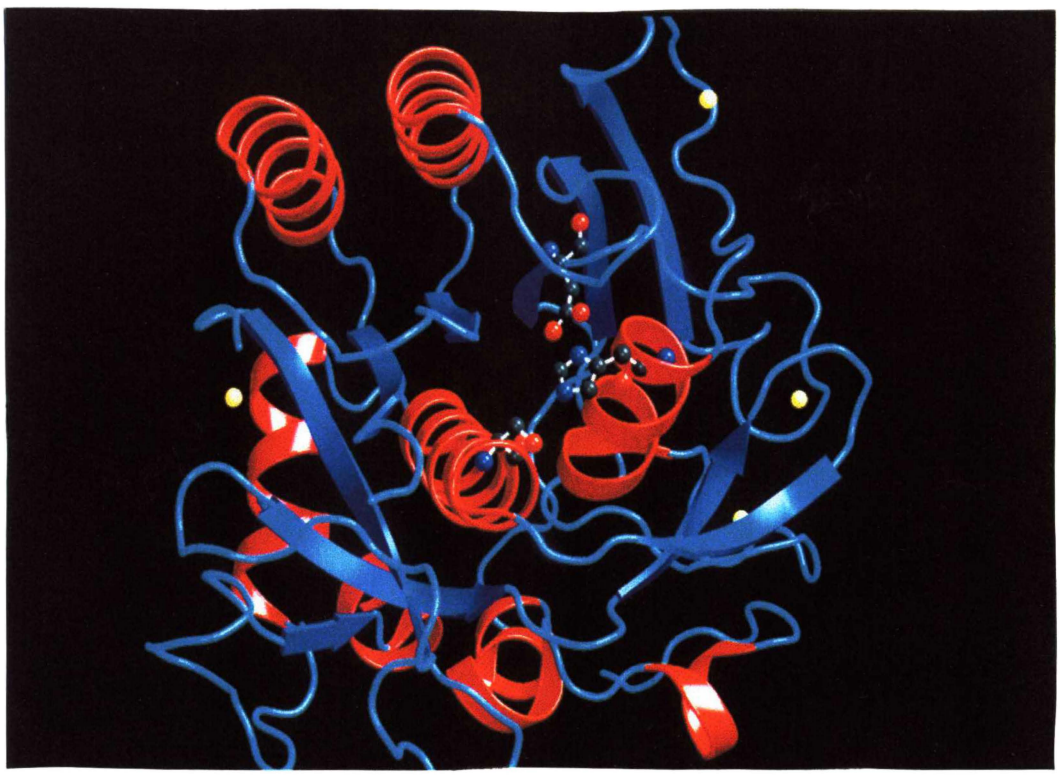


Figure 6.2 3-Dimensional Structure of Ak.1 Protease.

Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

1. *Calcium (1) and Calcium (4) Binding Sites*

Figure 6.3 shows the structures of the Ca(1) and Ca(4) binding sites. Ca(1) is thought to have the strongest binding constant as it is bound to the protein by 7 ligands. No water is associated with this Ca^{2+}. It is bound by both monodentate and bidentate aspartate ligands, as well as backbone carbonyl residues. It appears to be buried within the molecule. This same site in thermitase has a very high binding constant ($k_{diss} < 10^{-10}$ M), and is not removed by EDTA treatment (Briedigkeit & Frommel, 1989).

Ca(4) is bound to the protease by 4 ligands. It also binds to 3 water molecules. Like Ca(1), it binds via monodentate and bidentate aspartate ligands, as well as backbone carbonyl residues. It is more exposed to the environment than Ca(1).

Note the close proximity of Ca(1) and Ca(4). In fact one protein residue, Glu 83, binds to both Ca^{2+} ions. The backbone carbonyl group binds to Ca(1) (monodentate binding), while both oxygens in the R-group of glutamate bind to Ca(4) (bidentate, chelating, binding). Due to the close proximity of the two Ca^{2+} ions, their binding may be cooperative.

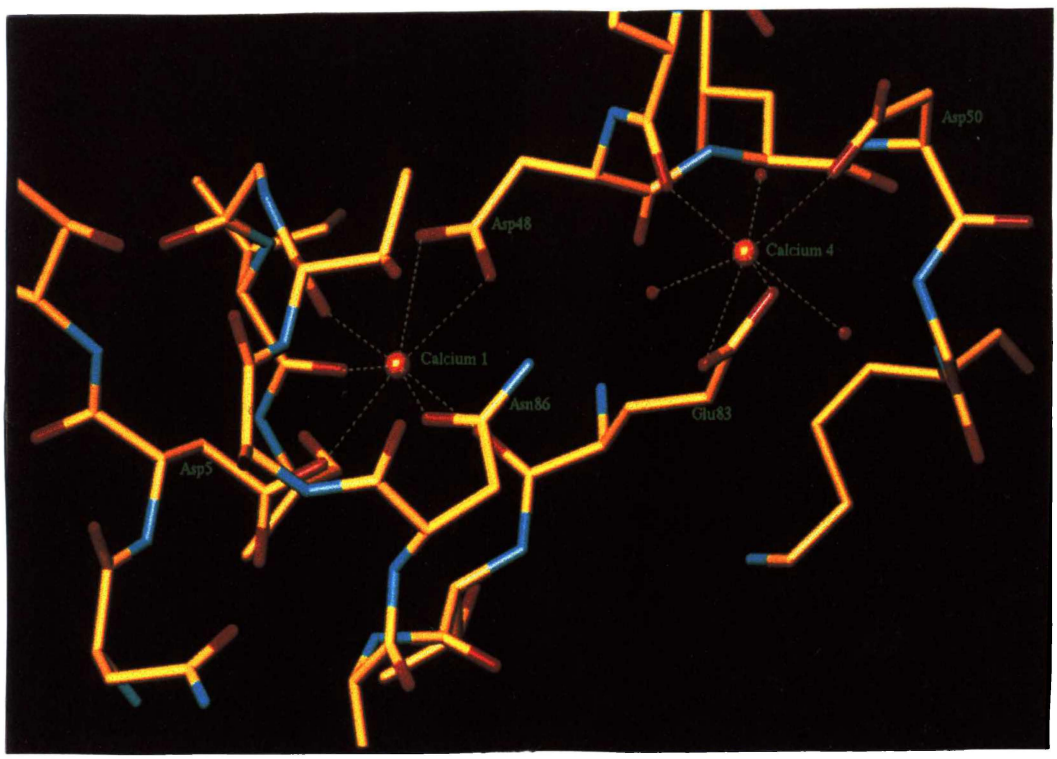


Figure 6.3 Structure of the Ca (1) and (4) Binding Sites of Ak.1 Protease.
Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

2. *Calcium (2) Binding Site*

Figure 6.4 shows the structure of the Ca(2) binding site. Like Ca(4), this Ca^{2+} ion binds via 4 protein ligands and 3 water molecules. It binds to 3 aspartate residues. It binds to Asp 58 and Asp 63 via their R-groups (monodentate and bidentate/chelating respectively), and to Asp 65 via the backbone carbonyl oxygen. This Ca^{2+} ion is more exposed to the solvent than Ca(1) and Ca(4). This site is not found in other subtilisins such as subtilisin BPN' and subtilisin Carlsberg (Briedigkeit & Frommel, 1989).

The comparable site in thermitase contains Ca^{2+} bound to the protein by 7 protein ligands and one water molecule (Gros, P. *et al.*, 1991). It has a dissociation constant of 10^{-10} M. Ca^{2+} in this site in thermitase is likely to bind tighter than in Ak.1 protease due to it binding to the protein by 7 sites in thermitase, as opposed to 4 sites in Ak.1 protease.

3. *Calcium (3) Binding Site*

Figure 6.5 shows the structure of the Ca(3) binding site. This is only *proposed* to be a Ca^{2+} binding site. The electron density at this site suggests several possible scenarios. Either a Na^{+} ion is bound at this site (as suggested by the bond lengths), or a Ca^{2+} ion is bound to the site in only a fraction of the molecules. The later was suggested as electron density obtained from x-ray crystallography is an average of the molecules in the crystal. Alternatively, Na^{+} ions could be bound to this site at low Ca^{2+} concentrations, with Ca^{2+} ions present at high concentrations.

This site corresponds to the weak binding site of thermitase ($k_{diss} = 10^{-4}$ M) and subtilisin BPN' (Briedigkeit & Frommel, 1989). Crystal structures of thermitase showed that Ca^{2+} was absent at this site in the presence of 5 mM Ca^{2+}, but present at 100 mM Ca^{2+} (Gros *et al.*, 1991). The crystal structure of Ak.1 protease was determined in the presence of 1 mM Ca^{2+}.

The "Ca(3)" in Ak.1 protease is bound by only 3 protein ligands and 3 water molecules. Therefore, it is coordinated to only 6 sites, compared to the 7 coordinate bonds in the other Ca^{2+} sites. In thermitase, Ca^{2+} at this site is bound to the protein by 3 ligands and 4 water molecules (Gros *et al.*, 1991).

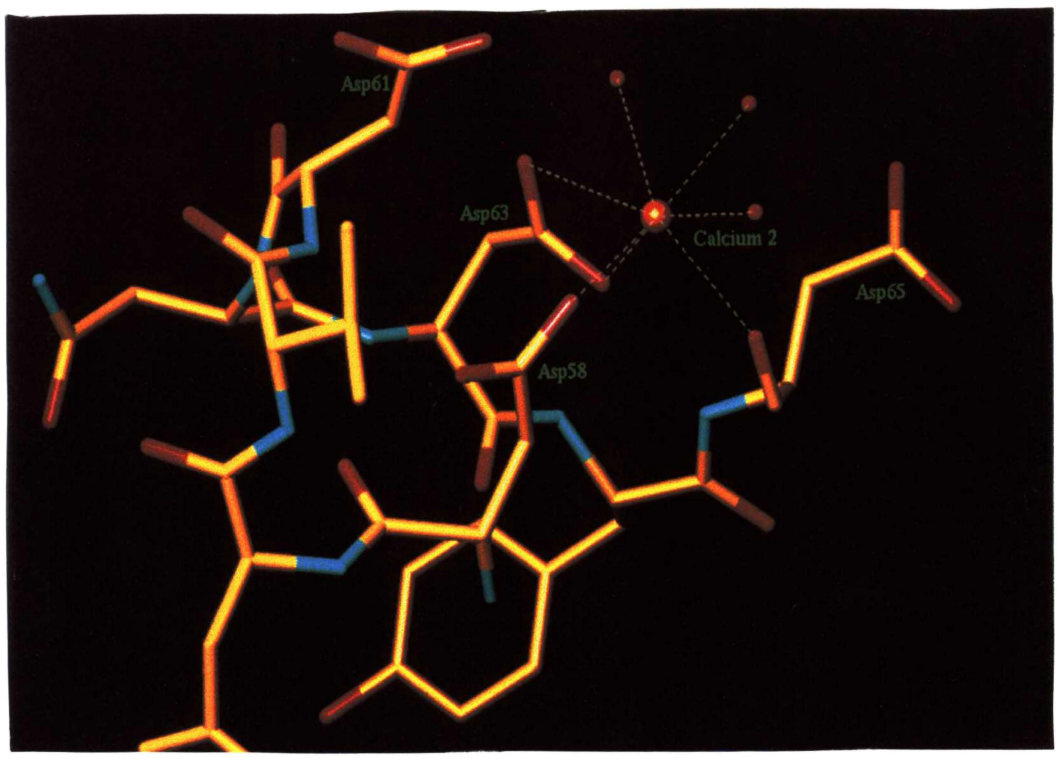


Figure 6.4 Structure of the Ca(2) Binding Site of Ak.1 Protease.

Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

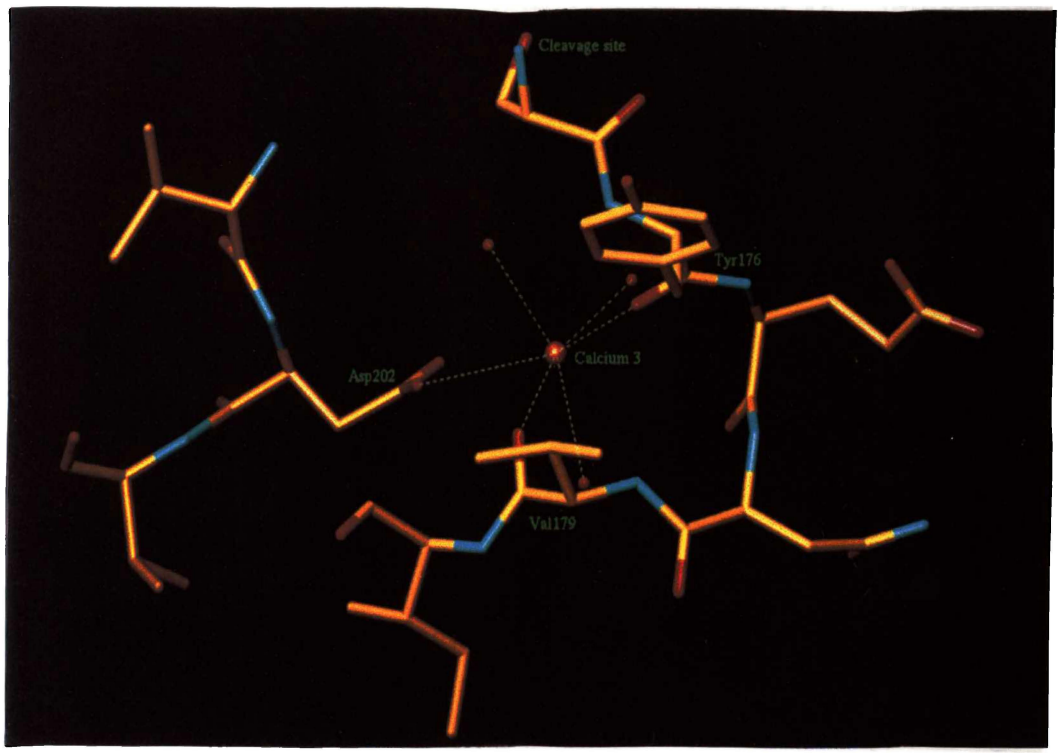


Figure 6.5 Structure of the Ca(3) Binding Site of Ak.1 Protease.

Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

6.3.2 Effect of Calcium Ions on Thermostability

6.3.2.1 Effect of Calcium on Thermostability

Figure 6.6 shows the relationship between the half life of Ak.1 protease at 70°C and the Ca^{2+} concentration. [Appendix 3 contains the thermostability graphs, as well as the first and second order plots of this data.] This shows that low calcium concentrations (<10 μM) have no significant effect on thermostability. At higher concentrations of Ca^{2+}, a substantial increase in thermostability is found as the Ca^{2+} concentration increases. Maximum thermostability was obtained with a Ca^{2+} concentration of > 5 mM. At this concentration, the half life of the protease is *4 orders of magnitude* greater at 70°C than at 1 μM Ca^{2+}.

Table 6.1 shows the effect of the Ca^{2+} concentration on the rates of denaturation and autolysis of the protease. This shows that at low Ca^{2+} concentrations ($\leq$30 μM), the most significant cause for the loss of activity at 70°C is thermal denaturation. At Ca^{2+} concentrations between about 50-200 μM Ca^{2+}, both denaturation and autolysis play key roles in the loss of activity.. At Ca^{2+} concentrations above this, autolysis is dominant. This suggests that Ca^{2+} ions stabilise Ak.1 protease by the prevention of *thermal denaturation*. Another possibility is that Ca^{2+} binding to a high-affinity binding site may protect the enzyme against autolysis, while Ca^{2+} binding to a lower-affinity binding site may stabilise the enzyme against thermal denaturation.

Figure 6.7 shows the log K_{obs} (second order rate constant for autolytic loss of enzyme activity) with respect to Ca^{2+} concentration (expressed as pCa). This relationship is not linear throughout the entire Ca^{2+} concentration range. An apparent K_s of approximately 100 μM for Ca^{2+} was obtained. This suggests that there may be more than one type of Ca^{2+} binding interaction involved in increasing thermostability in Ak.1 protease.

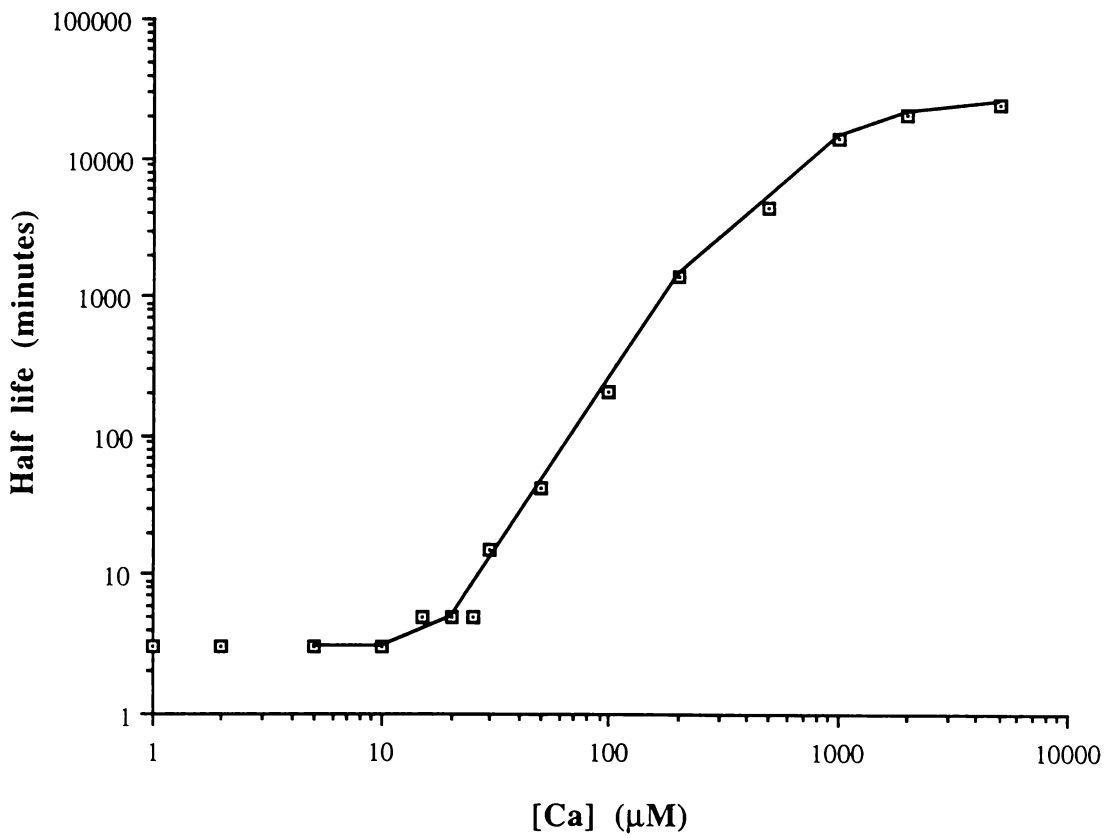


Figure 6.6 Half Life Vs Calcium Concentration at 70°C.

Ak.1 protease was incubated at 70°C for various time periods in the presence of 0-5mM Ca^{2+}. The half lives were determined by assaying for remaining activity with 0.2% azocasein as the substrate (section 6.2.4).

$[Ca^{2+}]$ μM	Denaturation	Autolysis
0	+	
0.5	+	
1	+	
2.5	+	
5	+	
10	+	
15	+	
20	+	
25	+	
30	+	
50	+	+
100	+	+
200	+	+
500		+
1000		+
2000		+
5000		+

Table 6.1 Major Cause for Loss of Activity at 70°C at Different Calcium Ion Concentrations.

Denaturation and autolysis were determined to be the most significant causes by looking for linearity in the first and second order thermostability plots respectively (Appendix 3). In the cases when the plots were either both almost linear, or both non-linear, it was assumed that they were equally significant at that calcium concentration.

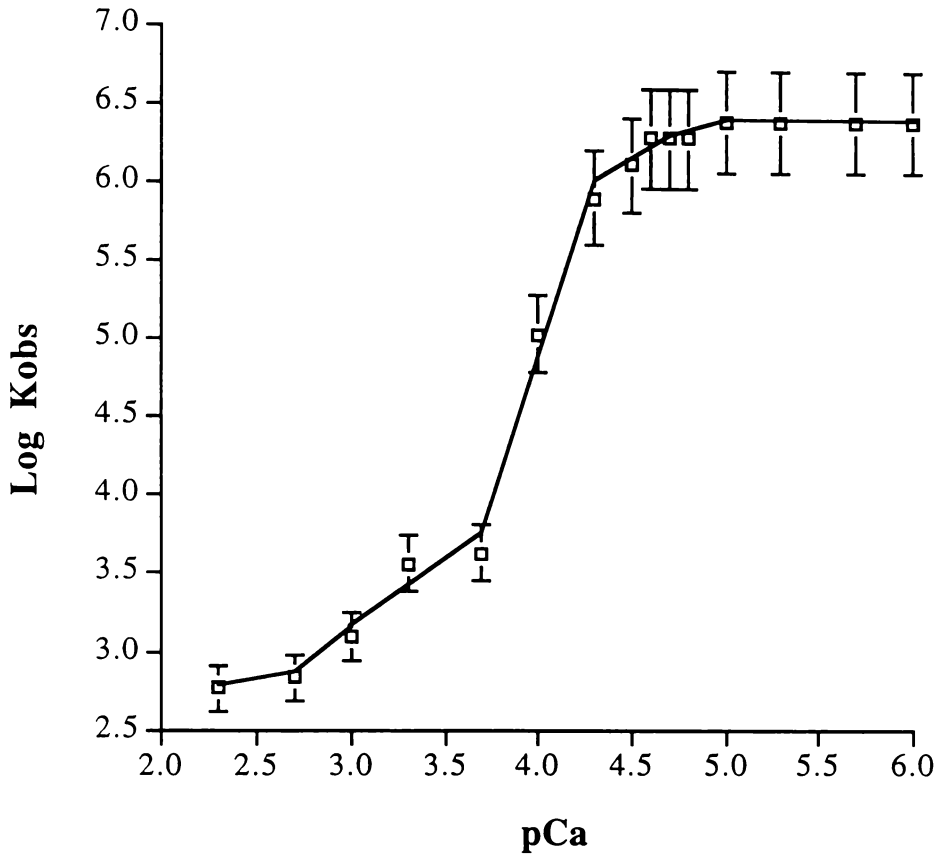


Figure 6.7 Log K_{obs} Vs pCa at 70°C.

Ak.1 protease was incubated at 70°C for various time periods in the presence of 0-5mM Ca^{2+}. The half lives were determined by assaying for remaining activity with 0.2% azocasein as the substrate (section 6.2.4). K_{obs} is the second order rate constant, or the rate of autolysis. pCa = -log[Ca].

Thermitase is dependent on Ca^{2+} for thermostability (e.g. Frommel and Sander, 1989). It binds three Ca^{2+} ions per enzyme molecule, but only one of the sites, Ca(3), is involved in increasing thermostability. Stability is only slightly affected by the two tightly bound Ca^{2+} molecules. A marked increase in thermostability is prevalent when the weaker Ca^{2+} site is filled.

Table 6.2 shows a comparison of the half lives of Ak.1 protease and thermitase (Frommel *et al.*, 1980) at 80°C. This shows that although thermitase is intrinsically more thermostable at 80°C, Ak.1 protease is significantly more stabilised by Ca^{2+}. Thus while Ak.1 protease has a 68% sequence similarity to thermitase, the stabilisation by Ca^{2+} is significantly different so its Ca^{2+} binding ability may be quite different from thermitase.

	Half Life (80°C)	
Enzyme	Ca^{2+}-Free	With Ca^{2+}
Ak.1 Protease	<1 minute	15 hours
Thermitase	9 minutes	18.9 minutes

Table 6.2 Effect of Ca^{2+} on the Half Lives of Ak.1 Protease and Thermitase.

Ak.1 protease was incubated at 80°C for several time periods and assayed for remaining activity with the substrate 0.2% azocasein at 75°C. The thermitase data was obtained from Frommel *et al.*, 1980.

Based on the structural and enzymatic data, the extra degree of stabilisation of Ak.1 protease by Ca^{2+} above that of thermitase-Ca^{2+} could be due to the presence of an extra Ca^{2+}-binding site in Ak.1 protease. This new site is located close to Ca(1), and could therefore change the binding properties of this site also. Another possibility is that the binding constants of one or more of the Ca^{2+}-binding sites could be higher for Ak.1 protease, as compared to thermitase. This could also increase the thermostability of the protease.

6.3.2.2 Estimation of the Binding Constants of Ak.1 Protease

The Ca (1) binding site of Ak.1 protease appears to be buried within the protein. In thermitase, this buried Ca^{2+} could not be removed by EDTA treatment (Briedigkeit & Frommel, 1989). If the same is true for Ak.1 protease, then the binding constant for the Ca (1) site would be smaller than the binding constant of EDTA ($k_{diss} < 10^{-10}$ M). Thus, the increase in thermostability of the protease in the presence of 30 μM-5 mM Ca^{2+} is not likely to be due to the binding of Ca^{2+} at this site.

The Ca (2) binding site in thermitase has a medium-strong binding constant ($k_{diss} = 10^{-10}$). The Ca^{2+} is bound to the protease by 7 protein ligands and 1 water molecule. In contrast, Ca^{2+} ions at this site, and at the Ca (4) site in Ak.1 protease are bound by only 4 protein ligands, and 3 water molecules. This suggests that these two Ca^{2+} binding sites are weaker than Ca (2) in thermitase. Their binding constants are probably within the range of 10^{-10} to 10^{-4} M.

The 3-dimensional structure of Ak.1 protease shows that like thermitase, in the presence of 1 mM $CaCl_2$, Ca^{2+} does not significantly bind to the Ca (3) binding site. Thus the binding constant at this site is likely to be high, possibly similar to thermitase ($k_{diss} \sim 10^{-4}$ M). The Ca (3) binding site in thermitase is only a weak binding site, yet it is the only site which confers significant stabilisation of the protease. Therefore, it is not surprising that the half life at 80°C has only doubled in the presence of Ca^{2+} as Ca^{2+} binds weakly to this site.

The majority of the increase in stabilisation of Ak.1 protease by Ca^{2+} occurs in the concentration range of 50μM-1mM $CaCl_2$. The change from denaturation to autolysis as being the major cause for loss of activity at 70°C occurs within this range. Due to the significant difference in the degree of stabilisation of Ak.1 protease by Ca^{2+} as compared to thermitase (Table 6.2), it is likely that more than one of the four Ca^{2+} binding sites are involved in increasing the thermostability of the protease as opposed to just one site in thermitase (Ca (3)).

The results of the thermostability studies of Ak.1 protease with Ca^{2+} suggests the presence of two types of binding sites involved in increasing thermostability. At low Ca^{2+} concentrations (high-affinity site), the protease appears to be protected from autolysis, while at high Ca^{2+} concentrations (low-affinity site), the protease appears to be protected from thermal denaturation (see section 6.3.2.1). It is possible that the potential high affinity site/s are the

Ca (2) and/or Ca (4) sites. The low affinity site could be due to Ca^{2+} binding to the Ca (3) site.

6.3.3 Effect of Lanthanide Ions on Thermostability

Like Ca^{2+}, lanthanide ions are "hard" acids. Thus, their interactions are primarily ionic in nature, and are more dependent on electrostatic interactions and *ionic radii* rather then electronic interactions (Evans *et al.*, 1979). They also prefer oxygen donors, rather than nitrogen or sulphur donors, and lack strong directionality in binding donor groups.

Table 6.3 shows the effective ionic radii of calcium, lanthanides (e.g. Tb & Eu) and other metal ions. This table shows that the lanthanides exhibit similar ionic radii and coordination numbers to calcium. As binding to the oxygen groups in proteins is more dependent on ionic radii than charge, the *trivalent* lanthanide ions tend to bind to *divalent* calcium sites better than other divalent cations such as magnesium and cobalt, which have much lower ionic radii (Martin, 1984; Evans *et al.*, 1979). Indeed, such results have been detected in a number of enzymes (e.g. Khoo *et al.*, 1984).

As Ca^{2+} significantly increases the thermostability of Ak.1 protease, experiments were conducted to see if the lanthanides could also stabilise the enzyme.

Figure 6.8 shows the half lives of Ak.1 protease in the presence of lanthanides at 5 mM concentrations. Note the *semi-log* scale of this graph. The thermostability graphs are located in Appendix 3. These results show that while Ca^{2+} has a dramatic effect on the stability of Ak.1 protease, the lanthanide ions had a much smaller effect on thermostability.

The first and second order plots (Appendix 3) in the presence of 5 mM lanthanide ions shows that in all cases, the predominant cause of loss of activity is due to autolysis. Thus, like 5 mM Ca^{2+}, these metals stabilise the protease by the prevention of thermal denaturation. This is not suprising as lanthanide ions are expected to have a similar effect as Ca^{2+} ions (Evans *et al.*, 1979).

Element	Ion	Coordination Number			
		6	7	8	9
Calcium	Ca^{2+}	1.00	1.06	1.12	1.18
Magnesium	Mg^{2+}	0.72	-	0.89	-
Manganese	Mn^{2+}	0.80	-	-	-
Cobalt	Co^{2+}	0.72	-	-	-
Copper	Cu^{2+}	0.69	-	-	-
Strontium	Sr^{2+}	1.12	-	-	-
Cadmium	Cd^{2+}	0.97	-	-	-
Barium	Ba^{2+}	1.34	-	-	-
Vanadium	VO^{2+}	-	-	-	-
Lanthanum	Ln^{3+}	1.03	1.10	1.16	1.22
Cerium	Ce^{3+}	1.01	1.07	1.14	1.20
Praseodymium	Pr^{3+}	0.99	-	1.13	1.18
Neodymium	Nd^{3+}	0.98	-	1.11	1.16
Samarium	Sm^{3+}	0.96	1.02	1.08	1.13
Europium	Eu^{3+}	0.95	1.01	1.07	1.12
Gadolinium	Gd^{3+}	0.94	1.00	1.05	1.11
Terbium	Tb^{3+}	0.92	0.98	1.04	1.10
Dysprosium	Dy^{3+}	0.91	0.97	1.03	1.08
Holmium	Ho^{3+}	0.90	-	1.02	1.07
Erbium	Er^{3+}	0.89	0.95	1.00	1.06
Thulium	Tm^{3+}	0.88	-	0.99	1.05
Ytterbium	Yb^{3+}	0.87	0.93	0.99	1.04
Lutetium	Lu^{3+}	0.86	-	0.98	1.04

Table 6.3 Effective Ionic Radii (å) of Some Cations.

Adapted from Khoo *et al* (1984) and Martin (1983).

5 mM concentrations of some metals are toxic to some enzymes, so any stabilisation against denaturation and autolysis may be partly masked by an inhibition of activity. To examine this, possibility, Ak.1 protease was incubated with 0.5 mM concentrations of these and other lanthanides for 5 hours at 70°C.

Figure 6.9 shows the % activity remaining of the protease in the presence of these metal ions at 70°C. These results show that most of the lanthanides tested showed some stabilisation, as compared to the apo-enzyme control. While the % activity remaining of the protease in the presence of these metals is very small, it must be remembered that the half life of Ak.1 protease in the absence of Ca^{2+} is only *3 minutes* at 70°C, and these samples were incubated for *5 hours* at 70°C. Thus, the lanthanides have stabilised the enzyme, but at a significantly lower degree than Ca^{2+} (<7%).

While the % activity remaining with the lanthanides is quite small, it is interesting to note that Pr^{3+} has the highest degree of stabilisation of the lanthanides, while Lu^{3+} has the lowest (no activity remaining). As these lanthanides have the ionic radii most and least similar, respectively, to the ionic radius of Ca^{2+}, this is perhaps not surprising.

Due to the general observation that lanthanides tend to bind to calcium-binding sites in proteins, it is quite surprising that the degree of stabilisation by the lanthanides is so small. However, this does not mean that the lanthanides are not binding to the protein.

Thermitase is known to bind terbium ions (Frommel *et al.*, 1980). It does so by binding to the two tightly-bound Ca^{2+} sites, rather than to the weak-binding site. Consequently, the binding of terbium has only a slight stabilising effect as the thermostability of thermitase is dependent significantly only on metal ion binding to the weak-binding site. Thus while Ak.1 was not significantly stabilised by the lanthanides, it may still be binding them at the other calcium binding sites.

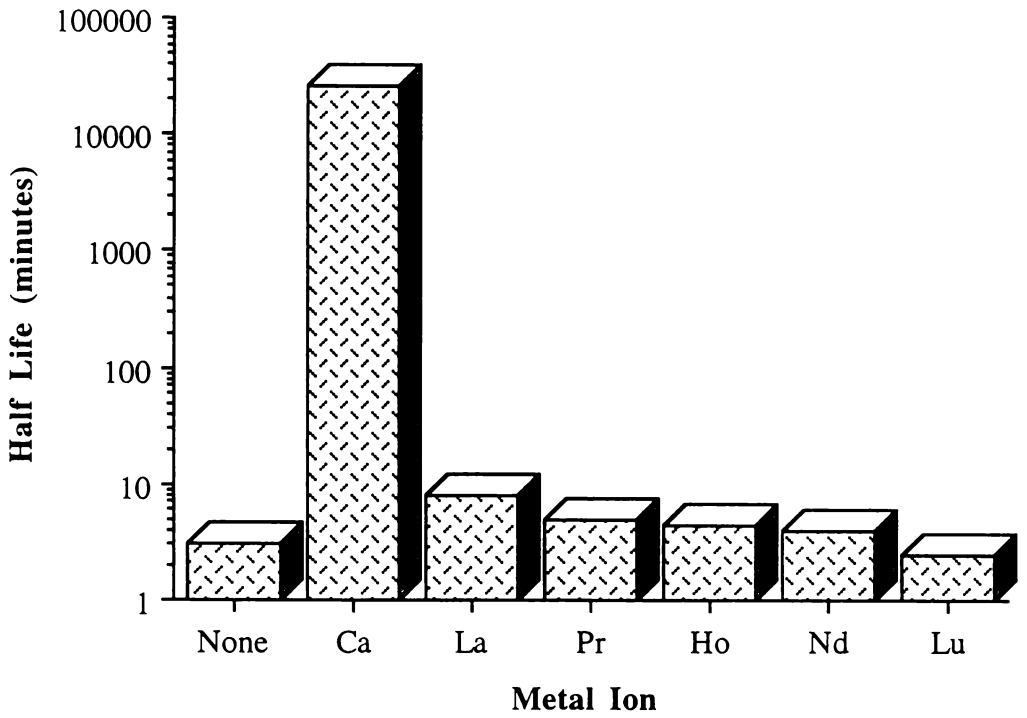


Figure 6.8 Thermostability of Ak.1 Protease at 70°C in the Presence of 5mM Lanthanide Ions.

The protease was incubated at 70°C in the presence of 5 mM lanthanide ions for various time periods, then assayed for remaining activity with 0.2% azocasein (section 6.2.4). Note the log scale of the half life axis.

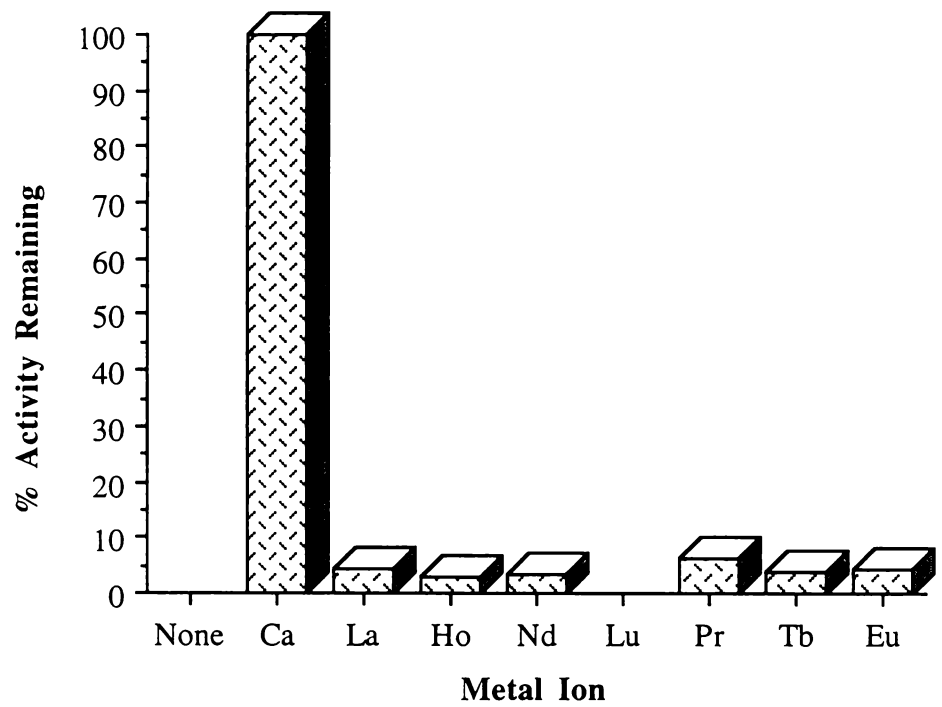


Figure 6.9 % Activity Remaining Vs Lanthanide Ion after 5 Hours at 70°C
The protease was incubated for 5 hours at 70°C in the presence of 0.5 mM lanthanide ions for various time periods, then assayed for remaining activity with 0.2% azocasein (section 6.2.4).

6.3.4 Effect of Other Cations on Thermostability

Figure 6.10 shows the half lives of Ak.1 protease in the presence of some divalent cations at 5 mM concentrations. The thermostability plots are located in Appendix 3. Note the *log scale* of the half life axis. These results show that while Ca^{2+} has a dramatic effect on the stability of Ak.1 protease, the other cations had a significantly lower effect. The only metal to afford significant stabilisation relative to Ca^{2+} is Sr^{2+}, with a half life of 18.5 hours at 70°C. However, this is still only 4% of the stabilisation by Ca^{2+} ions. It is interesting to note that Sr^{2+} belongs in the same group in the periodic table as Ca^{2+} (group IIA), as does Mg^{2+}. However, Mg^{2+} does not stabilise the protease as significantly as Sr^{2+}. Sr^{2+} and Mg^{2+} have larger and smaller ionic radii, respectively, than Ca^{2+}. Sr^{2+} ions appear to bind to the protease with a higher affinity than Mg^{2+}.

The first and second order plots (Appendix 3) show that the loss of activity in the presence of the metals Cd^{2+}, Mg^{2+} and Co^{2+} was primarily due to denaturation. Both denaturation and autolysis were significant in the presence of Mn^{2+} while autolysis was predominant in the presence of Sr^{2+}.

In spite of the large differences in the stabilising abilities of these cations compared with Ca^{2+}, there is still a significant increase in thermostability of Ak.1 protease by these metals. For example, Mg^{2+} increased the half life from 3 min to 25.5 minutes at 70°C. This is almost a *9-fold* increase in thermostability as compared to apo-Ak.1 (while being only 0.09% of the stabilisation of Ca^{2+}). As mentioned previously, Ca^{2+} causes only a *2-fold* increase in thermostability of thermitase, so stabilisation of Ak.1 protease by these metals is significant.

The lower degree of stabilisation by Mg^{2+}, Co^{2+} and Mn^{2+} could be attributed, in part, to the fact that each of these ions has an effective ionic radius considerably less than that of Ca^{2+} (see Table 7.2). Also, cations such as Mn^{2+} and Cd^{2+} possess a high degree of directional specificity, as opposed to Ca^{2+} and the lanthanides (Khoo *et al.,* 1984). Another problem with Mg^{2+} and Cd^{2+} is their preference to bind to nitrogen, rather than oxygen ligands. Cd^{2+} binds strongly to sulphur ligands. It exhibits considerable covalency in its bonds as opposed to the essentially ionic nature of the Ca^{2+} bonds (Martin, 1984).

Perhaps of more interest is the observation that 5 mM concentrations of these cations (except for Co^{2+}) increase the thermostability of Ak.1 protease significantly more than the lanthanides. It was expected that the reverse would

be true. It could be because either the lanthanides did not bind or are binding at sites not important for thermostability.

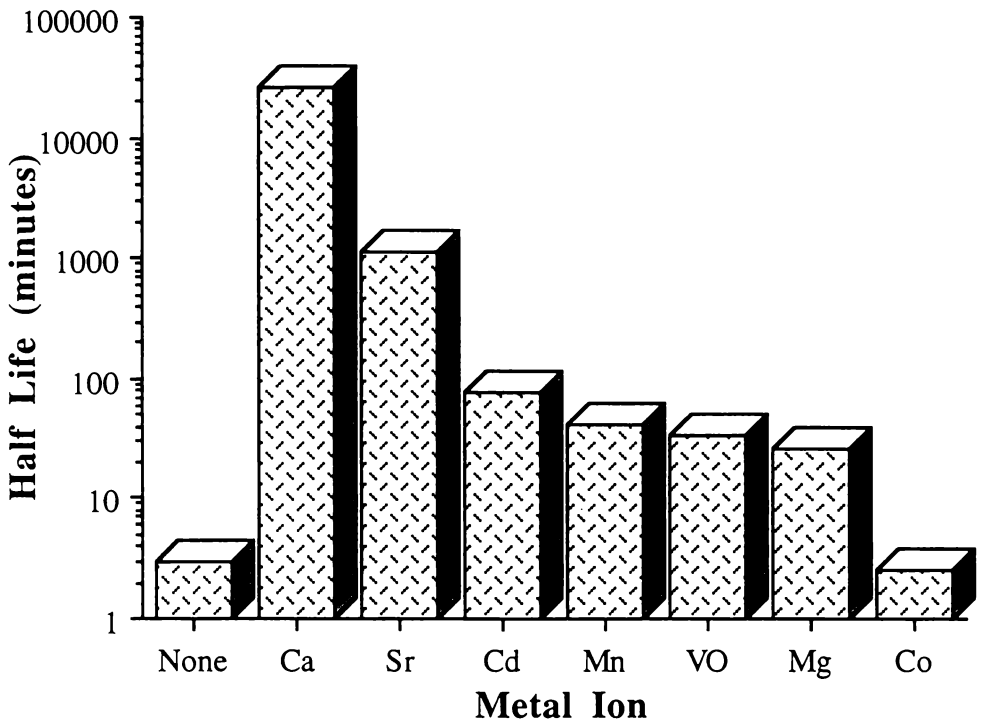


Figure 6.10 Thermostability of Ak.1 Protease at 70°C in the Presence of 5mM Cations.

The protease was incubated at 70°C in the presence of 0.5 mM metal ions for various time periods, then assayed for remaining activity with 0.2% azocasein (section 6.2.4). Note the log scale of the half life axis.

As with the lanthanides, 5 mM concentrations of some metals are toxic to some enzymes, so any stabilisation may be being masked by an inhibition of activity. Therefore, Ak.1 protease was incubated with 0.5 mM concentrations of these and other cations for 5 hours at 70°C as before.

Figure 6.11 shows the % activity remaining of the protease in the presence of these cations. These results show that the 10 fold reduction in Sr^{2+} levels resulted in very little stabilising effect on the protein. In contrast, Cd^{2+} and Mn^{2+} significantly stabilised the enzyme. This suggests that 5 mM concentrations of Cd^{2+} and Mn^{2+} either inhibited the activity of the protease, or destabilised it, or perhaps both.

Of interest is the small degree of stabilisation of the protease by Pb^{2+} ions. Heavy metals (e.g. Hg^{2+} and Pb^{2+}) and other metals such as Cd^{2+} and Zn^{2+} bind to proteins primarily at cysteine and methionine residues, often causing inhibition of enzymes. Yet these cations have actually stabilised the enzyme. It is possible that this stabilising influence is due to a decrease in autolysis of the protease in the presence of heavy metals.

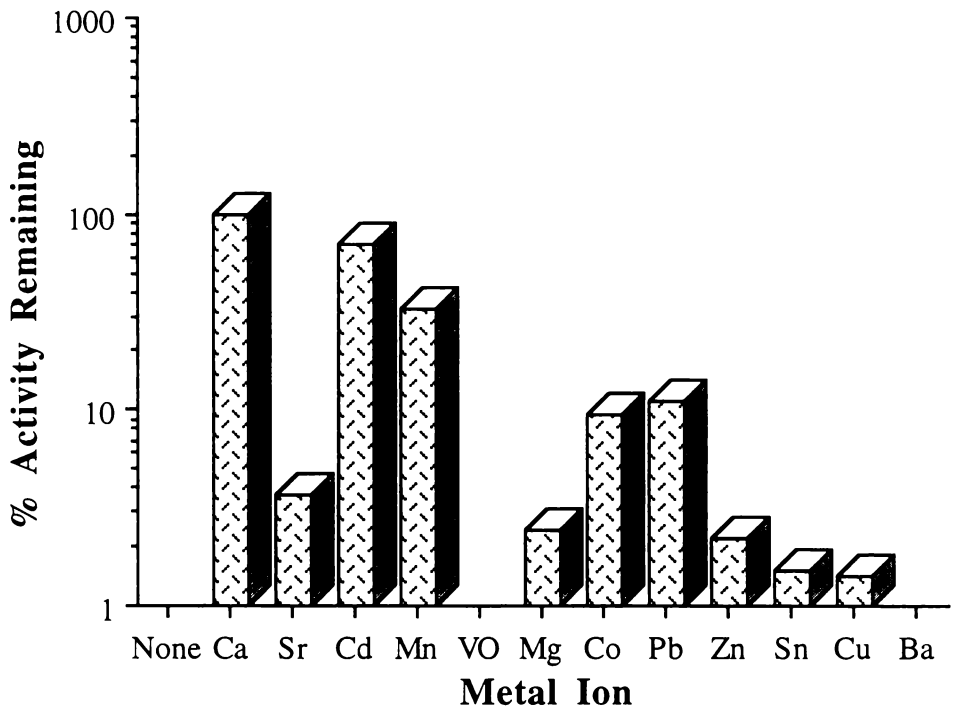


Figure 6.11 % Activity Remaining Vs Cation after 5 Hours at 70°C

The protease was incubated for 5 hours at 70°C in the presence of 0.5 mM metal ions for various time periods, then assayed for remaining activity with 0.2% azocasein (section 6.2.4).

6.3.5 Effect of Other Additives on Thermostability

Table 6.4 shows the effect of additives on the thermostability of Ak.1 protease. It should be noted that all these solutions are in the presence of 5 mM Ca^{2+}. These results show that the only significant stabilisers were 90% solutions of sorbitol, trehalose and glycerol. Figure 6.12 looks at the data of these three stabilisers more closely. Stabilisation by these solutions has been noted with other enzymes (e.g. Piller *et al.*, 1996).

Figure 6.12 shows that 90% sorbitol was the best stabiliser. Loss of stability with increasing temperature was initially slow, with 90% activity remaining after 15 minutes at 105°C. Above this temperature, the stability declined rapidly.

At these high temperatures (> 90°C), denaturation and autolysis are not the only possible causes of loss of activity. Degradative reactions such as deamidation, cysteine elimination and peptide bond hydrolysis are significant. All of these reactions require water, so a significant reduction in water activity (e.g. by the addition of salts and polyols) could possibly stabilise the enzyme.

The rate of these reactions are dependent on the conformational stability of the protein at a given temperature (Daniel, 1996). Thus, the prevention of unfolding could reduce the rate of these reactions. So 5 mM $CaCl_2$ was added to the buffer to minimize denaturation/autolysis of the enzyme and therefore increase its thermostability.

It should be noted that a lower than expected thermostability of the protease control (just 5 mM $CaCl_2$) was detected. This could be due to the use of tricine as the buffer. Tris buffers tend to chelate Ca^{2+} ions. If this was the case here, then the lower than expected thermostability could be due to the lowering of the [Ca^{2+}] available to the protease by the buffer. HEPES buffer was not used here as it did not have sufficient buffering capacity at pH 7.5 at temperatures > 90°C.

Additive	% Activity Remaining				
	90°C	95°C	100°C	105°C	115°C
Buffer only (control)	3	1	-	-	-
0.01% Triton X-100	3	0	-	-	-
1% Triton X-100	2	0	-	-	-
90% Sorbitol	99	103	90	86	0
90% Trehalose	97	96	-	-	-
90% Glycerol	92	97	80	62	2
40 µg/ml BSA	2	3	-	-	-
1% BSA	6	5	-	-	-
500 µg/ml Gelatin	4	0	-	-	-
5% Dextran	3	2	-	-	-
0.001% Tween-80	2	2	-	-	-
50% $(NH_4)_2SO_4$	12	5	-	-	-
1% Dithiothreitol	0	2	-	-	-
15% Betaine	17	2	-	-	-
0.8M NaCl	1	1	-	-	-
50 mg/ml Mannitol	5	1	-	-	-

Table 6.4 The Effects of Several Additives on the Stability of Ak.1 Protease.

The enzyme was incubated in 50 mM Tricine pH 7.5 + 5 mM $CaCl_2$ with the above additives for 15 minutes at the temperatures indicated, then cooled rapidly on ice. The remaining activity was determined by assaying the enzyme with 0.2% azocasein in 50 mM HEPES/NaOH pH 7.5 + 5 mM $CaCl_2$ for 20 minutes at 75°C. - = Not determined.

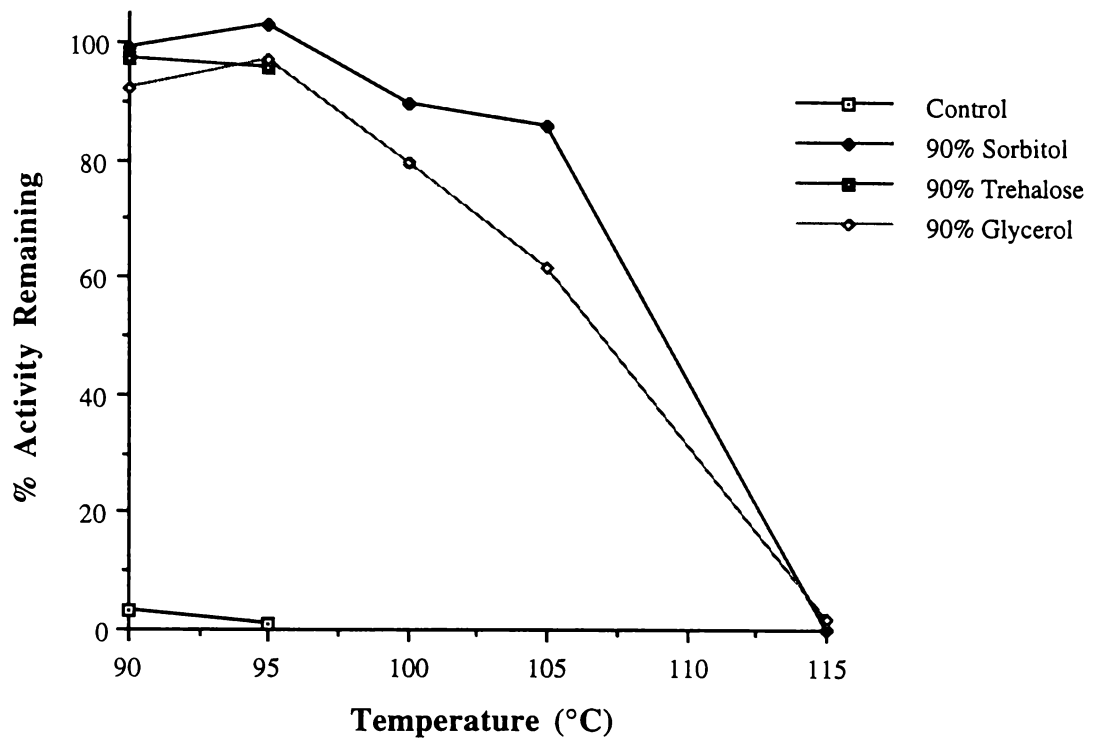


Figure 6.12 % Activity Remaining Vs Temperature of Ak.1 Protease With 90% Sorbitol, Trehalose and Glycerol.

The protease was incubated for 15 minutes at the above temperatures in the presence of 90% solutions of sorbitol, trehalose and glycerol. The remaining activity was determined using 0.2% azocasein at 75°C (section 6.2.4). Due to the tendancy of 90% trehalose solutions to precipitate once cooled down, only a few of the data points could be determined accurately.

Table 6.5 shows the half life of Ak.1 protease at 105°C in the presence and absence of 90% sorbitol. These results show that the half life has increased from <<1 min to 104 minutes at 105°C. The loss of activity is virtually instantaneous in the absence of sorbitol. This is a large increase in the thermostability of the protease. The first and second order plots show that denaturation is the major cause of loss of activity in the presence of 90% sorbitol. This is not surprising as there is a significant reduction in water activity, and water is required for the deacylation step of catalysis (and therefore autolysis).

Conditions	Half Life (min)
Control (buffer only)	~0
90% sorbitol in buffer	104

Table 6.5 Half Life of Ak.1 Protease at 105°C in the Presence and Absence of 90% Sorbitol.

The protease was incubated in sealed glass capillary tubes at 105°C for various time periods, then assayed for remaining activity with 0.2% azocasein (section 6.2.4). The 'buffer' consisted of 50 mM Tricine pH_{95} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100.

Yang *et al* (1996) investigated the effect of polyols such as methoxypolyethylene glycol (PEG) on the stability of subtilisin Carlsberg. The PEG-subtilisin had a higher thermostability than the native enzyme, but the transition temperature was not greatly changed. The kinetics of inactivation of the protease with temperature indicated that the loss of activity was due to a decrease in the rate of autolysis of the enzyme.

Gekko & Timasheff (1981) investigated the mechanism of stabilisation of the protease chymotrypsin by glycerol. In this case, it was found that the glycerol increased the melting temperature of the protease, effectively decreasing the rate of denaturation at a given temperature. It appears to be affecting the enzyme by stabilising hydrophobic interactions, which could result in either an acceleration of protein folding or an increase in the activation energy of unfolding.

What was surprising was that concentrated solutions of proteins (1% BSA) did not stabilise the enzyme. It was expected that protein solutions would stabilise the protease as enzymes are often stabilised by their substrates.

Ammonium sulphate stabilised the protease about 6 fold at 90°C. This property has been found with other proteases by others (Piller *et al.*, 1996). In these cases, stabilisation was due to the inhibition of autolysis of the protease.

6.4 CONCLUSIONS

Ca^{2+} stabilises proteins by typically binding into pockets of the protein. This effectively 'locks-up' loop/s of the protein that might otherwise be more mobile. These mobile loops are potentially more susceptible to localised denaturation and/or autolysis.

Ak.1 protease is very significantly stabilised by Ca^{2+} ions. The half life at 70°C increases from 3 minutes in the absence of Ca^{2+}, to 18 days in the presence of 5 mM Ca^{2+}. It was found that as the concentration of Ca^{2+} ions increased, the degree of denaturation decreased. At high Ca^{2+} concentrations, the major cause of loss of activity was due to autolysis.

Based on the structural and enzymatic data, the extra degree of stabilisation of Ak.1 protease by Ca^{2+} above that of thermitase-Ca^{2+} could be due to the presence of an extra Ca^{2+}-binding site in Ak.1 protease. This new site is located close to Ca(1), and could therefore change the binding properties of this site also. Due to the thermostability of Ak.1 protease increasing over 4 orders of magnitude, Ca^{2+} is a very important stabiliser of this enzyme. Another possibility is that the binding constants of one or more of the Ca^{2+}-binding sites could be higher for Ak.1 protease, as compared to thermitase. This could also increase the thermostability of the protease.

Lanthanide ions stabilised the protease, though to a much smaller degree than Ca^{2+}. Like 5 mM Ca^{2+}, they stabilised the protease by the prevention of denaturation. Other cations stabilised the protease to a small degree, especially Sr^{2+}. Different cations had different effects on the stability of the enzyme.

Other significant stabilisers were 90% solutions of sorbitol, trehalose and glycerol. At 105°C, 90% sorbitol increased the thermostability of the protease

from <<1 minute to 104 minutes. It appears to do so by the prevention of autolysis.

CHAPTER 7

SUBSTRATE SPECIFICITY OF AK.1 PROTEASE

7.1 INTRODUCTION

Subtilases are members of the clan (superfamily) of subtilisin-like serine proteases (Siezen & Leunissen, 1997). Over 200 members are known, 170 of which are are fully sequenced, including thermitase. Ak.1 protease was identified as a member of this clan by its sequence homology to thermitase. Siezen & Leunissen (1997) analysed the known subtilases and compared the sequences of the residues at the active site, a conserved region of proteases. They identified 6 families: Subtilisin, Thermitase, Proteinase K, Lantibiotic Peptidase, Kexin and Pyrolysin families. Ak.1 protease belongs to the thermitase family. Other members of this family include proteases from the bacterium *Thermoactinomyces* sp. E79 and archaea such as *Natriaba asiatica* 172 P1 and *Haloferax mediterranei* R4 (Siezen & Leunissen, 1997).

Figure 7.1 shows the amino acid sequence of Ak.1 protease and 9 other subtilisins. The shaded columns show the conserved amino acids.

The substrate specificity of many subtilases has been described previously (e.g. Rothe *et al.*, 1989). Three dimensional structures of some members of this clan have been determined, such as subtilisin Carlsberg and thermitase (e.g. Katz & Kossiakoff, 1990; Gros, *et al*, 1989). This enables a detailed analysis and comparison of the active site clefts of subtilases. This in turn can help to explain the substrate specificities seen.

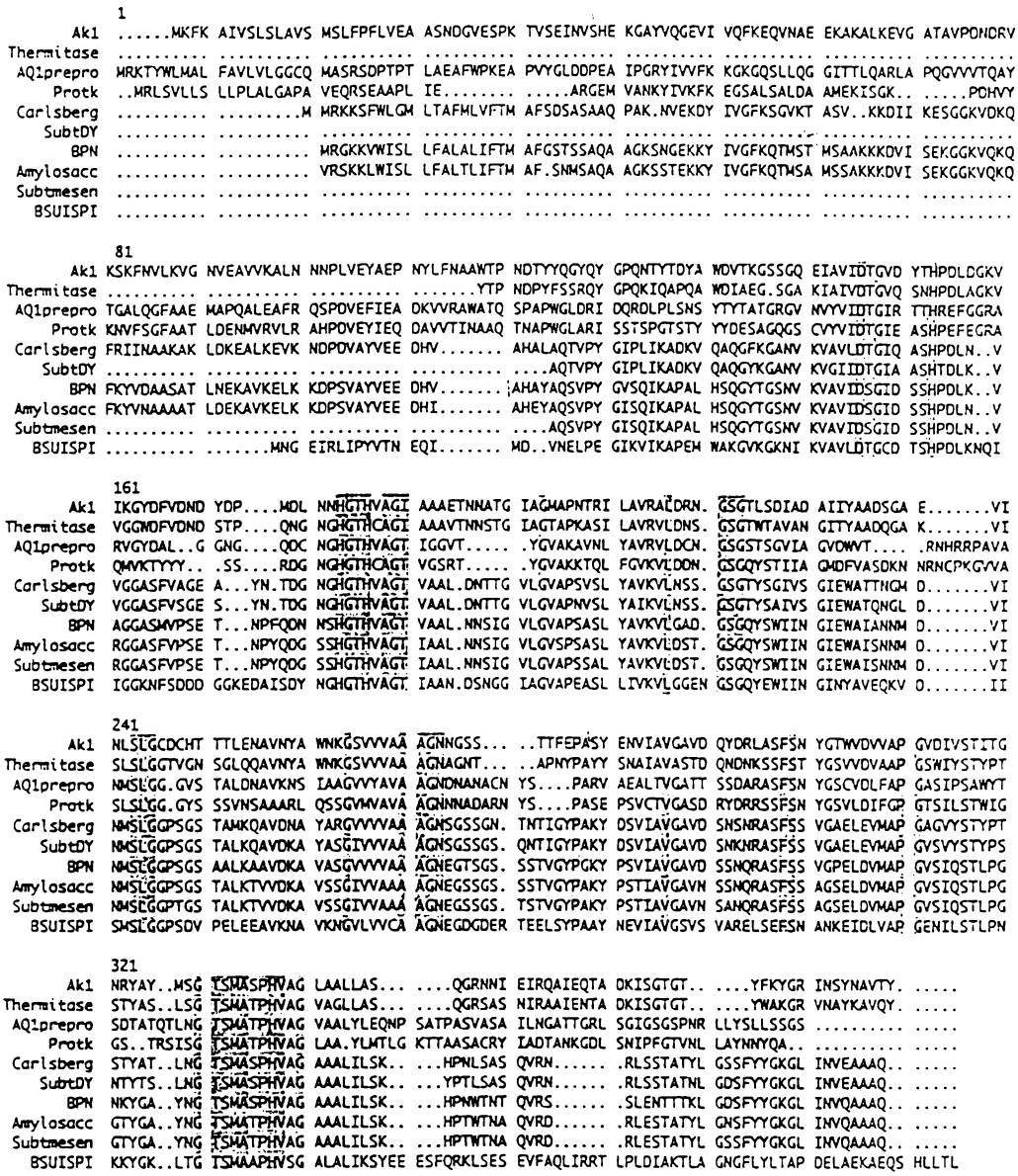


Figure 7.1 Sequence Comparison of Ak.1 Protease With 9 Other Subtilisins.
From Maciver *et al.*, 1994. The shaded columns indicate the conserved amino acids. Ak1 = protease from *Bacillus* st. Ak.1; thermitase = protease from *Thermoactinomyces vulgaris*; AQ1prepro = protease from *Thermus aquaticus* YT-1; Protk = protease from *Tritirachium album* Limber; Carlsberg = protease from *B. licheniformis*; SubtDY = protease from *B. subtilis* DY; BPN = protease from *B. amyloliquefaciens*; Amylosacc = protease from *B. amylosacchariticus*; Subtmesen = protease from *B. mesentericus*; BSUISPI = major serine protease from *B. subtilis*. Only Ak.1 protease and thermitase belong to the 'thermitase group' in Figure 7.1.

7.2 MATERIALS AND METHODS

7.2.1 Protein Substrates

Ak.1 protease was assayed with 0.2% concentrations of the proteins azocasein, cytochrome *c*, bovine serum albumin (BSA) and keratin in 50 mM Hepes/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C according to the method in section 2.2.1.

7.2.2 Peptide Substrates

The peptide substrates tested belonged to the following groups:

1. peptide-*p*-nitroanilide (pNA) section 2.2.2.1
2. peptide-p-nitrophenol (pNP)-ester section 2.2.2.2
3. peptide-methyl ester (OMe) or peptide-ethyl ester (OEt) section 2.2.2.3
4. Ac-Ala-αnapthyl ester (αNA) section 2.2.2.4
5. Furylacryl-peptide (FA) section 2.2.2.5

Activity towards a variety of synthetic peptide substrates was determined in a Perkin-Elmer Lambda #B spectrophotometer fitted with a thermoelectric cell holder. The assay mixture consisted of 1 ml of 1.0 mM substrate (50 μM for furylacryl-linked substrates) in 50 mM HEPES/NaOH pH 7.5 (at the specified temperature) containing 5 mM $CaCl_2$. The substrate was preincubated at the required temperature for 10 minues. 10 μl of protease solution was added, mixed and the absorbance change was monitored continuously using the Perkin-Elmer Computerised Spectroscopy Software. Further details, e.g. wavelengths and unit definitions can be found in the sections listed above.

For substrates not completely soluble in water (e.g. *p*-nitrophenol-ester-linked substrates) 50% acetonitrile was included in the substrate buffer. These substrates were assayed at 40°C due to the increased risk of flammability of the solvent at higher temperatures. Methyl- and ethyl-ester-linked substrates were assayed at 20°C as these substrates decompose significantly at higher temperatures.

7.2.3 Michaelis Menten Kinetics

Activity towards the peptide-pNA substrates was determined according to the method described in section 2.2.2.1. The assay mixture consisted of 1 ml of different concentrations of substrate in 50 mM HEPES/NaOH pH 7.5 (at the required temperatures) containing 5 mM $CaCl_2$ and 0.01% Triton X-100 (in later experiments, the substrate volume was 100 µl, and the calculations were adjusted accordingly). The substrate was preincubated at the required temperature for 10 minues. 10 µl of protease solution was added, mixed and the absorbance change was monitored continuously at 400 nm. The results were expressed grapically on Michaelis Menten and Lineweaver Burk plots, and Direct Linear plots were used to determine the K_m and V_{max}.

Activity of Ak.1 protease with SAAPFpNA and SAAPApNA was determined in the presence of 100 mM NaCl, 50% methanol and 50% sorbitol. The same method as above was used, except the temperature was 40°C and the assay solution were mixed more extensively to overcome the problems of high viscosity.

7.3 RESULTS AND DISCUSSION

7.3.1 Structure of the Active Site

The 3-dimensional structure of Ak.1 protease revealed the nature of the active site (see Figures 6.2 and 8.1; Smith *et al.*, 1998)). It exhibits a typical subtilisin-like arrangement of the active site residues. Figure 7.2 shows the backbone structure of the active site. The active site residues (Ser 266, His 72 and Asp 39) are shown more fully.

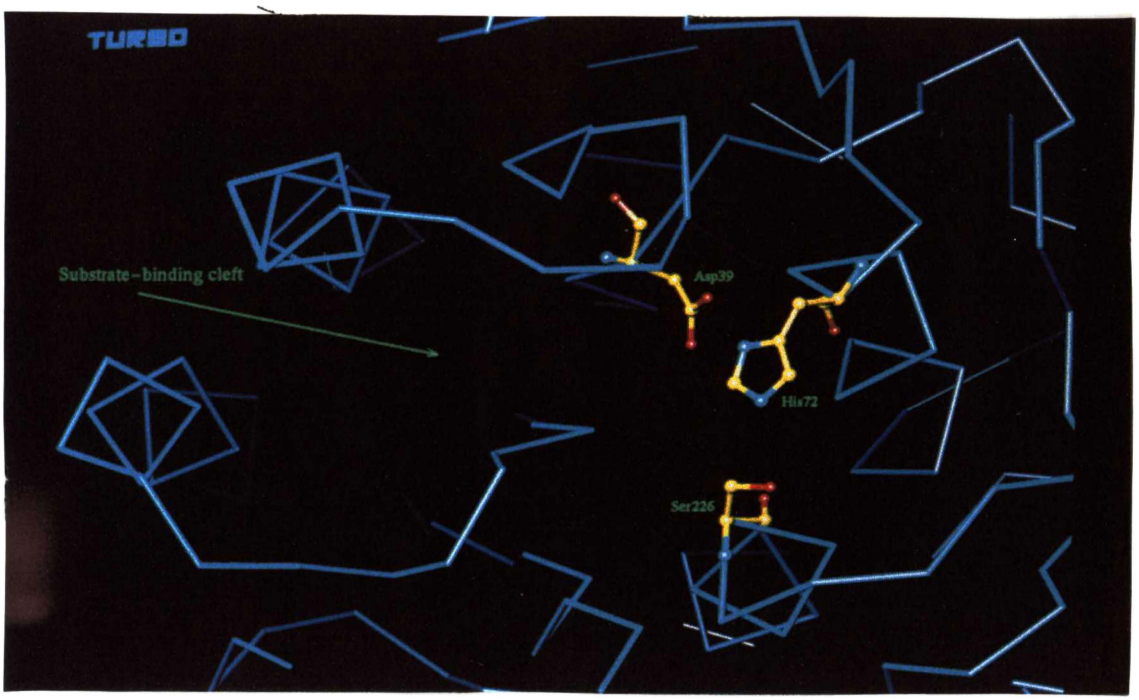


Figure 7.2 Backbone Structure of the Active Site of Ak.1 Protease.
Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

The active site has the appearance of a "cleft" on the surface of the enzyme. It can accomodate an extended chain of substrate (up to S_4-S_5). The Ser 226 and His 72 residues are flanked by α-helices. The active site cleft is very hydrophobic. Figure 7.3 is a space-filling model of the substrate SAAPFpNA in the active site of the protease.

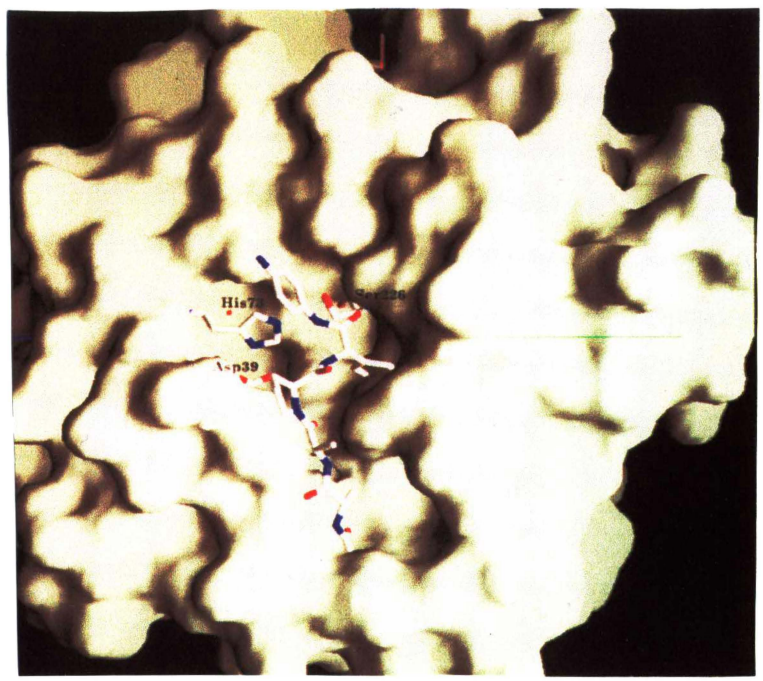


Figure 7.3 Model of the Substrate Suc-Ala-Ala-Pro-Phe-pNA in the Active Site of Ak.1 Protease.

Photograph supplied courtesy of Dr Clyde Smith (Auckland University, New Zealand).

The models show that the active site is a groove on the surface of the enzyme. The active site residues, and the S_1-S_2 sites, are located in a large bottle-shaped pocket. The residues at this site are neutral to acidic. The pNA group (leaving group) appears to point outwards, away from the protein, while the P_1 R-group fits in the pocket. There is a marked bend near Asp 39, corresponding to the P_2 subsite, requiring the substrate to bend at this point. However, it must be stressed that the position of the substrate in Figure 7.14 was not determined crystallographically, rather it was positioned by molecular modelling (Smith *et al.*, 1998).

The S_3 binding site resembles a narrow neck, or crevasse. The S_4 site is in another pocket on the surface of the molecule. An interesting difference between Ak.1 protease and other subtilisins' is the location of the disulphide bond. It forms a tight loop in the substrate-binding cleft. The disulphide bond is in the S_4 position. More will be discussed about the structure around the disulphide bond in Chapter 8. The S_5 site is flanked by two α-helices.

When the crystal structure was determined (Smith *et al.*, 1998), it was discovered that 9 amino acids (residues 166-174) were apparently "missing" from the structure. It appears that the protease had undergone autolysis. Amino acid sequencing showed that there had been a cleavage in the protease between residues 174-175. However, the residues 166-174 were still present in the molecule, as only one cleavage had occurred. Thus, it appears that the residues 166-174 apparently have a high mobility, so there was not sufficient electron density to map their positions in the molecule.

Figure 7.4 shows the sequence of the protease around the "missing" amino acids. This break is located near the Ca(3) binding site (see Figure 6.5) and the disulphide bond.

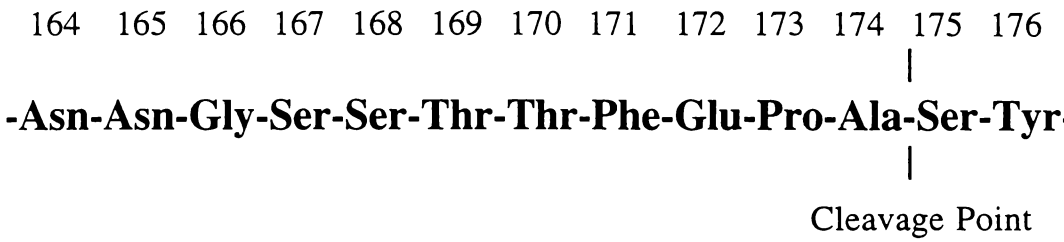


Figure 7.4 Sequence of Ak.1 Protease Around the Cleavage Site.
Partial amino acid sequence of Ak.1 protease around the autolytic site. Cleavage of the protease was between residues 174-175. Residues 166-174 were not seen in the 3D structure

7.3.2 Activity of Ak.1 Protease Towards Protein Substrates

Table 7.1 shows the specific activity of the protease towards several protein substrates. All the assays were conducted in the same way, and the activities were all determined at 280 nm to allow direct comparisons of the activities with the different proteins. This shows that the protease is more specific for azocasein above the other protein substrates tested. It should be noted that azocasein was tested rather than unmodified casein due to the solubility problems of native casein. Ak.1 protease has a very low activity towards keratin.

Appendix 1 shows the relationship between absorbance at 280 nm and enzyme concentration with protein substrates. This relationship is usually linear only over a small absorbance change (e.g. 0.1-0.2). However, when cytochrome *c* is the substrate, the relationship is linear up to an absorbance of about 1.5. This was also seen with an aspartic protease from *Bacillus* st. Wp22.A1 (Toogood, 1995). This makes cytochrome *c* a desirable protein substrate for proteases.

Substrate	Specific Activity (U/mg)
Azocasein	520
Cytochrome *c*	32
Bovine Serum Albumin (BSA)	10
Keratin	1.2

Table 7.1 Specific Activity of Ak.1 Protease Towards Protein Substrates.
Ak.1 protease was assayed with 0.2% concentrations of the proteins azocasein, cytochrome *c*, bovine serum albumin (BSA) and keratin in 50 mM Hepes/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C according to the method in section 2.2.1. 1 Unit is described as the amount of enzyme required to produce an OD280 nm of 1.0 per minute.

7.3.3 Activity of Ak.1 Protease Towards Peptide Substrates

7.3.3.1 Reasons for the Use of 1mM Peptide Substrates

The results in Chapters 7 and 8 show a comparison of the specific activities of Ak.1 protease with a variety of peptide substrates at 1 mM concentrations. In later experiments, activity was determined mostly with 1 mM Suc-Ala-Ala-Pro-Phe-pNA (SAAPFpNA). This concentration is approximately equal to the K_m of Ak.1 protease at 75°C with this substrate, though it is approximately x10 the K_m at 20°C. Ideally, 10-20 mM SAAPFpNA should have been used, as the enzyme should be in the presence of excess substrate. There are 3 reasons why 1 mM SAAPFpNA was used.

(1) Solubility: The maximum concentration of SAAPFpNA obtainable without the addition of large quantities of organic solvents is approximately 4 mM. Organic solvents, such as acetonitrile and methanol, significantly affect some properties of the protease (e.g. K_m), so it was desirable that they were not present.

(2) Cost: The substrate is very expensive. Due to the large numbers of assays required, and the high cost of the substrate, a lower substrate concentration was used.

(3) Stability of the Substrate: At the higher concentrations of SAAPFpNA, > 2 mM, the background activity due to the instability of the substrate became quite significant. This increases the experimental errors considerably, and prevents accurate determinations of low protease activity.

However, in most cases, after activity had been determined with 1 mM SAAPFpNA, this was followed up with the determination of the K_m and V_{max} of the protease. Thus, the results were analysed according to changes in the K_m and V_{max} of the protease rather than basing interpretations solely on specific activity differences with 1 mM substrates. In a few cases, such as determining the thermostability of Ak.1 protease in the presence of dithiothreitol (Chapter 8), there were no follow up kinetics experiments. This occurred as it was important that SAAPFpNA was used as the substrate, rather than a protein substrate due to uncertainty of the effect/s of DTT on the protein substrate.

7.3.3.2 Effect of the Size of Substrates on Specific Activity

Table 7.2 shows the specific activities of Ak.1 protease towards a variety of peptide-*p*-nitroanilide (-pNA) substrates. The substrates are ordered according to decreasing size. Table 7.3 shows the specific activities of the protease with a variety of peptide-ester substrates. The substrates are ordered according to decreasing activity.

These results show that except with Ala-αNE, there was no activity of Ak.1 protease with substrates which occupy only the P_1-P_1' sites (e.g. Ala-pNA and Phe-pNA). However, activity was detected with a few substrates which occupy the P_2-P_1' sites. The best of these substrates is *CBZ*-Leu-pNP. Activity was also detected with *CBZ*-Nle-pNP and *CBZ*-Ala-pNP, but not with *CBZ*-Ile-pNP and other *CBZ*-amino acid-pNP substrates.

This shows that Ak.1 protease has a preference for hydrophobic, branched-chain amino acids in the P_1 site of the protease. This is not surprising as the active site cleft is very hydrophobic, thus favouring the binding of hydrophobic substrates. This also shows that the size of the substrate is an important factor determining the specific activity of the protease with a substrate. This suggests that the protease has a requirement for the P_2 site to be occupied before significant binding of the substrate can be achieved. .

For example, activity was detected with Phe at the P_1 site. This is typical of chymotrypsin-like proteases. However, while activity was detected when phenylalanine was at the P_1 position of two of the substrates, SAAPFpNA and SFAAFpNA, there was no activity against several Phe-endgroup substrates e.g. Phe-pNA and Phe-pNP ester. This could be due to the differences in the chain lengths of the substrates.

A comparison of the alanine-based pNA substrates (Table 7.2) shows that the specific activity increases with increasing chain length. This was seen when comparing the specific activities of Ala-Ala-pNA & Ala-pNA, and also the pair of substrates *Suc*-Ala-Ala-Ala-pNA and *Suc*-Ala-Ala-pNA. This was also seen with thermitase (Kleine, 1982).

Substrate	Temp (°C)	Specific Activity (U/mg)
No Acetonitrile		
Suc-Ala-Ala-Pro-**Phe**-pNA	75	313.79
Suc-Ala-Ala-Pro-**Leu**-pNA	75	103.03
Suc-Ala-Ala-Pro-**Ala**-pNA	75	25.00
Suc-Ala-Ala-**Ala**-pNA	75	16.89
Suc-Ala-**Ala**-pNA	75	0.56
Ala-**Ala**-pNA	75	0.75
Glu-**Phe**-pNA	75	< 0.10
CBZ-**Phe**-pNA	75	< 0.10
Suc-**Phe**-pNA	75	< 0.10
Ac-**Phe**-pNA	75	< 0.10
Phe-pNA	75	< 0.10
Gly-pNA	75	<0.10
Ala-pNA	75	< 0.10
50% Acetonitrile		
Suc-Ala-Ala-Ala-Ala-**Ala**-pNA	40	0.59
Suc-Phe-Ala-Ala-**Phe**-pNA	40	2.82
Suc-Ala-Ala-**Ala**-pNA	40	0.22
Val-Leu-**Lys**-pNA	40	< 0.10
Suc-Ala-**Ala**-pNA	40	< 0.10
Ala-Ala-**Phe**-pNA	40	0.18
Z-Lys-**Arg**-pNA	40	< 0.10
CBZ-**Arg**-pNA	40	< 0.10
Bz-**Arg**-pNA	40	< 0.10
Bz-**Tyr**-pNA	40	< 0.10

Table 7.2 Substrate Specificity of Ak.1 Protease with p-Nitroanilide Substrates in Order of Decreasing Size.

Assays at 75°C were conducted with 1 mM peptide-pNA in 50 mM Hepes/NaOH pH75 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100. Assays at 40°C were conducted with 1 mM peptide-pNA in 50 mM Hepes/NaOH pH75 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100 + 50% acetonitrile. Units = µmol pNA/min/mg. Extinction coefficients = 10500 and 9400 for 0 and 50% acetonitrile buffers, respectively.

Substrate	Temp (°C)	Specific Activity (U/mg)
p-Nitrophenol Ester		
CBZ-**Leu**-pNP	40	1.38
CBZ-**Nle**-pNP	40	0.45
CBZ-**Ala**-pNP	40	0.42
CBZ-**Val**-pNP	40	< 0.10
CBZ-**Ile**-pNP	40	< 0.10
CBZ-**Gly**-pNP	40	< 0.10
CBZ-**Tyr**-pNP	40	< 0.10
CBZ-**Trp**-pNP	40	< 0.10
CBZ-**Pro**-pNP	40	< 0.10
CBZ-Benz-**Asp**-pNP	40	< 0.10
CBZ-Benz-**Cys**-pNP	40	< 0.10
Methyl Ester		
Ac-Ala-Ala-**Ala**-OMe	20	28.94
Ac-Ala-**Ala**-OMe	20	42.04
Asp-**Phe**-OMe	20	< 0.10
Phe-OMe	20	< 0.10
Ac-**Tyr**-OMe	20	< 0.10
Gly-OMe	20	< 0.10
Ethyl Ester		
Ac-**Phe**-OEt	20	< 0.10
Ac-**Trp**-OEt	20	< 0.10
Tos-**Arg**-OEt	20	< 0.10
Bz-**Arg**-OEt	20	< 0.10
Other Substrates		
Ala-αNE	40	873.82

Table 7.3 Substrate Specificity of Ak.1 Protease with Peptide-Ester Substrates.

1 mM peptide-pNP ester in 50 mM Hepes/NaOH pH_{75} 7.5 + 0.01% Triton X-100 + 50% acetonitrile. Units = μmol pNP/min/mg; 1 mM peptide-methyl or ethyl-ester in 5 mM Hepes/NaOH pH_{20} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100 + 0.0025% bromothymol blue. Units = OD_{615} of 1.0/min/mg; 1 mM Ala-α-napthyl ester in 50 mM Hepes/NaOH pH_{40} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100. Units = μmol NAP/min/mg.

To investigate this further, the specific activity of some of these substrates were compared to that of *Suc*-Ala-Ala-Ala-Ala-Ala-pNA (SA_5pNA). Due to the insolubility of the later substrate, 1mM of the substrates were tested in 50 mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 50% acetonitrile at 40°C. Figure 7.5 shows the specific activities of Ak.1 protease towards 3 peptide substrates, which differ only by the number of alanine residues in the substrate. This shows that the specific activity does indeed increase with chain length.

Table 7.4 compares the specific activities of two of the substrates in the presence and absence of 50% acetonitrile. These results show that the specific activity dramatically decreases in the presence of the cosolvent.

	Specific Activity (µmol/min/mg)	
Substrate	No Acetonitrile	50% Acetonitrile
Suc-Ala-Ala-Pro-Ala-pNA	10.1	0.52
Suc-Ala-Ala-Ala-pNA	7.5	0.22

Table 7.4 Effect of Acetonitrile on the Specific Activity of Ak.1 Protease with 2 Substrates at 40°C.

1 mM peptide-pNA in 50 mM Hepes/NaOH pH_{75} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100 +/- 50% acetonitrile at 40°C. Units = µmol pNA/min/mg;

Absorbance change with respect to time was curved with the substrate SA_5pNA even at low changes of absorbance (e.g. 0.05). Thus, the very initial rates (first 5-10 seconds) were determined. This may be due to cleavage within the peptide, at a site other than at the P_1 site, generating a smaller -pNA substrate. As the protease exhibits lower specific activity towards smaller substrates, the curved relationship may be due to the production of increasing amounts of smaller -pNA substrates which the protease cleaves with a lower specific activity. This curved relationship was not detected with smaller peptide substrates (except at very high enzyme concentrations where the substrate is limiting or where the substrate concentration is very low). This suggests that a substrate containing 6 potential 'amino acids' (e.g. SA_5pNA) has exceeded the size of the binding cleft.

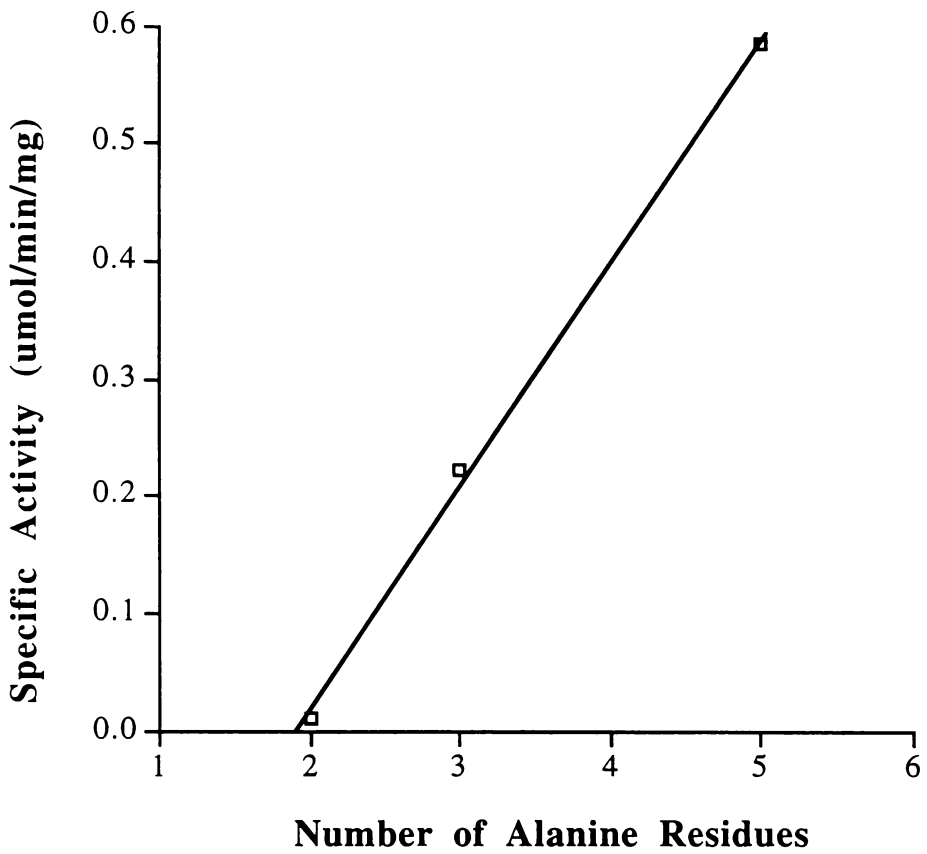


Figure 7.5 Relationship Between the Specific Activity of Ak.1 Protease and the Chain Length of the Substrate.

Ak.1 protease was assayed with the substrates SAApNA, SAAApNA and SAAAAApNA. 1mM substrate was in 50 mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 50% acetonitrile. 1 minute assays were performed at 40°C.

The stability of the protease in 50% acetonitrile was tested to determine if the nonlinearity of the assay with SA_5pNA was at least partly due to the instability of the protease under these conditions. Figure 7.6 shows there is a small loss of activity over a 15 minute time period in 50% acetonitrile at 40°C. As the protease is in the presence of the solvent for only 1 minute, it is likely that the enzyme has suffered not much activity loss during this time.

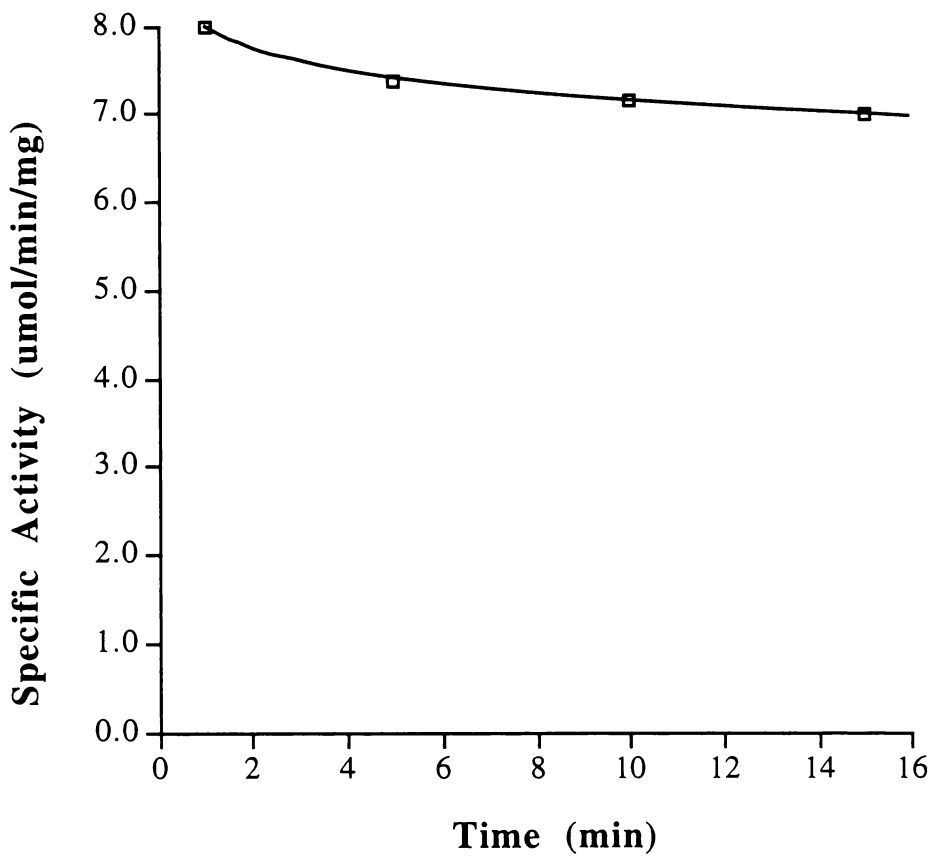


Figure 7.6 Stability of Ak.1 Protease to 50% Acetonitrile at 40°C.
Ak.1 protease was incubated with 50% acetonitrile at 40°C for various time periods, then assayed immediately for remaining activity at 20°C with 1 mM SAAPFpNA according to the method in section 7.2.2.

7.3.3.3 Effect of P1-P5 Amino Acids on Specific Activity

Table 7.2 shows a comparison of the specific activities of Ak.1 protease with 1 mM amino acid/s-*p*-nitroanilide (aa-pNA) substrates at 75°C in order of decending specific activity. Ak.1 protease shows significant activity towards SAAPFpNA, a typical subtilisin-type substrate. It also exhibits significant activity towards SAAPLpNA and SAAPApNA, which differ in structure only at the P_1 amino acid. This is consistant with the autolytic site of the protease (Figure 7.4). The two amino acids before the cleavage site (i.e. -S_2-S_1-) are -Pro^{173}-Ala^{174}-.

Figure 7.7 shows the structures of the amino acids phenylalanine, leucine and alanine. The R-group of Phe consists of a methyl group and a benzene ring. Leu has an isopropyl group, a structure similar to half the R-group of Phe. Ala only has the methyl group. Thus, the decrease in the specific activity of the protease could be partly due to the decrease in hydrophobicity of the P_1 amino acid.

This shows that as the hydrophobicity and size of the R group of the P_1 amino acid increases, the specific activity of the protease increases. This supports the idea that the protease has a preference for hydrophobic and/or branched-chain amino acids at the P_1 site.

The protease showed activity towards a variety of multiple alanine-based substrates, but activity was detected with Ala-pNA only after an incubation of 24 hours at 75°C. Ak.1 protease also showed significant activity towards alanine-based methyl esters, in spite of the temperature of assay being 55°C below the normal assay temperature of the protease.

The protease showed the best activity with -PNA substrates containing Ala-Ala-Pro in the P_4-P_2 sites of the binding cleft. Thermitase shows a preference for *hydrophobic* groups at the P_3-P_4 sites. Ak.1 protease readily cleaves substrates containing alanine at these sites. It is unclear whether the decrease in activity with smaller substrates is solely due to a decrease in chain length, or in part due to the introduction of a *hydrophilic* succinyl-group at the P_2-P_4 sites in smaller substrates.

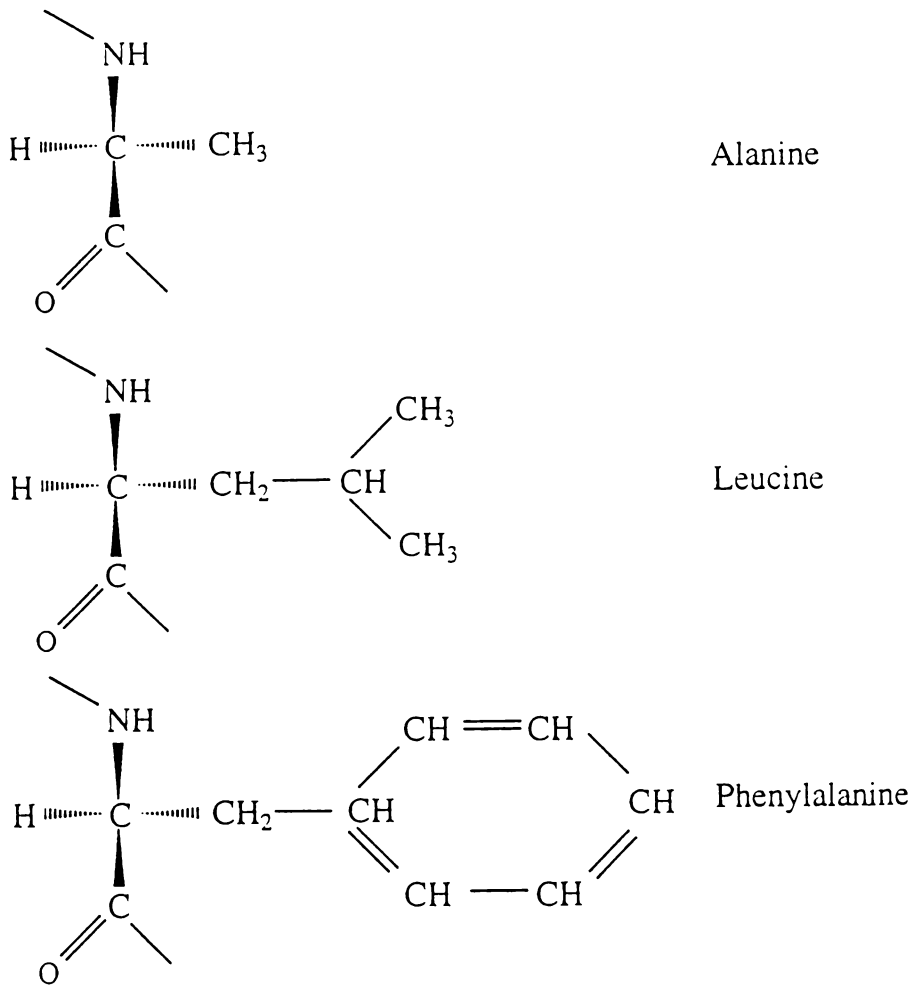


Figure 7.7 Comparison of the R-Groups of Three Amino Acids.

It was unable to cleave a number of trypsin and chymotrypsin-type substrates which contain arginine or tyrosine at the P_1 site (Table 7.3). However, these were all small substrates, containing only one or two amino acids. As the protease prefers long-chained substrates, activity with these amino acids at the P_1 site cannot be excluded. It was also unable to cleave the substrates *FA*-Leu-Gly-Pro-Ala, *FA*-Gly-Leu-NH_2 and *FA*-Phe-Gly-Gly.

7.3.4 Michaelis Menten Kinetics

7.3.4.1 Substrate Specificity of Ak.1 Protease at 75°C

Table 7.5 shows the K_m, V_{max} and specificity data (V_{max}/K_m) of Ak.1 protease with 5 substrates in the temperature range of 5-75°C. Figures 7.8-7.9 shows an example of Michaelis Menten/Lineweaver Burk plots, and a Direct Linear plot using the results with the SAAPFpNA substrate at 40°C. The remainder of the plots are located in Appendix 4.

Both SAAPFpNA and SAAPApNA have similar K_m's, while the other substrates (SAAPLpNA, SAAApNA and SAApNA) have higher K_m's. SAApNA has a relatively high K_m of about 17 mM. This suggests that it binds to the protease weaker than the other substrates. Earlier results (section 7.3.3.2) suggested that the protease has a requirement for the P_2 site to be occupied before significant cleavage of the substrate can occur. The later substrate occupies the sites P_3-P_1'. Thus, it appears that Ak.1 protease does not significantly cleave smaller substrates as they bind with a low affinity to the enzyme.

Ak.1 protease has a higher specificity (V_{max}/K_m) for substrates containing Pro at the P_2 position. Proline is an unusual amino acid which causes a marked bend in the substrate. There is also a bend in the protease near Asp 39 (Figure 7.3) which is at the P_2 position. Therefore, it is not surprising that the protease has a high specificity for Pro at the P2 position as the bend in the substrate may allow it to fit better in the binding cleft than a substrate without Pro in this position. However, it must be stressed that the orientation of the substrate in Figure 7.3 was not determined crystallographically, rather it was positioned by molecular modelling (Smith *et al.*, 1998).

The V_{max} of the protease decreases as the size of the substrate decreases, as does the specificity.

Substrate	K_m			V_{max}			V_{max}/K_m		
	5°C	40°C	75°C	5°C	40°C	75°C	5°C	40°C	75°C
Suc-Ala-Ala-Pro-Phe-pNA	0.07	0.30	1.00	0.8	69.6	580	11.3	235	580
Suc-Ala-Ala-Pro-Leu-pNA	0.68	1.01	3.37	2.1	47.6	442	3.1	47.1	131
Suc-Ala-Ala-Pro-Ala-pNA	0.33	0.33	0.92	1.2	13.2	68	3.2	40.0	73.5
Suc-Ala-Ala-Ala-pNA	0.28	0.63	2.25	1.2	12.3	54	4.2	19.5	21.2
Suc-Ala-Ala-pNA	-	-	16.7	-	-	20	-	-	1.2

Table 7.5 Km, Vmax and Specificity of Ak.1 Protease with Several Substrates at 3 Temperatures.

1 ml of substrate in 50 mM Hepes/NaOH pH 7.5 (at the temperatures specified) + 5 mM $CaCl_2$ + 0.01% Triton X-100. Vmax values expressed as µmol pNA/min/mg.

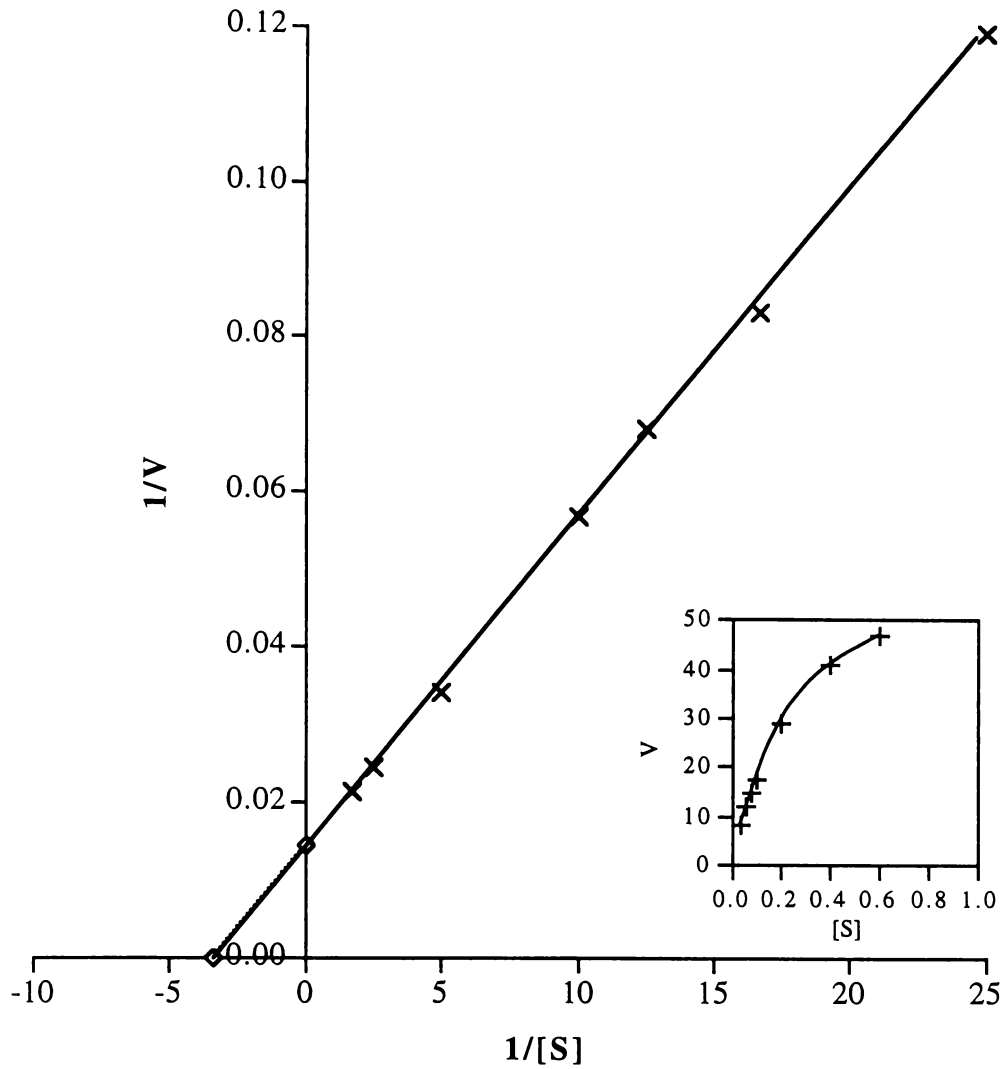


Figure 7.8 Lineweaver Burk Plot of Ak.1 Protease With SAAPFpNA at 40°C (inset is the Michaelis-Menten Plot).

Ak.1 protease was assayed at 40°C with different concentrations of the substrate SAAPFpNA in 50 mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 (section 7.2.3).

*The diamond-shaped data points on the x and y axes were obtained from the K_m and V_{max}, respectively, derived from the Direct Linear Plots. These points were plotted to enable the correct line to be drawn between the other points on the Lineweaver Burk Plot.

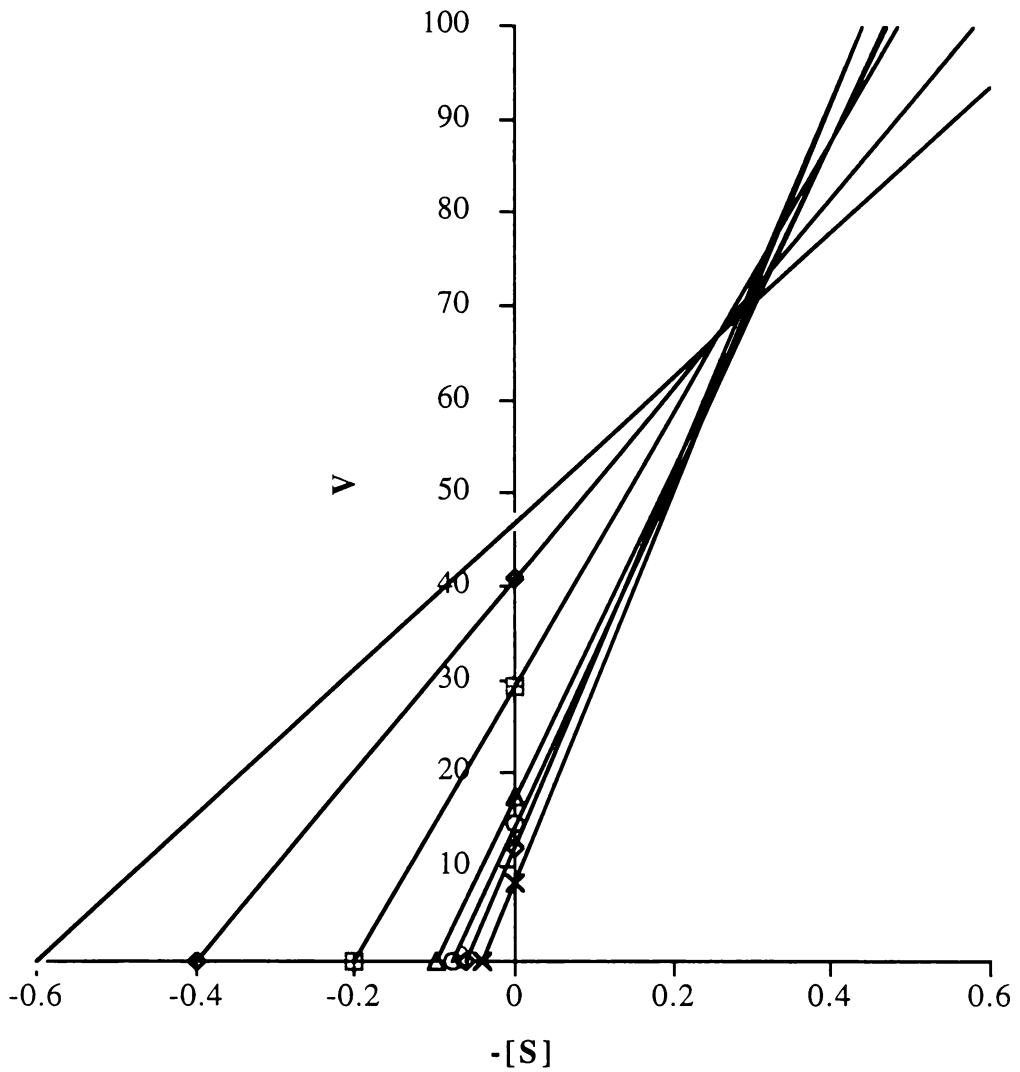


Figure 7.9 Direct Linear Plot of Ak.1 Protease With SAAPFpNA at 40°C
Ak.1 protease was assayed at 40°C with different concentrations of the substrate SAAPFpNA in 50 mM Hepes/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 (section 7.2.3).

7.3.4.2 Effect of Temperature on Km and Vmax

i) Effect of Temperature on the K_m of Ak.1 Protease

Table 7.5 shows the K_m, V_{max} and specificities of several of the substrates at 3 different temperatures. Figure 7.10 shows the % change in the K_m of the protease with 4 substrates with respect to temperature.

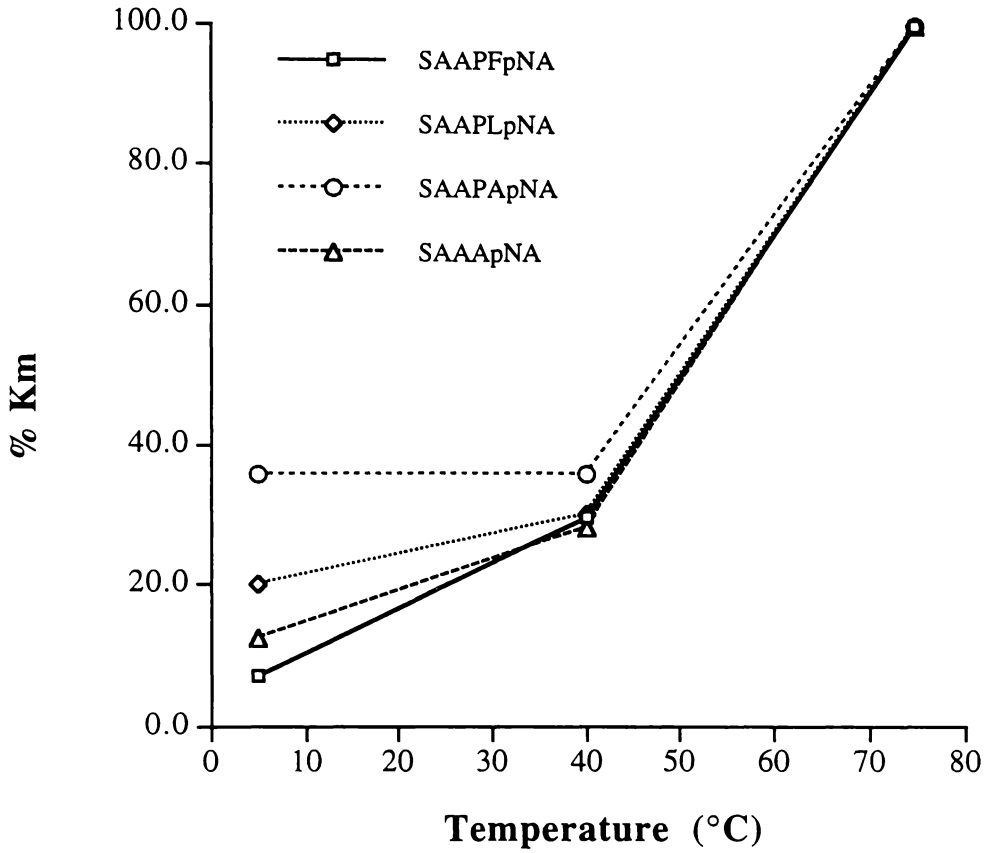


Figure 7.10 %Change in K_m with Respect to Temperature.

Ak.1 protease was assayed in the temperature range of 5-75°C with different concentrations of 4 substrates in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 (section 7.2.3).

The data shows that as a general rule, as the temperature decreases, the K_m decreases. The most significant change in the K_m occurs in the temperature range of 40-75°C. However, the % decrease in the K_m is different with different substrates. A comparison of the decrease in the K_m of the substrates SAAPXpNA, where X = Phe, Leu and Ala respectively, shows that as the size and hydrophobicity of the R-group of the P_1 amino acid decreases (Phe->Ala), the % change in the K_m decreases. In the case of SAAPApNA, there is no change in the K_m between 5-40°C. This shows that *the specificity of the protease changes with temperature.*

As the temperature decreases, the flexibility of a protein also decreases. Thus, a substrate in the binding cleft of an enzyme at 5°C is likely to be bound tighter than at 75°C, where the enzyme and substrate are more flexible. Therefore, it is not unexpected that the K_m of an enzyme decreases with temperature.

While the K_m of the protease with SAAPApNA is unchanged in the temperature range of 5-40°C, there is a significant decrease in the K_m of the protease with SAAApNA from 40-5°C. Both these substrates containing Ala at the P_1 position. This shows that the change in K_m with temperature is not solely dependent on just the P_1 amino acid, but is likely to be dependent on the structure of the whole substrate.

Mahan *et al* (1990) investigated the effect of temperature on the apparent K_m of glutathione reductases from the mesophiles spinach, corn and cucumber. They found that the K_m was the lowest at the lower end of the 'thermal kinetic window' (average range of temperatures the organism lives in). In other words, at temperatures lower or higher than the average temperature range of the organism, the K_m increased. For example, corn has an average growth range of 23-32°C, while that of cucumber was between 35-41°C. The K_m of their respective glutathione reductases was at it lowest point within this range. At temperatures above and below this range, the K_m steadily increased. They concluded that the K_m of enzymes is conserved and minimum around the habitat temperature.

However, the situation is very different for Ak.1 protease. *Bacillus* st. Ak.1 is grown optimally around 70-75°C, yet the K_m of Ak.1 protease decreases significantly at much lower temperatures. Possibly, the above mesophilic enzymes have adapted to large fluctuations in their habitat temperature.

ii) Effect of Temperature on the V_{max} of Ak.1 Protease

Figure 7.11 shows the % change in the V_{max} of the substrates with respect to temperature. These results show that the V_{max} decreases with decreasing temperature. It also shows that the % change in the V_{max} is approximately the same for all 4 substrates.

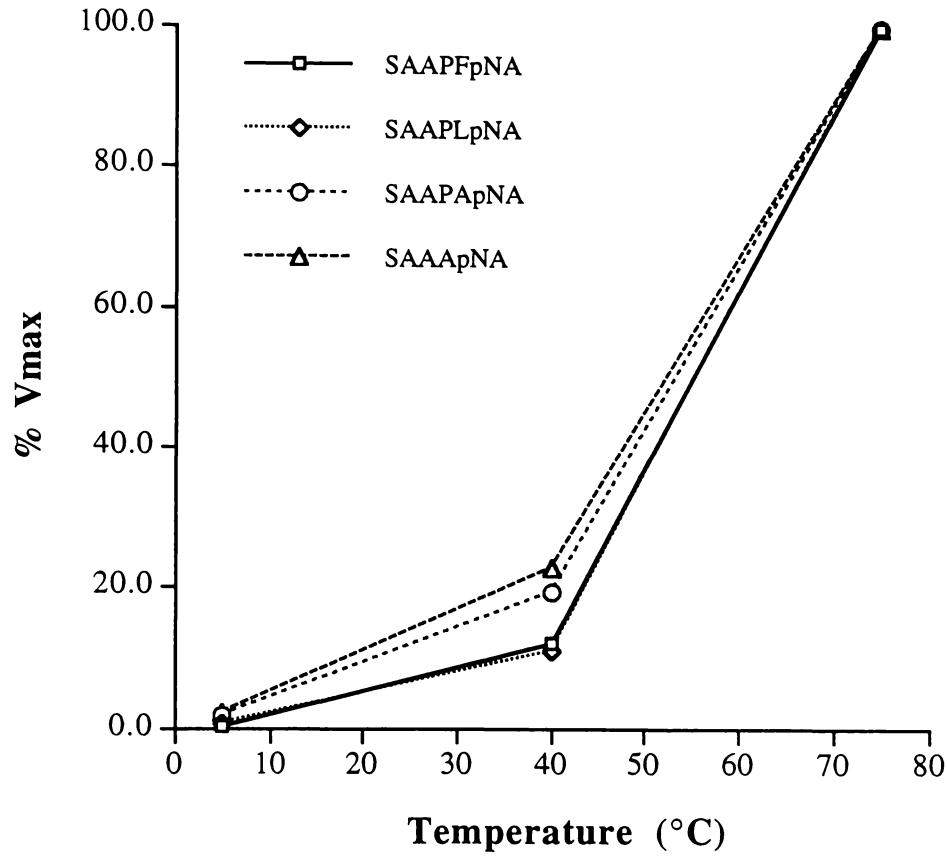


Figure 7.11 %Change in V_{max} with Respect to Temperature.

Ak.1 protease was assayed in the temperature range of 5-75°C with different concentrations of 4 substrates in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 (section 7.2.3).

The higher the temperature, the greater the flexibility of the enzyme and substrate, and the more strained the chemical bonds within molecules. As the temperature decreases, the flexibility of the enzyme decreases, making it more difficult to cleave the substrate. Therefore, the V_{max} of an enzyme tends to decrease with decreasing temperature.

The V_{max} of the protease with SAAPFpNA is the highest of all the substrates tested at 75°C, yet is the lowest at 5°C. As the K_m with this substrate is also the lowest at 5°C, it still has the highest specificity. The specificities (V_{max}/K_m) of Ak.1 towards SAAPLpNA and SAAPApNA are quite similar at 5-40°C, but differ considerably at 75°C.

The greatest change in the V_{max} occurs within the temperature range of 40-75°C, the same temperature range where the greatest change in K_m occurs. This data shows that the specificity change of the protease with temperature is mostly due to the differences in the binding of different sized substrates at different temperatures rather than a change in the V_{max}.

7.3.4.3 Effect of Additives on the Km and Vmax

i) Effect of Additives on the K_m of Ak.1 Protease

Figure 7.12 shows the effects of 3 additives on the K_m of Ak.1 protease at 40°C with 2 substrates. These results show that the only additive that had any significant effect on the K_m of the protease was 50% methanol. This dramatically increased the K_m by about 64 fold with both substrates. Others (e.g. Fink, 1973) have found that in almost all cases, cosolvents (e.g. methanol) exert a negative effect on the substrate binding. This is possibly due to a hydrophobic partitioning effect on the substrate. The presence of water helps to drive substrate binding as the substrate and the binding-cleft of the protease are both hydrophobic, and tend to repel water. A solution of 50% methanol in water is more hydrophobic than 100% water. Thus the substrate would tend to have a lower affinity for binding to the protease as the cosolvent is also hydrophobic.

It is interesting to compare the effect of methanol on the K_m of the protease with the K_m Vs temperature data. The K_m of the protease increases with temperature with all the substrates tested. At higher temperatures, hydrophobic interactions increase (Brandts, 1967). The addition of methanol has increased the hydrophobicity of the solvent, causing a decrease in substrate binding to the protease. This suggests that substrate binding is driven by hydrophobic interactions.

100 mM NaCl and 50% sorbitol had very little effect on the K_m of either substrate. Higher concentrations of salt may be required to affect the K_m of the enzyme.

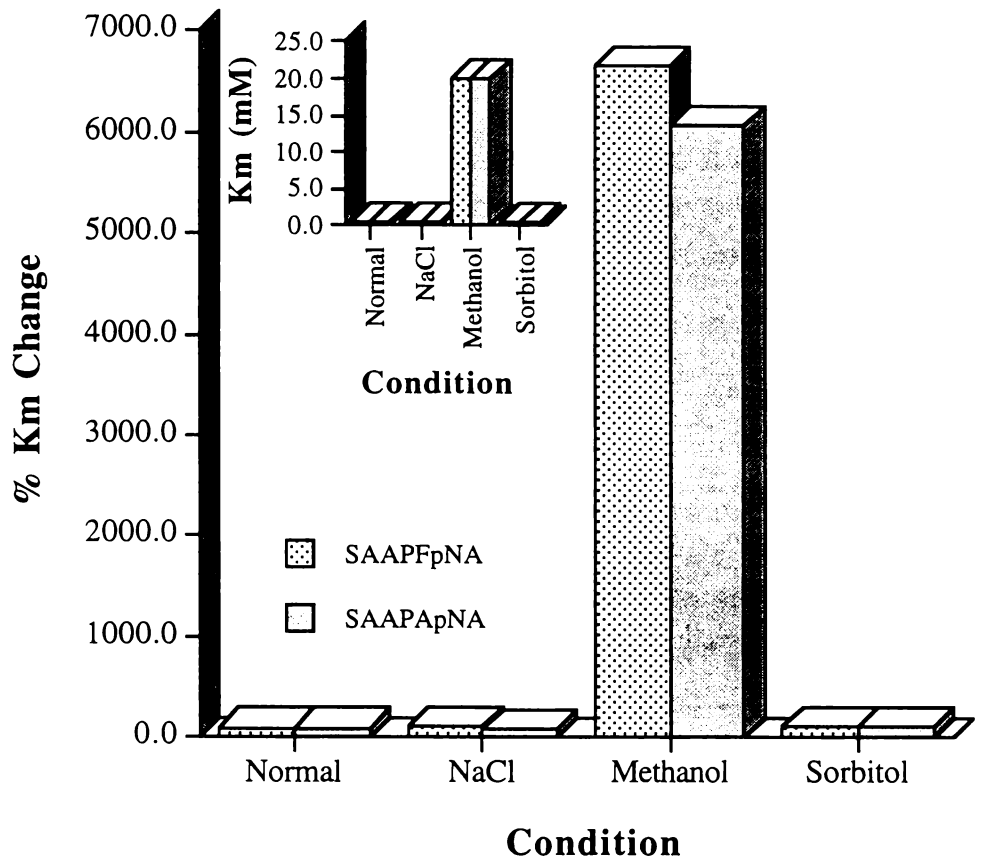


Figure 7.12 Effect of Additives on the K_m with 2 Substrates (Inset Shows the Actual K_m Changes).

Normal: 50 mM HEPES/NaOH pH_{40} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100; NaCl: 100 mM NaCl in the above buffer; Methanol: 50% methanol in the above buffer; Sorbitol: 50% sorbitol in the above buffer.

ii) Effect of Additives on the V_{max} of Ak.1 Protease

Figure 7.13 shows the effects of 3 additives on the V_{max} of Ak.1 protease at 40°C with 2 substrates. This shows that the effect of additives on the V_{max} is more variable with the two substrates. In all cases, the three additives decreased the V_{max} of the protease. However, this decrease was more significant with the substrate SAAPFpNA. There was little change in the V_{max} with SAAPApNA (max loss with NaCl of 1.6 fold) as compared to the losses with SAAPFpNA.

The most dramatic difference in V_{max} is in the presence of 50% methanol. Methanol has decreased the V_{max} with SAAPFpNA by 3.5 fold, compared to 1.3 fold with SAAPApNA. These substrates differ in sequence by only the P_1 amino acid. Phe is more hydrophobic than Ala as it contains a phenyl group. As methanol affects the hydrophobicity of the solvent, it appears that the more hydrophobic the substrate is, the more the activity of the protease is affected.

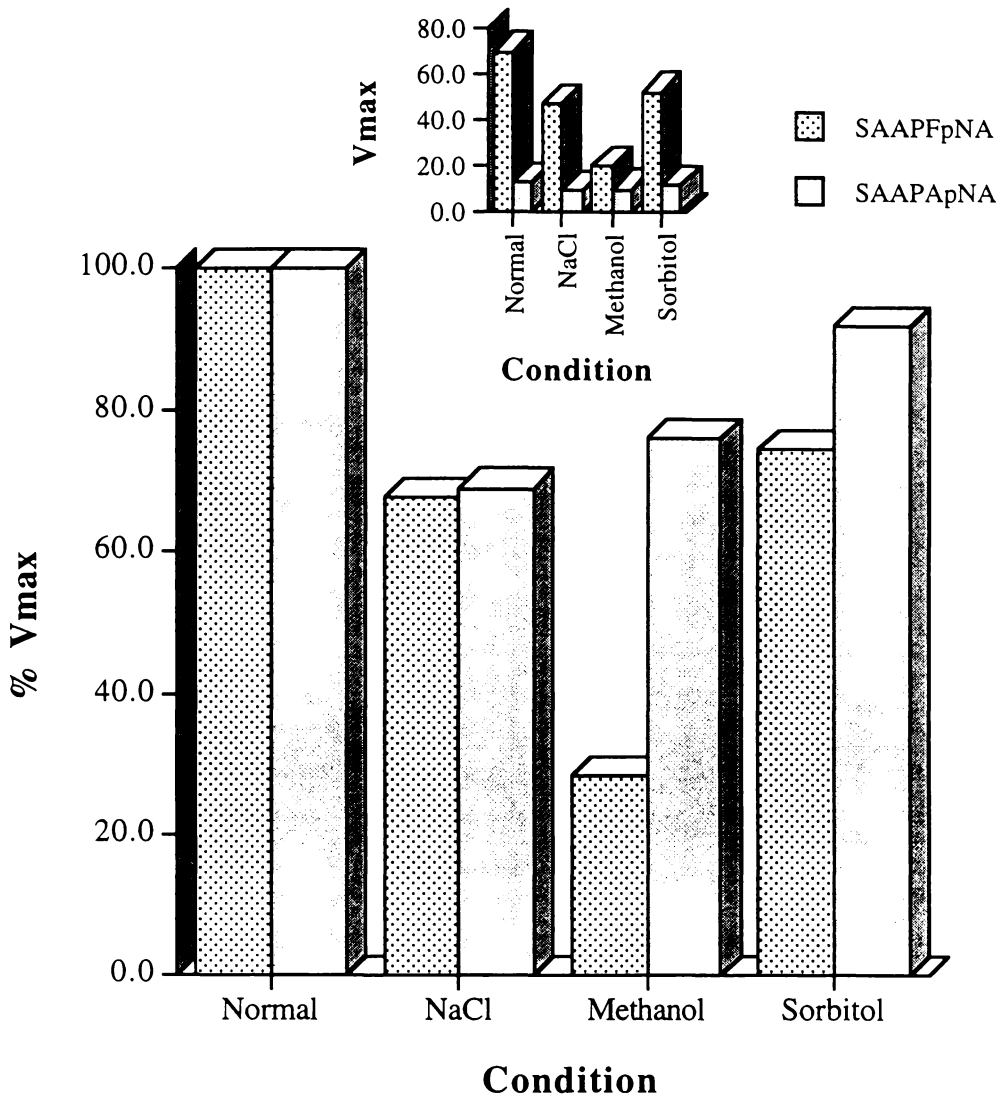


Figure 7.13 Effect of Additives on the V_{max} with 2 Substrates (Inset Shows the Actual V_{max} Changes).

Normal: 50 mM HEPES/NaOH pH_{40} 7.5 + 5 mM $CaCl_2$ + 0.01% Triton X-100; NaCl: 100 mM NaCl in the above buffer; Methanol: 50% methanol in the above buffer; Sorbitol: 50% sorbitol in the above buffer.

7.3.5 Comparison of the Substrate Specificity of Ak.1 Protease, Thermitase and Other Subtilisins

7.3.5.1 Comparative Sequences at the Active Site of Subtilisins

An alignment of the amino acid sequences of 126 subtilases (subtilisins), including Ak.1 protease and thermitase, shows that very few amino acids are universally conserved (Siezen and Leunissen, 1997). These include the catalytic triad residues Ser221, His64, Asp32, and a non-catalytic glycine residue (Gly219). Four other glycine residues (Gly34, 65, 83 and 154) are varied only once or twice. Gly34 and Gly154 contain main-chain torsion angles that are not able to be achieved by amino acids with side chains. Therefore, it is not surprising that these residues are so well conserved. Residue Asn155 is also highly conserved. Its role is to stabilise the oxyanion generated in the tetrahedral transition state of catalysis (Carter and Wells, 1990). Many more residues are totally conserved within the individual families of subtilisins (Siezen and Leunissen, 1997).

Figure 7.14 shows the structure of the active site of Ak.1 protease with the substrate SAAPFpNA modelled in it. It also contains the structure of thermitase (green ribbon) superimposed on it. The yellow bond indicates the presence of the disulphide bond of Ak.1 protease. This shows that there are significant differences in the structures of the substrate binding cleft of the two proteases. Further discussion of the significance of the disulphide bond in this position will be discussed in Chapter 8.

7.3.5.2 Comparison of the Substrate Specificity of Ak.1 Protease and Other Subtilisins

In general, subtilisins exhibit a broad specificity. They prefer to cleave substrates containing neutral residues at the P_1 site (Keil, 1992). This is consistant with Ak.1 protease and thermitase, which cleaves substrates containing Leu and Ala at the P_1 site. These proteases also have a high specificity for Phe at the P_1 site (Bromme & Kleine, 1984).

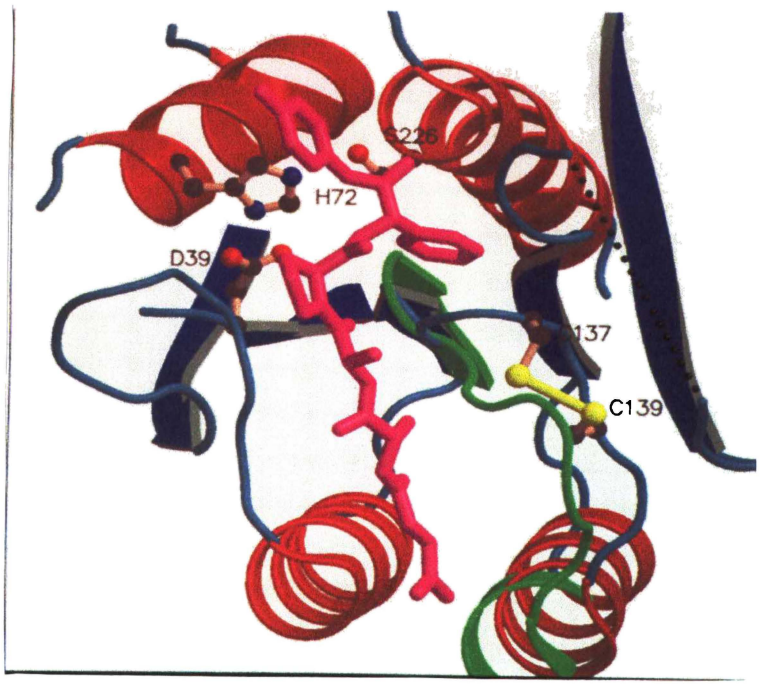


Figure 7.14 Structures of Ak.1 Protease and Thermitase in the Region of the Substrate Binding Cleft.

Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand). The dotted line to the right of the photograph indicates the likely position of the "missing" amino acids. The pink structure is the substrate SAAPFpNA modelled into the active site. The green ribbon indicates part of the structure of thermitase. The rest of the structure of thermitase was not included as it resembles the structure of Ak.1 protease more closely.

Thermitase, like Ak.1 protease, contains P_1 and P_4 sites that are large and hydrophobic (Siezen & Leunissen, 1997). Substrate binding is predominately determined by the binding of the substrate at these two sites in thermitase. Thermitase prefers substrates containing aromatic or large non-polar amino acids at these sites. It cleaves a variety of polyalanine peptides (Bromme *et al.*, 1986). Figure 7.15 shows the cleavage sites of several alanine-based peptides by these thermitase and subtilisin BPN'.

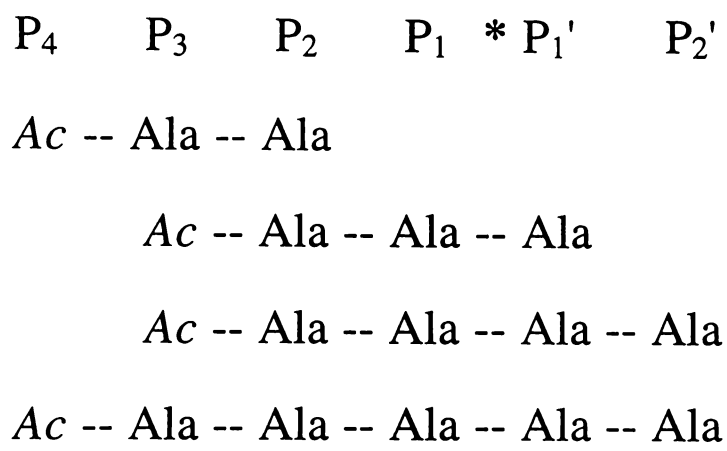


Figure 7.15 Cleavage Sites of Thermitase and Subtilisin BPN'.
(Adapted from Bromme *et al.*, 1986). * indicates the cleavage site.

A comparison of the K_m and k_{cat} values of thermitase with the substrates *Ac*-Ala-Ala-Ala-Ala and *Ac*-Ala-Ala-Ala-Ala-Ala shows that the K_m decreases and the K_{cat} increases with the addition of an extra alanine residue at the N-terminal side of the substrate (Bromme *et al.*, 1986). This was also seen with Ak.1 protease with the substrates *Suc*-Ala-Ala-Ala-pNA and *Suc*-Ala-Ala-pNA (Table 7.5). Thus, increasing the chain length of the substrate in the P_1-P_5 region increases both the binding affinity and the catalytic efficiency for the substrates.

Thermitase and subtilisin BPN' show a preference for substrates which will occupy subsites P_3-P_2'. This was seen as an increase in K_{cat}, with not much effect on K_m, of the enzyme as the P_1' then P_2' sites were occupied. Proteinase K exhibits a similar profile, except that it also prefers to have site P_3' occupied also (Bromme *et al.*, 1986). The effect of the occupancy of the P1'-P3' subsites with Ak.1 protease was not investigated.

Like Ak.1 protease, thermitase (and to a lesser extent elastase and subtilisin BPN') show a preference for multiple alanine-based peptide-pNA substrates with larger numbers of alanine residues (Kleine, 1982). Note that Ac-Ala-Ala is a competitive inhibitor of these enzymes. In spite of activity towards these substrates, alanine at the P_1 position is not favoured in these proteases (Bromme *et al.*, 1986). Ak.1 protease also cleaves di and tri-alanine substrates, though with a low specificity.

Thermitase shows a preference for *hydrophobic* groups at the P_3-P_4 sites (Siezen *et al.*, 1991). Ak.1 protease readily cleaves substrates containing alanine at these sites. It is unclear whether the decrease in specificity with smaller *Suc*-Ala_n-pNA substrates, where n = 1-5, is solely due to a decrease in the chain length, or in part due to the introduction of a *hydrophilic* succinyl-group at the P_2-P_4 sites in smaller substrates. The putative P_5 site is flanked by 2 α-helices, and can easily accomodate the succinyl-group in SAAPXpNA (X = Phe, Leu or Ala). In the case of thermitase, the formation of the [E.S]-complex and the deacylation rate constant (k_{cat}) decreases with the substitution of benzyloxycarbonylated (Bz)-substrates with succinyl-substrates (Kleine, 1982). The opposite was true for pancreatic elastase and proteinase K. This shows that the specificity of catalysis is influenced by the size and/or charge of N-protecting group on the substrates and potentially the position of this group in the binding cleft (Kleine, 1982).

Rothe *et al.* (1981) studied the hydrolysis of Z-dipeptide-OMe substrates with thermitase. They found that the best substrate was Z-Val-Phe-OMe, with a K_{cat}/K_m of 365. The conclusions were that the P_1 and P_2 sites are hydrophobic, with mutual interactions occuring between the side chains of the S_1 and S_2 amino acids for optimal hydrolysis. Bromme and Kleine (1984) carried out studies with thermitase on a variety of substrates, such as Z-Gly-Pro-Leu-Gly-Pro and Bz-Gly-His-Leu. They concluded that thermitase has a preference for hydrophobic residues at the P_1 position, and small neutral residues at P_2 (e.g. glycine, valine or proline). Bromme *et al.* (1986) concluded that thermitase has

a preference for proline at positions P_2 and P_4. Ak.1 protease has a preference for Pro at the P_2 site.

Unlike Ak.1 protease, thermitase cleaves a variety of chymotrypsin-like substrates (e.g. Ac-Tyr-OEt), though not trypsin-like substrates (e.g. Tos-Arg-OMe) (Kleine, 1982).

Serine proteases are an example of 'histidine enzymes' which carry out nucleophilic covalent catalysis (Kleine, 1982). This type of enzyme has a charge-relay system. A typical feature of this type of enzyme is that the efficiency of hydrolysis of amide bonds (e.g. -pNA substrates) is lower than for esterolysis (e.g. -OMe substrates). Ak.1 protease shows higher esterolytic activity over amide hydrolysis, as do thermitase, subtilisin BPN' and elastase (Kleine, 1982).

Peek *et al* (1993) determined the cleavage sites of oxidised insulin B chain by Ak.1 protease. Figure 7.16 shows the cleavage sites of Ak.1 and other subtilisins. The most obvious difference in the cleavage of oxidized insulin B chain by these proteases is the high specificity of Ak.1 protease above the other proteases. It cleaves significantly at only 3 sites. The major cleavage site is Leu^{15}-Tyr^{16}, the same as for the other subtilisins. Other major hydrolysis sites are Gln^4-His^5 and Glu^{13}-Ala^{14} (Peek *et al.*, 1993). Subtilisins such as thermitase, pancreatic elastase and subtilisin BPN' do not cleave at the latter site, though proteinase B cleaves it to a minor degree (Bromme and Kleine, 1984).

The cleavage at Leu^{15}-Tyr^{16} by Ak.1 proteases is consistant with the preference of hydrophobic amino acids at the P_1 site. The cleavage of the sites Gln^4-His^5 and Glu^{13}-Ala^{14} is more difficult to understand. It was expected that the protease would be more likely to cleave the peptide with preferentially hydrophobic amino acids at this site, e.g. Leu^{11}-Tyr^{12}, Leu^{17}-Val^{18} and Phe^{24}-Phe^{25}. However, there was no significant cleavage at any of these sites. The substrate specificity data for Ak.1 protease does not include substrates containing Gln and Glu at the P_1 site, so cleavage at these sites may be typical for this enzyme. Alternatively, activity at these sites in the substate may be a reflection of the importance of the amino acids at the P_2-P_5 and/or P_1'-P_3' sites.

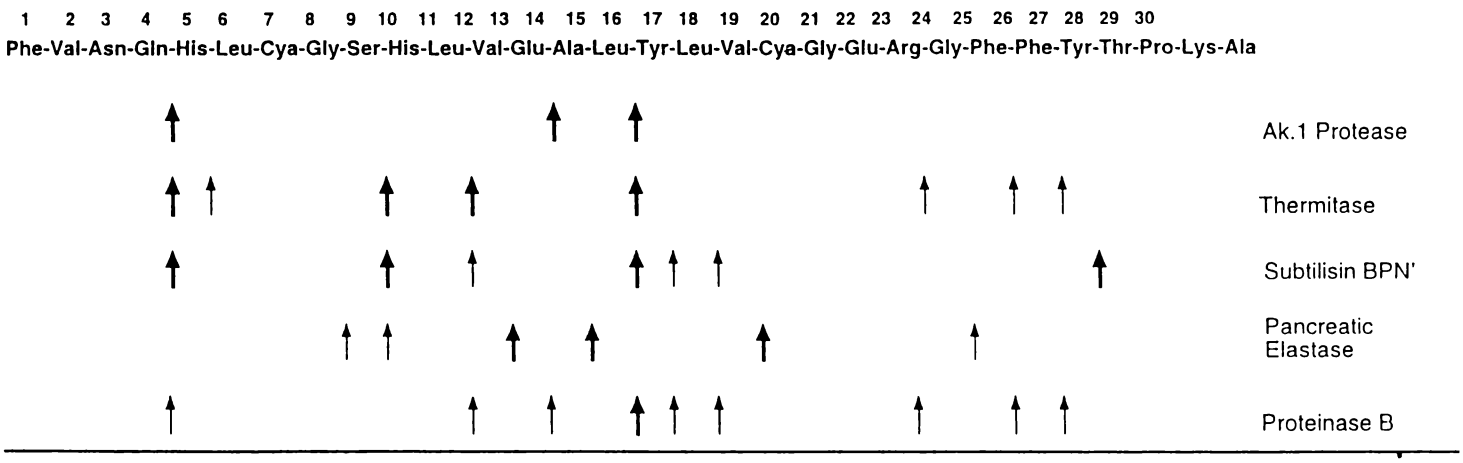


Figure 7.16 Cleavage of Oxidised Insulin B Chain by Several Subtilisins.
Adapted from Bromme & Kleine (1984) and Peek *et al* (1993).

Thermitase cleaves sites such as Leu^{15}-Tyr^{16}, Leu^{11}-Val^{12}, Gly^{4}-His^{5}, and Ser^{9}-His^{10}. Subtilisin BPN' cleaves at the sites Gly^{4}-His^{5}, Leu^{11}-Val^{12}, Leu^{15}-Tyr^{16} and Tyr^{26}-Thr^{27}. This shows a preference for branched-chain and aromatic amino acids at the P_1 site. This is similar to Ak.1 protease, except for that Ak.1 protease does not prefer Tyr at the P_1 site.

7.4 CONCLUSIONS

The active site is a cleft, composed of hydrophobic amino acids in the substrate-binding cleft. It is similar to other subtilisins, but differs in the presence of a disulphide bond. The space-filling model of the protease with the substrate SAAPApNA in the active site shows the cleft 'bends' at the P_2 site. This is easily accomodated for by proline at this position, as proline causes a bend in the substrate. This can explain the preference for proline at the P_2 site.

The protease has a limited substrate specificity, preferring to cleave substrates containing neutral or hydrophobic amino acids, such as valine, alanine or phenylalanine, at the P_1 site. It has a preference for proline at the P_2 site, and alanine at the P_1-P_4 sites. It has esterase activity, being able to cleave methyl, ethyl and p-nitrophenyl esters. Studies with *Suc*-Ala_n-pNA substrates (n=2-5) shows that the specific activity of the protease increases with increasing chain length, though a substrate containing 5 alanine residues appears to be cleaved significantly at more than one site.

An analysis of the effect of temperature on the K_m and V_{max} of Ak.1 protease with several substrates revealed that the specificity of the protease (V_{max}/K_m) changes with temperature. The K_m and V_{max} decreased with decreasing temperature, but not to the same degree with all substrates.

A comparison of the K_m and V_{max} of the protease to the substrates *Suc*-Ala-Ala-Pro-Xaa-pNA, where Xaa = Phe, Leu or Ala, shows that the larger and more hydrophobic the P_1 amino acid is, the higher the specificity of the protease for that substrate.

If the protease is assayed with substrates in the presence of 50% methanol, the K_m tends to increase dramatically. This is possibly due to a hydrophobic partitioning of the solvent. The V_{max} of the protease decreases under these conditions.

CHAPTER 8

ROLES OF THE DISULPHIDE BOND

8.1 INTRODUCTION

Maciver *et al* (1994) sequenced Ak.1 protease, and found that it contained two cysteine residues. At the time, they concluded that it was unlikely that a disulphide bond would be present as these two residues were separated in the sequence by only one amino acid. Such a bond would possibly be strained, and easily broken. However the crystal structure of the protease revealed that it did indeed contain a disulphide bond (Smith *et al.*, 1998).

An analysis of the Brookhaven Protein Database revealed that there are over 500 proteins known to contain cysteine residues separated by only one amino acid. However a preliminary search of some of these proteins has found no evidence that a disulphide bond between cysteine residues separated by one amino acid exists in these proteins. Indeed, it is the current opinion among some protein crystallographers that such an occurence is extremely rare, if not unique for Ak.1 protease (Baker, E. & Smith, C.A., personal communication). Thus the confirmation that a disulphide bond does exist in Ak.1 protease in solution by biochemical means is necessary.

Figure 8.1 shows a representation of the structure of the protease. The green bond indicates the position of the disulphide bond. This figure shows that the disulphide bond is located in the substrate binding cleft. It is located near Ca(3) (maroon ball), and the cleavage point of the protease. Due to this location, it is possible that it may have some influence on the activity of the protease.

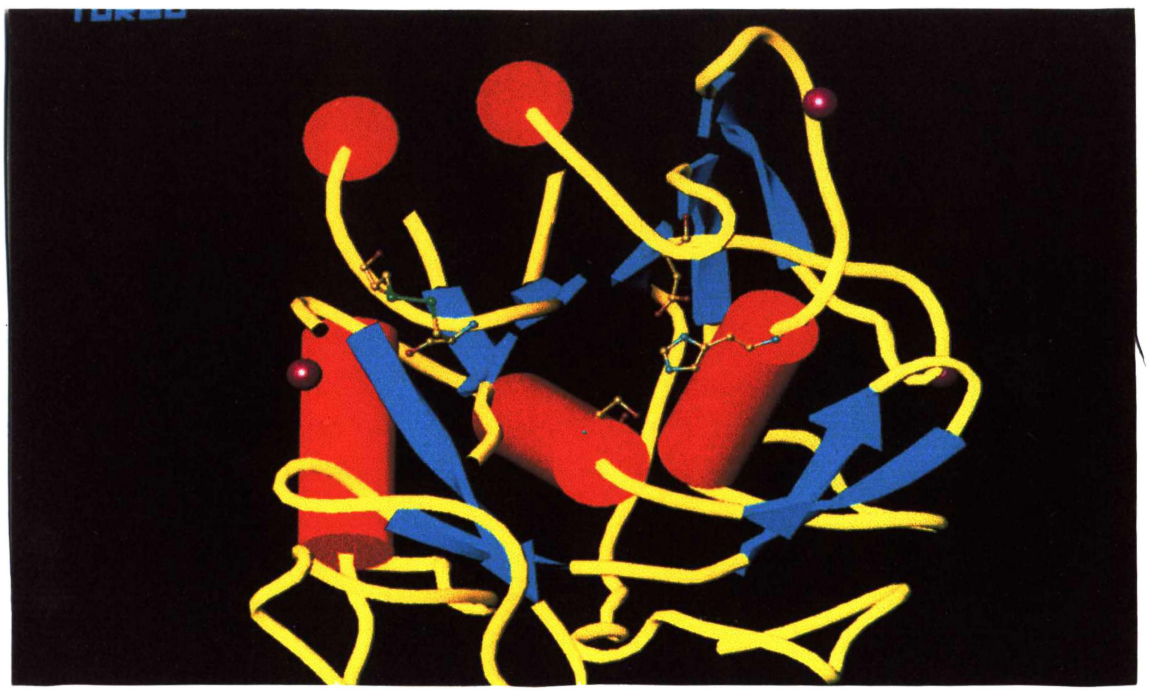


Figure 8.1 Three-Dimensional Structure of Ak.1 Protease
Photograph supplied courtesy of Dr Clyde Smith (Massey University, New Zealand).

Many subtilases are known to contain naturally occuring disulphide bonds such as proteinase K (Siezen & Leunissen, 1997). Disulphide bonds are known to contribute to the overall stability of an enzyme. Indeed, some researchers have tried to increase the stability of a protease by the introduction of disulphide bonds by site-directed mutagenesis, with mixed success (e.g. Matsumura *et al.*, 1989; Wells & Powers, 1986; Katz & Kossiakof, 1990). Thus, the presence of a disulphide bond in Ak.1 protease could possibly contribute to its thermostability.

Thermitase contains one cysteine residue, Cys75, located near the active site residues (Hausdorf *et al.*, 1980). It has been shown that this residue plays a role in catalysis. However, the cysteine residues in Ak.1 protease are located in a different position in the molecule. Indeed, the position of this disulphide bond appears to be unique to Ak.1 protease. A comparison of the sequences of 126 subtilisins shows that no other subtilisin contains a disulphide bond in this position. In fact, only one other protease, Catfish virus protease from Herpes virus 1, contains one of these two cysteine residues, which corresponds to the Cys137 in Ak.1 protease (Siezen & Leunissen, 1997). However, this protease has only been partially sequenced, so it is not certain if this protease is actually a subtilisin. The question arises, therefore, whether this disulphide bond contributes to the specific properties of Ak.1 protease.

8.2 MATERIALS AND METHODS

8.2.1 Reagents and Equipment

The DTT, βME and iodoacetic acid (IAA) were made up to 10 mM concentrations in RO water. The metal salts used were mercuric nitrate, lead nitrate, cadmium chloride and zinc chloride. All the metal ions were dissolved in RO water to a final concentration of 200μM. Double strength substrates were prepared in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100. The substrate was diluted by half in the metal ion solutions. Fluorescein mercuric acetate (FMA) was dissolved in a few drops of acetone, then 50 mM borate pH 9.0 (to prevent interactions of FMA with groups other than -SH) was added to a final concentration of 1mM FMA. The solution was filtered through an 0.22μ filter.

The fluorimeter used was a Perkin Elmer Luminescence Spectrophotometer LS 50B, using the software FLDM. The excitation and emission slit widths were 10 nm. Emission filters were used. The scan speed was 500.

8.2.2 Enzyme Pre-treatment

The purified enzyme was initially in a solution of 20 mM TES/NaOH pH_{75} 7.5 containing 1 mM $CaCl_2$ and 0.01% Triton X-100. As TES buffer contains sulphur, this may interact with the thiol reagents (e.g. Ellman reagent) in these experiments. Thus the buffer needed to be removed. For the Ellman assays, the enzyme was pretreated as described in section 2.7.2. The enzyme was also pretreated in the presence of urea, NaCl and EDTA to denature the enzyme, but do DTT was added. This was to check for the presence of buried free cysteine residues. For the NTSB reaction, the enzyme was bound onto a Sep-Pak column. The enzyme was eluted with 50% acetonitrile according to the method in section 2.6.1. The acetonitrile was removed by extensive ultrafiltration (Amicon, YM-10) against RO water. The enzyme in the presence of TES buffer was also tested.

Ak.1 protease was incubated with 10 mM concentrations of the reducing and SH-modifying reagents for 1 hour at room temperature prior to the assays. Samples of the DTT-protease were dialysed against water at 4°C for 2 days, changing the water 5 times, to remove the DTT. The water was continuously flushed with nitrogen gas, in an attempt to keep the protease in a reducing environment. A control Ak.1 sample (no DTT treatment) was also dialysed, as

before, except it was not flushed with nitrogen. Another sample was flushed with O_2 continuously for 1 hour, followed by an incubation overnight in a high O_2 environment. This was an attempt to reform the disulphide bond. These samples were subjected to free cysteine analysis and activity determination.

8.2.3 Cysteine and Disulphide Bond Assays

8.2.3.1 Ellman Assay

The quantitation of the number of cysteine residues/disulphide bonds indicated by the protein sequence and structure was confirmed by the Ellman assay as described in section 2.7.2.

8.2.3.2 Reaction of Thiols with Disodium 2-nitro-5-thiosulphobenzoate (NTSB)

Ak.1 protease of known concentrations, was assayed for disulphide bond presence with NTSB by the method described in section 2.7.2. Samples of Ak.1 protease in the presence and absence of TES buffer were tested to see if the TES buffer interferred with the assay (TES contains sulphur). Further samples of Ak.1 protease were tested in the presence of 2M guanidine thiocyanate to unfold the protein in case the NTSB reagent was unable to access the disulphide bond. No DTT was added to the expose the sulphydryl groups of the protein as the sodium sulphide in the NTSB solution reduces the bond, while not reacting with NTSB. The concentration of disulphides is calculated by determining the concentration of CNT in solution. As the reaction with NTSB produces only 1 mole of CNT per disulphide bond (Thannhauser *et al.*, 1984), the concentration of CNT is equivalent to the concentration of disulphide bonds.

8.2.4 Assays With Peptide Substrates

To determine the effect of the reagents on *activity*, the DTT-Ak.1 was tested with 1 mM SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 10 mM reductant/modifier at 20°C according to the method described in section 2.2.2. To determine the effect of the concentrations of DTT and fluorescein mercuric acetate (FMA) on activity, the protease was preincubated with 0-1mM concentrations of these reagents, then assayed for remaining activity with 1 mM SAAPFpNA as above.

Ak.1 protease was preincubated in the presence of 100μM of the heavy metals for 1 hour at room temperature. The control contained 5 mM $CaCl_2$. The protease samples were then assayed for activity at 25°C with 1 mM SAAPFpNA substrate (final concentration) in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 100μM metal ion according to the method in section 2.2.2.1 (100μl assay volume). Azocasein substrate was not used as the effect of the metal ions on this substrate was unknown.

8.2.5 Michaelis Menten Kinetics

The protease was assayed with the following substrates:

SAAPFpNA	see section 2.2.2.1
SAAApNA	see section 2.2.2.1
SAApNA	see section 2.2.2.1
CBZ-Gly-pNP	see section 2.2.2.2

To test the effect of DTT on K_m and V_{max}, the enzyme was tested with several concentrations of the above substrates under the same conditions. To test the effect of DTT on the V_{max} of the protease with SAAPFpNA over a wide temperature range, the protease was assayed, as above, in the temperature range of 5-75°C. Some of these experiments were repeated with the dialysed enzyme samples. No reductant/modifying agent was present in the assays. This was to check that the reductants/modifying agents were not influencing the assay (as opposed to modifying the protease). The "reduced" enzyme after sephadex G25 and ultrafiltration (i.e. for use in the Ellman assay) was assayed at 20°C with SAAPFpNA as before to determine the K_m and V_{max}.

To determine the effect of the heavy metals on the K_m and V_{max} of the protease, the enzyme samples were assayed for activity at 20°C with a variety of concentrations of SAAPFpNA substrates before.

8.2.6 Fluorescence Spectroscopy

The protease (0.65 μM), pretreated via Sep-Pak (section 2.6.1) was incubated with FMA (0.3 and 300 μM final concentration). Another sample of protease was pretreated with 10 mM DTT for 1 hour at room temperature to open the disulphide bond before being treated with Sep-Pak and FMA. Part of the samples were extensively ultrafiltered (Amicon, YM-10) against RO water. Both the retentate (enzyme) and final permeate were retained. The samples

were subjected to excitation scans (emission λ = 517 nm) and emission scans (excitation λ = 258 nm). Scans were also performed on RO water, borate buffer, FMA, FMA + DTT and protease samples. The protease-FMA complex was detected by an increase in the peak area at 517 nm compared to the same concentration of free FMA in solution.

The protease-FMA complexes were incubated with 10 mM DTT for 1 hour at room temperature. The DTT/FMA (free in solution) was removed by extensive ultrafiltration (Amicon, YM-10) against RO water, retaining the retentate (enzyme) and final permeate. Both retentate and the permeate were scanned, as above, and the results compared to the protease-FMA sample. The permeates were tested to determine the amount of fluorescence due to trace amounts of FMA remaining free in solution after ultrafiltration.

8.2.7 Determination of Thermostability

The protease was preincubated in the presence of either 10 mM DTT, 5% βME or 10 mM IAA, or combinations of the above, for 1 hour at room temperature. Samples of each condition were withdrawn and stored on ice until assayed (non-incubated controls). The remainder of the samples were incubated for 1 hour at 85°C, then cooled on ice. The activity remaining was determined by assaying the samples with 0.2mM SAAPFpNA at 20°C (section 2.2.2.1; 100µl assay). The % activity remaining was calculated by comparing the activity of the incubated sample to that of its non-incubated control.

To determine the half life of the protease in the presence and absence of a disulphide bond, the protease was preincubated in 10 mM DTT for 1 hour at 20°C, then incubated at 85°C. Samples were withdrawn at various time periods, cooled on ice and assayed for remaining activity with 1 mM SAAPFpNA at 20°C.

8.2.8 Protein Assay

The concentration of the protease was determined in these experiments by the modified Lowry method (see 2.3.1).

8.3 RESULTS AND DISCUSSION

8.3.1 Reduction and Reoxidation of the Disulphide Bond

8.3.1.1 Ellman Assay

Table 8.1 shows the results of the Ellman assay for free thiols. Untreated enzyme, i.e. no reductant added, gave no colour response with the Ellman reagent, as did the denatured enzyme. As the Ellman reagent reacts with free sulphydryl groups (thiols), as opposed to disulphide bonds, this supports the crystallographic data which suggested the presence of a disulphide bond in the native enzyme.

Enzyme Pretreatment	Mol -SH/Mol Protease
None	0
Urea & NaCl/Sephadex G25/EDTA	0
DTT/SepPak or Dialysis under N_2	<1
DTT/SepPak/O_2 Treatment	<1
DTT/Urea & NaCl/Sephadex G25/EDTA	1.7

Table 8.1 Detection of Cysteine Residues in Ak.1 Protease After Several Treatments.
The presence of free thiols was detected by a modification of the Ellman assay as described in section 2.7.2.

The pretreatment method for opening the disulphide bond had a large influence on the results. The use of SepPak C_{18} or dialysis under N_2 was not sufficient to maintain the enzyme in the free thiol form. The free cysteine residues may have been oxidised (covalent modification) preventing the reformation of the disulphide bond. Alternatively, the disulphide bond could be partially reforming in the absence of the reductant. The Ellman assay is dependent on detecting free thiols rather than disulphide bonds. Therefore, it was not possible to determine which of the two oxidation reactions above (if not both) were causing the decrease in the detection of free thiols. Thus, it is not known how readily the disulphide bond can reform in this protease.

The use of urea to denature the enzyme and the presence of EDTA to minimize reformation of the disulphide bond in the absence of the reductant was successful in maintaining the cysteine residues in the free thiol form. EDTA minimizes reformation of the disulphide bond by chelating the metal ions which are catalysts for the oxidation reactions. This treated sample produced a ratio of *1.7 cysteine residues per enzyme molecule.* As no free thiols were present in the native enzyme, these residues must exist naturally as a disulphide bond.

It was initially thought that once opened, the disulphide bond would be unlikely to be reformed (easily). Table 8.2 shows a comparison of the bond lengths and angles involved in the disulphide bond in Ak.1 protease as compared to average values. These results show that there is no significant difference in the bond lengths and angles of the disulphide bond of Ak.1 protease as compared to average values. Therefore, it is likely that the disulphide bond could be reformed once the reductant is removed.

8.3.1.2 Reaction with Disodium 2-nitro-5-thiosulphobenzoate(NTSB)

In spite of repeated attempts, no colour response was obtained in the presence of Ak.1 protease. Enzyme samples were in the presence of either Tris buffer or no buffer (after Sep-Pak and ultrafiltration) to exclude any possible interferences in the assay. To remove the possibility that the disulphide bond was buried in the molecule out of reach of the reagent, guanidine thiocyanate (2M) was added to unfold the protein. Still no colour response was obtained. Protein and activity assays confirmed the presence of the enzyme in approximately 20μM concentrations. Standards of cysteine (-SH) and cystine (-S-S-) of 10-100μM concentrations showed a easily detectable colour response This experiment was repeated with trypsin, a protein known to contain disulphide bonds. No colour response was obtained with this enzyme either. It is not known why the enzymes did not react with the reagent.

Bonds	Bond Length		Bond Angle	
	Ak.1	Avg	Ak.1	Avg
C-S	1.85 1.79	1.810	-	-
S-S	2.04	2.036	-	-
C-S-S	-	-	104.95 105.42	104.5

Table 8.2 Comparison of the Bond Lengths and Bond Angles in Disulphide Bonds.

The average data was obtained from Vijayan (1976).

8.3.2 Effect of Reducing Agents on the K_m and V_{max} of Ak.1 Protease

8.3.2.1 Introduction

Reducing agents such as DTT cleave disulphide bonds by the following general reaction (White, 1972):

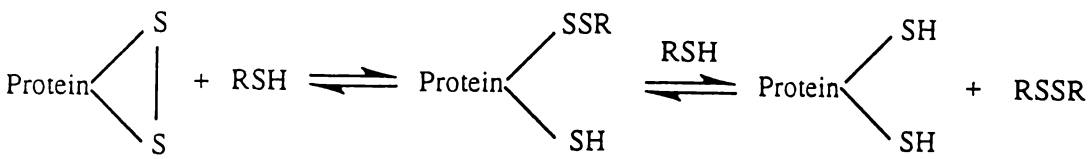


Figure 8.2 Reaction of Reducing Agents with Disulphide Bonds
From Creighton (1989)

As the disulphide bond is located in the substrate-binding cleft, does it have any effect on the *activity* of the enzyme? Does the shape of the binding cleft change significantly upon the opening of this bond? If so, this should be detectable as changes on the K_m and/or V_{max} of the enzyme with appropriately sized substrates.

8.3.2.2 Effect of Reducing and SH-Modifying Agents on Activity

Table 8.3 shows the effect of the reagents on the activity of Ak.1 protease. Both DTT and βME reduce the disulphide bond, while IAA alkylates free cysteine residues. These results show that opening the disulphide bond caused a significant reduction in activity of the protease towards the substrate SAAPFpNA. In contrast, IAA had only a slight effect on activity. This is because IAA acts on free cysteine residues, rather than on disulphide bonds. The combination of both a reducing agent and IAA shows a decrease in activity

equivalent to the decrease due to the reductant alone. Thus, the presence of a disulphide bond is important for the activity of the protease.

Reagent	% Activity Remaining
Non-dialysed Samples	
None (Control)	100
Dithiothreitol (DTT)	43
β-mercaptoethanol (βME)	54
Iodoacetic acid (IAA)	95
DTT + IAA	42
βME + IAA	52
DTT + βME	43
Dialysed Sample	
"Reduced" enzyme	72

Table 8.3 Effect of Reductants and Modifying Agents on the Activity of Ak.1 Protease.
The non-dialysed samples contained 10 mM of the reagents in the assay, while the dialysed sample did not.

The 'reduced' enzyme sample was prepared by treatment with DTT, followed by its removal by exhaustive dialysis under N_2. As shown with the Ellman assay, this method is not sufficient to maintain the cysteine residues in the reduced form. The activity of the dialysed sample is in between the native and DTT-containing samples. This suggests that either a portion of the protease molecules have reformed the disulphide bond, or the presence of the oxidised species (modified cysteine residues) affects the activity of the protease.

8.3.2.3 Effect of DTT on Km and Vmax

Different concentrations of the substrate SAAPFpNA were tested with Ak.1 protease, pretreated with DTT, and compared to an untreated sample of protease. It was found that the K_m was increased 3 fold, while the V_{max} was decreased by 2.6 fold (see Table 8.4). This is consistant with the positioning of the disulphide bond in the substrate-binding cleft. Opening this bond may be causing the cleft to change in shape, possibly opening it up, causing weaker interactions with the substrate.

As this bond is in the region of the P_4 subsite, it was proposed that perhaps the difference in the K_m and/or V_{max} may be reduced with smaller substrates which do not interact in this area. Thus the effect of DTT on the K_m/V_{max} of the protease with smaller-sized substrates was investigated.

Substrate	K_m		V_{max}		Specificity	
	Control	DTT	Control	DTT	Control	DTT
Suc-Ala-Ala-Pro-Phe-pNA	0.145	0.44	14.26	5.57	98.3	12.66
Suc-Ala-Ala-Ala-pNA	0.32	0.58	3.25	2.02	10.16	3.48
Suc-Ala-Ala-pNA	4.00	6.67	0.50	0.50	0.125	0.07
Cbz-Gly-pNP	8.30	8.30	5.00	6.67	0.6	0.8

Table 8.4 Substrate Specificity of Ak.1 Protease with Peptide Substrates at 20°C in the Presence and Absence of DTT.
Units 1 = µmol pNA/min/mg; units 2 = µmol pNP/min/mg.

Table 8.4 shows the results of these experiments. [The Michaelis Menten/Lineweaver Burk plots are located in the appendix.] This shows that as the size of the substrate decreases, so does the *change* in the K_m of the protease. The K_m went *up* when DTT was present, suggesting that the substrate may be more loosely bound to the enzyme. In fact activity with the smallest substrate, CBZ-Gly-pNP, showed no change in the K_m of the protease. Thus, it appears that the opening of the disulphide bond has only a localised effect on the shape of the active site.

The effects of the size of the substrate with DTT on the V_{max} is more variable. However, on average there was a decrease in the V_{max} of the protease in the presence of DTT. The smaller the substrate, the less the decrease in activity detected. The V_{max} with CBZ-Gly-pNP ester with DTT was higher than the control. However, this assay contained 50% acetonitrile (as opposed to <1% for the others) to enable the substrate to dissolve. This may have had an effect on the results.

The effect of DTT on the activity of the protease over a range of temperatures was tested. The K_m and V_{max} with SAAPFpNA was determined

over the temperature range of 5-75°C. Figure 8.3 shows the effect of DTT on the V_{max} of Ak.1 protease with SAAPFpNA at different temperatures. The Michaelis Menten and Lineweaver Burk plots are located in the appendix. This shows that as the temperature increases, the difference between the V_{max} of the control and DTT-enzyme increases. The V_{max} of the protease decreases with the opening of the disulphide bond at all the temperatures tested.

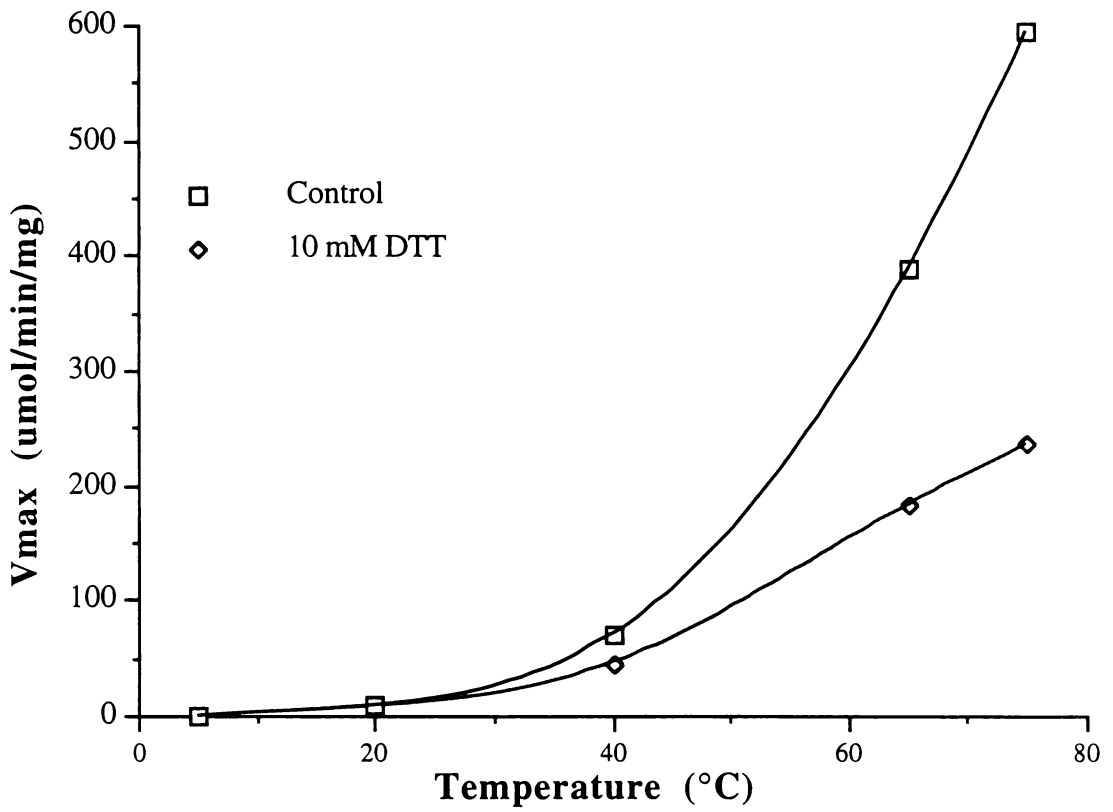


Figure 8.3 Effect of DTT on the V_{max} with SAAPFpNA at Different Temperatures.

Ak.1 protease was assayed in the temperature range of 5-75°C with different concentrations of SAAPFpNA in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and +/- 10 mM DTT (section 8.2.5).

This shows that as the temperature increases, the difference in the V_{max} proportionately increases. This could be due to the increasing flexibility of the enzyme at higher temperatures. Figure 8.4 shows Log V_{max} vs 1000/Temperature (K) (i.e. a variation on the arrhenius plot). The slopes are slightly different at the higher temperatures. At 20°C (55°C below the normal assay temperature), the enzyme would be significantly more rigid than at 75°C. Thus at 20°C, opening the disulphide bond may not cause much of a change in the structure of the S_4 binding site. At higher temperatures, there may be a more significant change in the structure of the binding cleft due to a greater flexibility at this temperature. The significance of this will be discussed further in section 8.3.5.

Figure 8.5 shows the effect of DTT on the K_m of the protease with SAAPFpNA at different temperatures. This shows that the K_m of the protease with an open disulphide bond varies significantly from the control (bond intact). It is interesting to note that for both the control and DTT-containing samples at 65-75°C, there is very little difference in their respective K_m in this temperature range. However, there is a large difference in the K_m's of the control and DTT samples at a given temperature. This is a reflection of the relative abilities of the substrate to bind to each form of the enzyme. If DTT has caused an opening of the substrate-binding cleft, the substrate may not be able to bind as efficiently.

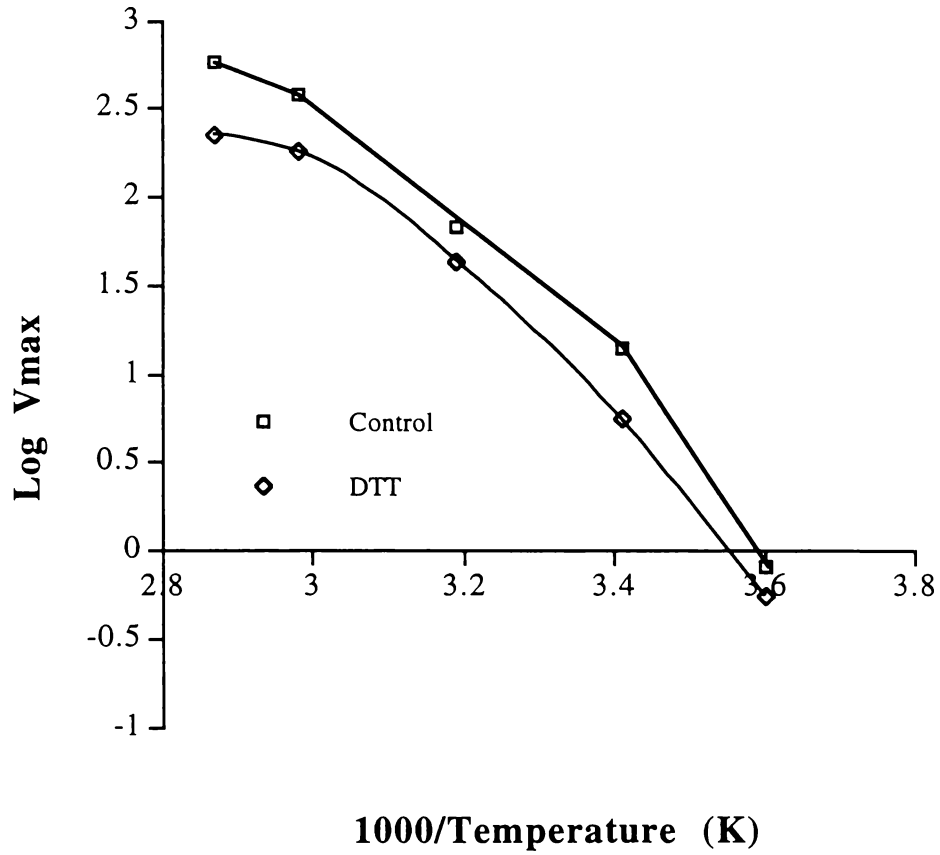


Figure 8.4 Effect of Temperature on the V_{max} With and Without DTT with SAAPFpNA.

Ak.1 protease was assayed in the temperature range of 5-75°C with different concentrations of SAAPFpNA in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and +/- 10 mM DTT (section 8.2.5).

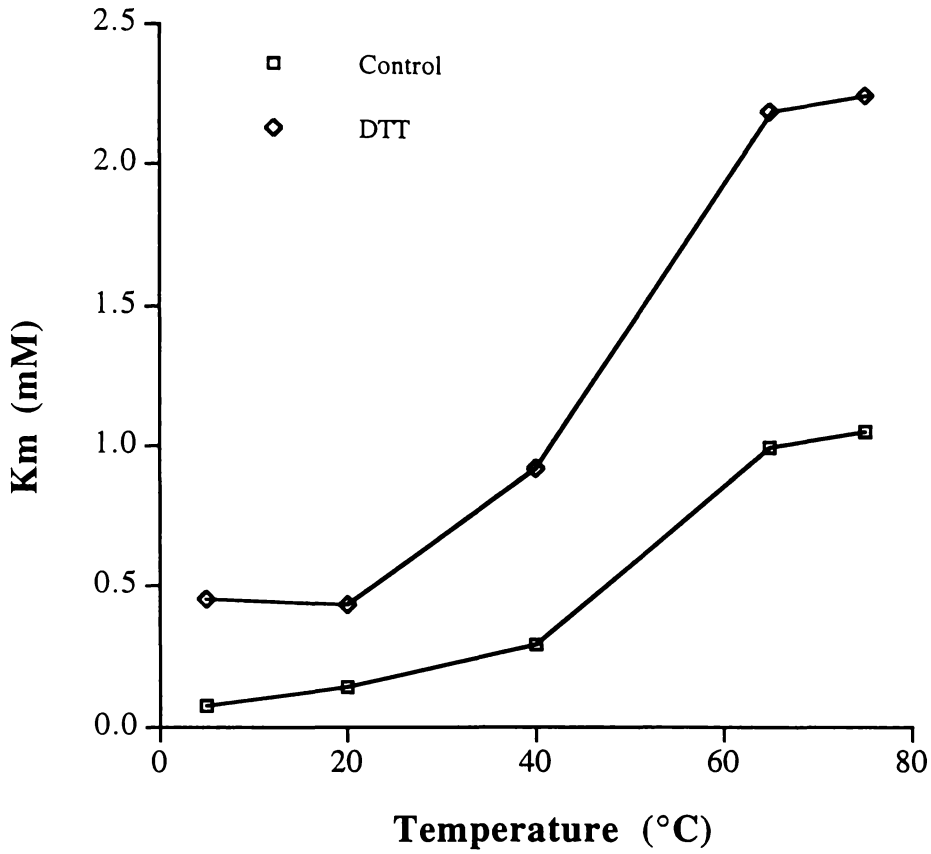


Figure 8.5 Effect of DTT on the K_m with SAAPFpNA at Different Temperatures.

Ak.1 protease was assayed in the temperature range of 5-75°C with different concentrations of SAAPFpNA in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and +/- 10 mM DTT (section 8.2.5).

Table 8.5 shows the K_m and V_{max} of the protease samples (a) unmodified enzyme, (b) enzyme with 10 mM DTT and (c) sample b with the DTT removed. This shows that the values of the K_m and V_{max} of the so-called 'reduced' enzyme mentioned previously (tested with the Ellman reagent) are in between that of the unmodified and DTT-containing enzyme. This is further evidence to suggest that either a proportion of the 'reduced' enzyme has reformed the disulphide bond, or the cysteine residues have been covalently modified, forming a species with lower activity..

Enzyme Sample	K_m	V_{max}
Unmodified Protease	0.145	14.3
Protease + 10 mM DTT	0.440	5.6
"Reduced" Protease (DTT Removed)	0.186	7.2

Table 8.5 K_m and V_{max} of Ak.1 Protease After Several Treatments.
Ak.1 protease was assayed 20°C with different concentrations of SAAPFpNA in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 (section 8.2.5).

8.3.2.4 Effect of Disulphide Bonds on the Activity of Other Enzymes

Recently, other enzymes/receptors have been reported that contain disulphide bonds located in regions important for function/activity. Cook & Eidne (1997) found that the presence of a disulphide bond between the conserved residues Cys114 and Cys195 in gonadotrophin-releasing hormone receptor is essential for the maintenance of receptor function. It does so by maintaining the correct receptor structure required for ligand binding (Cook & Eidne, 1997).

Dimeric alkaline phosphatase from *Escherichia coli* contains 2 intramolecular disulphide bonds between Cys168-Cys178 and Cys286-Cys336 (Sone *et al.*, 1997). One of the disulphide bonds (Cys286-Cys336) is required to correctly position the active site region of the enzyme, while the other is important for conformational stability.

Proteases are also known to contain disulphide bonds in the active site/binding cleft of the enzyme. The S_1 binding site of trypsin is cross-linked by the conserved Cys191-Cys220 disulphide bond. Wang *et al* (1997) substituted these cysteine residues with alanine and serine residues. They found that these

mutants had a decreased *acylation rate*. The substrate-binding and deacylation rate were unaffected by the loss of the disulphide bond. The stability of the mutant trypsins' were unchanged from the wild-type.

Factor VII, a glycoprotein serine protease involved in blood clotting, contains disulphide bond-linked heavy and light chains (James *et al.*, 1997). Cys310 binds to Cys329, forming a disulphide bond within the catalytic domain. A mutant form of this enzyme with Phe substituted for Cys310 had lower activity. It is proposed that the disulphide bond is essential for maintaining the correct orientation of the catalytic triad residues His193, Asp242 and Ser344 (James *et al.*, 1997).

The opening of the disulphide bond in Ak.1 protease has caused a decrease in the V_{max} of the protease with some of the substrates. This suggests that it has affected either the acylation and/or deacylation rates of the protease.

8.3.2.5 Comparison of the Structures of Ak.1 Protease and Thermitase

Figure 7.3 (earlier) shows an overlay of the structures of Ak.1 protease and thermitase in the region of the disulphide bond of Ak.1 protease, with the substrate SAAPFpNA modelled in the binding cleft. This shows that there is a significant difference in the structures of the S_4 binding site of the proteases where the disulphide bond is located.

In Ak.1 protease, there is an extra loop caused by the disulphide bond that is positioned in the S_4 site of the binding cleft. Thermitase does not have this loop. The typical S_4 binding site of subtilisins are large and hydrophobic. The side chains of the residues on either side of this site vary, which alters the size of the S4 site with different proteases (Siezen & Leunissen, 1997). Figure 7.3 shows that thermitase (green ribbon in the figure) appears to have a smaller binding cleft at the S_3-S_5 sites than Ak.1 protease. The disulphide bond in Ak.1 protease appears to decrease the size of the binding cleft at the S4 binding site, making similar size to thermitase in this region. It is proposed that when DTT opens the disulphide bond in Ak.1 protease, the S_4 binding site resembles a more open structure. Opening up this area could result in weaker binding of the substrate in this area, potentially increasing the K_m of the substrate.

Figure 8.6 shows the B values (average main-chain temperature factors) with respect to each amino acid in the sequence of Ak.1 protease (Smith *et al.*, 1998). This shows that there are localised regions in the structure with relatively high mobility. For example, Ser226, a member of the catalytic triad, has a high mobility.. The other two catalytic residues have low-medium mobilities. This is in contrast to thermitase, which has low mobility for all three catalytic triad residues (Gros *et al.*, 1989).

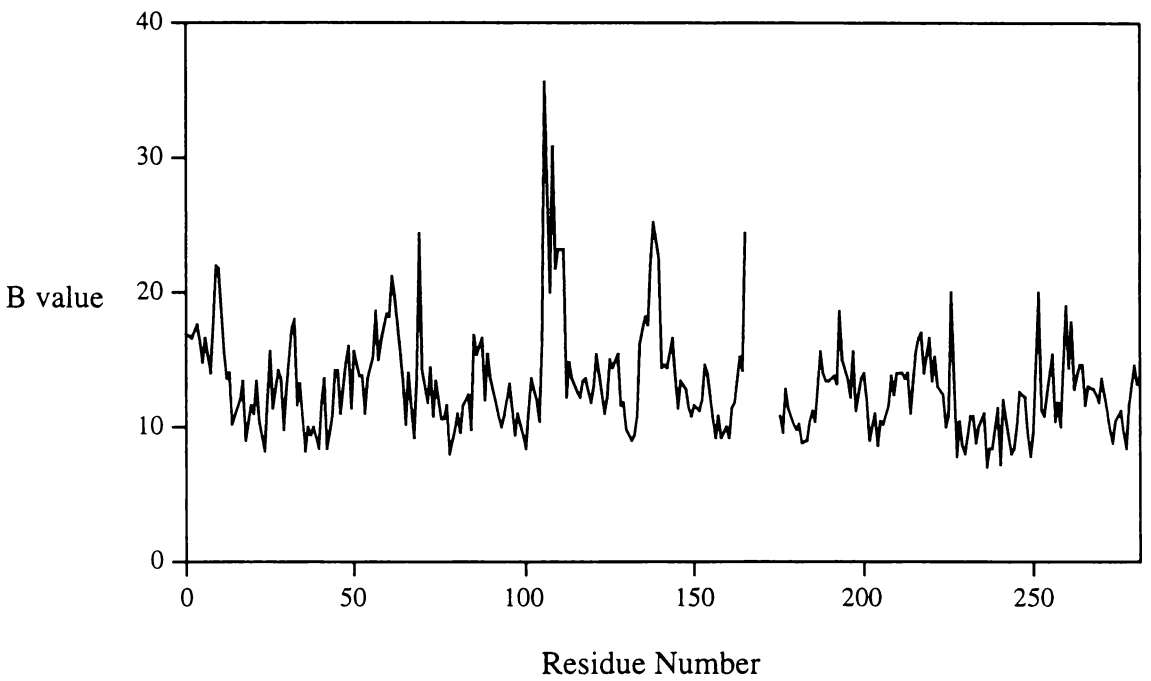


Figure 8.6 The Average Main-Chain Temperature Factors (A^2) Vs Residue Number for Ak.1 Protease.

The main-chain temperature factors for each residue have been averaged over the N, C^{α}, C and O atoms. The gap in the graph at residues 166-174 is due to the lack of electron density in the structure for these residues ('cleavage site' of the protease).

Two other portions of the sequence of Ak.1 protease that have relatively high mobility are the residues 106-111 and 137-139. Residues 137 and 139 are the cysteine residues, located on one side of the S_4 binding site. The sequence between residues Leu106-Thr111 is located on the other side of the S_2-S_5 binding site. Residue Leu106 has a very high mobility. In thermitase, there is a relatively low mobility in the residues 106-111 region (Ak.1 protease numbering; Gros *et al.*, 1989). However, there is high mobility in the region around residues 137-139 (Ak.1 protease numbering). This shows that the amino acids of Ak.1 protease forming the substrate binding cleft are relatively mobile compared to the rest of the molecule, especially around the S4 binding site. Thermitase appears to be less flexible than Ak.1 protease in this region (Gros *et al.*, 1989). Therefore, it is proposed that the opening of the disulphide bond results in a localised opening of the substrate binding cleft.

8.3.3 Effect of Heavy Metals on the K_m and V_{max} of Ak.1 Protease

8.3.3.1 Introduction

A variety of metals are known to bind to thiol groups in cysteine residues. They include mercury, lead, cadmium, arsenic, antimony, bismuth, zinc, copper and selenium. Figure 8.7 shows general diagrams of the interactions between cysteine residues and metals.

Ak.1 protease contains a surface-exposed disulphide bond which influences the activity of the protease. Heavy metals such as mercurous acetate are known to bind to sulphur atoms involved in disulphide bonds (Martin, 1979), forming mercurate disulphide bonds (R-S-Hg-S-R'). Thus the effect of heavy metals on activity was determined to see if these metals have any effect.

8.3.3.2 Effect of Metals on the Activity of Ak.1 Protease at 20°C

Figure 8.8 shows the effect of the 4 metal ions on the activity of Ak.1 protease at 25°C. These results show that these metals do indeed inhibit the activity of the protease. The order of decreasing inhibition is Hg^{2+}> Pb^{2+}> Zn^{2+}> Cd^{2+}.

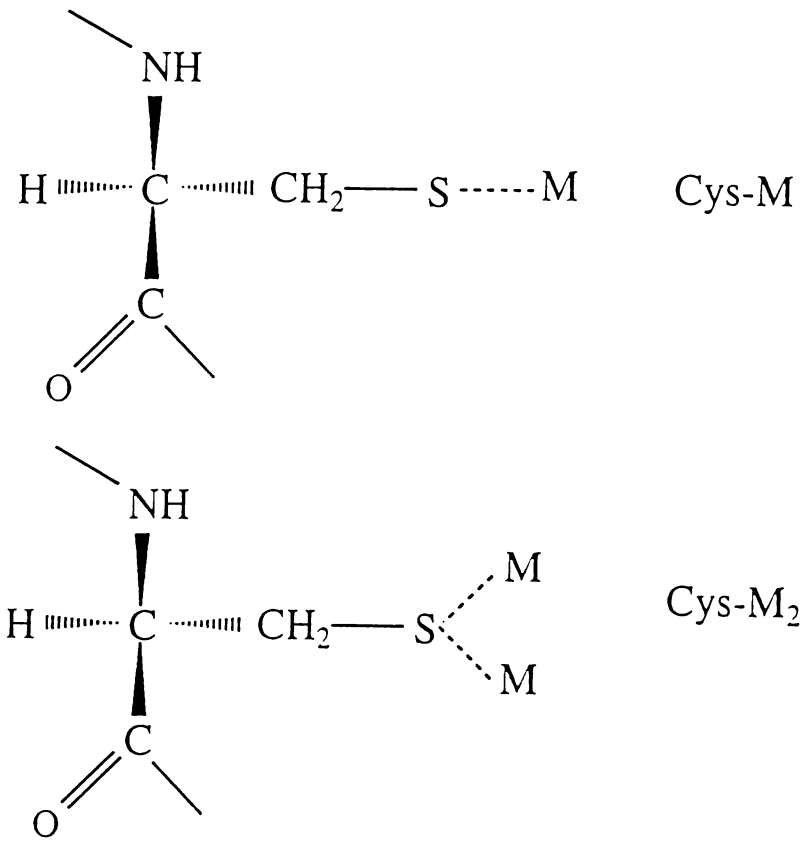


Figure 8.7 General Interactions Between Metals and Cysteine

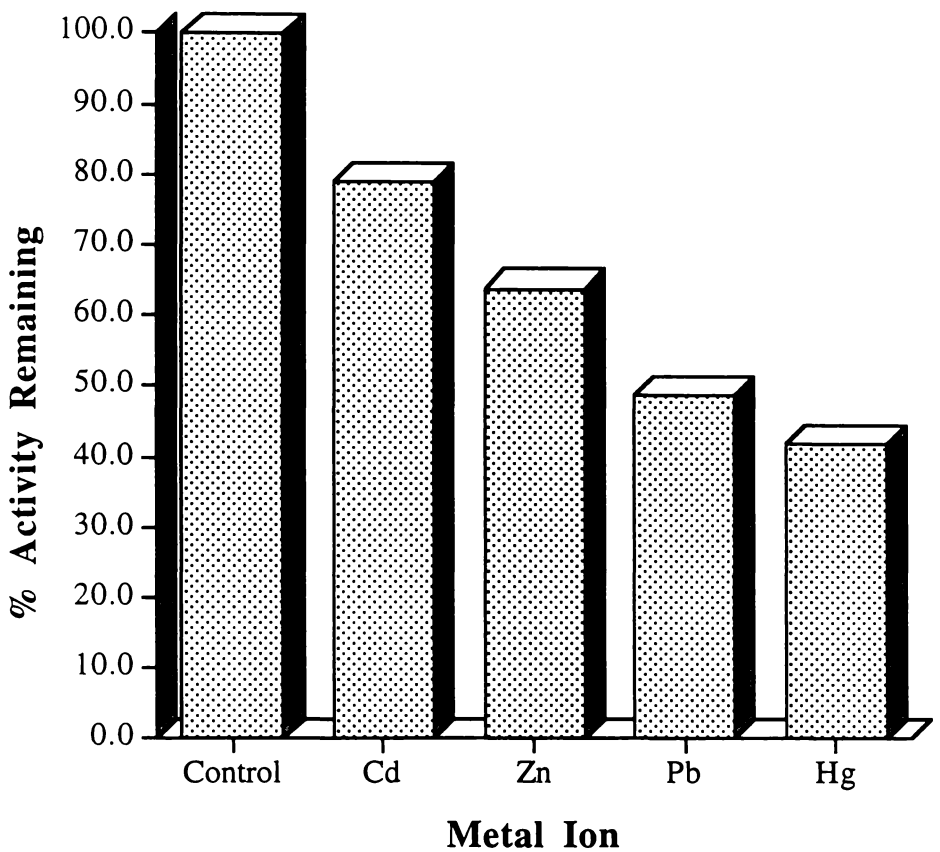


Figure 8.8 Effect of Four Metal Ions on the Activity of Ak.1 Protease.
Ak.1 protease was preincubated with 100 μM concentrations of the metal ions for 1 hour at room temperature, and the remaining activity was detected by assaying with 1 mM SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 and 100 μM metal ion at 20°C according to the method in section 2.2.2.1.

8.3.3.3 Effect of Metals on the Km and Vmax

Figure 8.9 shows the K_m of the protease with the substrate SAAPFpNA at 20°C in the presence of a reductant and metal ions. These results show that the sulphur-binding metal ions have caused an almost insignificant change in the K_m as compared to DTT. Thus these metal ions are affecting the activity of the protease in a different manner from DTT.

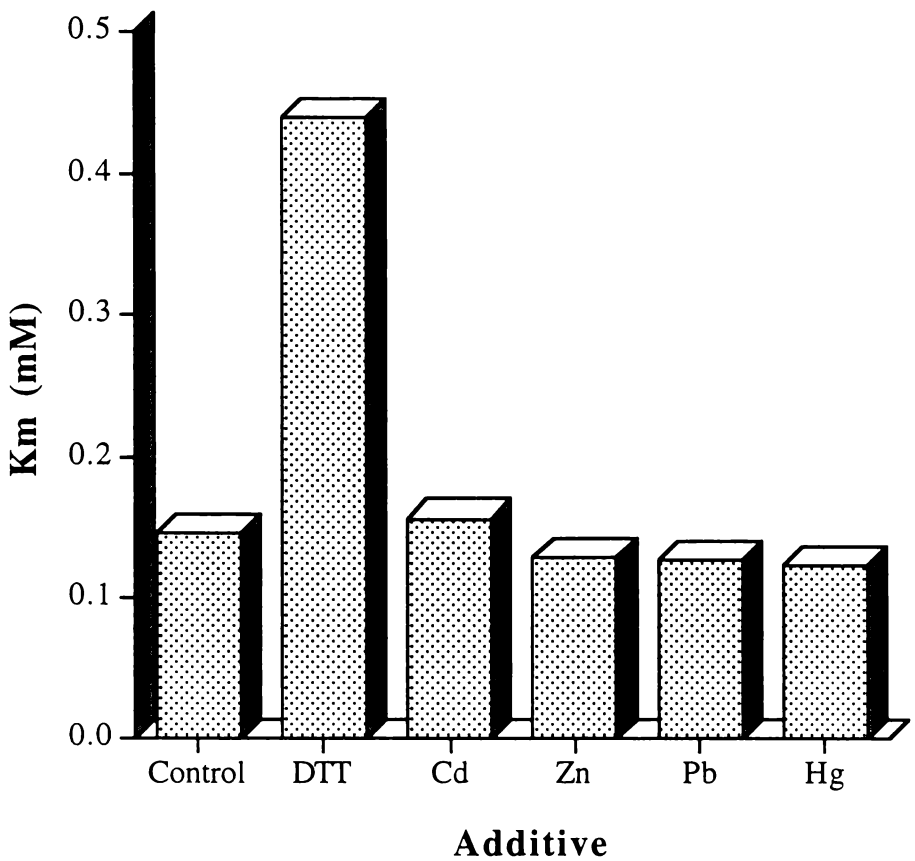


Figure 8.9 Effect of 100 μM Additives on the Km of Ak.1 Protease with SAAPFpNA Substrate.

Ak.1 protease was preincubated with 100 μM concentrations of the metal ions and/or DTT for 1 hour at room temperature. The protease samples were then assayed with the appropriate concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 and 100 mM metal ion at 20°C according to the method in section 2.2.2.1.

Figure 8.10 shows the V_{max} of the protease with the substrate SAAPFpNA at 20°C in the presence of a reductant and metal ions. These results show that the metal ions, (except for Hg^{2+}) have had very little effect on the V_{max} of the protease unlike DTT. Only Hg^{2+} ions have shown a significant decrease in V_{max} like DTT. Most of the additives have caused only a slight decrease in the V_{max} of the protease. Cd^{2+} has caused an increase in the V_{max}. Thus, the slight inhibition of the protease by Cd^{2+} appears to be due to the increase in the K_m partly offset by the increase in V_{max}. The inhibition by Pb^{2+} is due to a decrease in the V_{max}, partly offset by a decrease in the K_m. Thus, in summary, the decrease in activity by these metal ions is generally due to minor changes in the K_m and V_{max} (except Hg^{2+}). These metals had a stabilising effect on the protease (Chapter 6). The increase in stability may have been caused by a decrease in the autolytic rate.

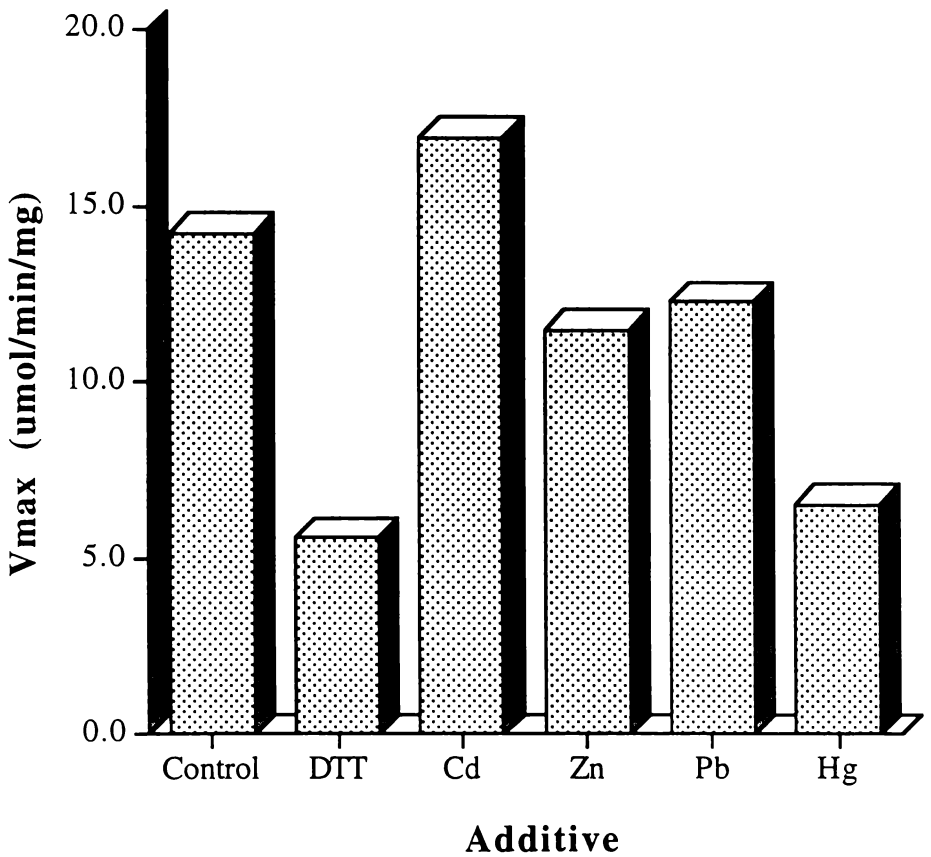


Figure 8.10 Effect of 100 μM Additives on the Vmax of Ak.1 Protease with SAAPFpNA Substrate.

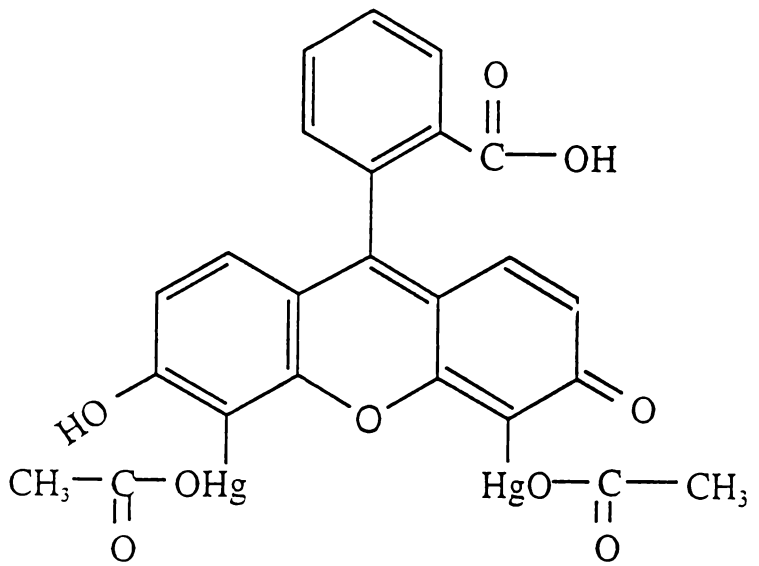
Ak.1 protease was preincubated with 100 μM concentrations of the metal ions and/or DTT for 1 hour at room temperature. The protease samples were then assayed with the appropriate concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 and 100 mM metal ion at 20°C according to the method in section 2.2.2.1.

8.3.4 Fluorescent Labelling of the Cysteine Residues of Ak.1 Protease

8.3.4.1 Introduction

Molecules excited by light of different wavelengths can relax back to the ground state by a variety of methods such as vibrational, rotational and translational relaxation. The *least* preferred way is by the loss of a photon of light (fluorescence). Thus while all molecules have the potential to fluoresce, only a fraction of them do. Molecules, such as biphenyl, do not fluoresce as their flexibility allows them to relax to the ground state by the loss of vibrational energy to the solvent. Rigid molecules, e.g. fluorene, have a more restricted mobility, so have fewer vibrational states to lose energy by, making it more likely to fluoresce.

Fluorescein mercuric acetate (FMA) is a dye for fluorescence and electron microscope detection of sulphydryl residues (Biggiogera & Pellicciari, 1988). It is a useful non-radioactive probe of proteins which contain accessible cysteine residues. Figure 8.11 shows the structure of FMA. When FMA binds to proteins, it becomes less flexible, and therefore the degree of fluorescence increases. Thus this makes FMA an ideal reagent for detecting if mercury binds to the protease.



Fluorescein Mercuric Acetate (FMA)

Figure 8.11 Structure of Fluorescein Mercuric Acetate (FMA)

8.3.4.2 Detection of Protease-FMA

Excitation and emission scans of FMA alone were determined to find the optimal excitation λ and emission λ. These were found to be 238 nm and 517 nm respectively. The major protease emission was at 343 nm.

Figure 8.12 shows a comparison of 3 emission scans (excitation λ = 238 nm). The scans shown are 0.3 μM FMA, 0.65 μM Ak.1 protease and the two combined. These results show that the degree of fluorescence of FMA at 517 nm has increased significantly in the presence of Ak.1 protease. As the enzyme does not emit significantly at 517 nm, the increase in fluorescence of FMA appears be due to a reduction in flexibility of FMA by its binding to the protease. Thus, FMA appears to be a fluorescent label of the enzyme.

In order to check these results, 0.65 μM protease samples preincubated in the presence of 1 mM FMA were extensively ultrafiltered to remove the free FMA in solution. The permeate (enzyme-FMA), final retentate (trace free-FMA) and RO water were scanned, as before, to determine the presence of any protease-FMA. Figure 8.13 shows a comparison of the emission scans of these samples.

These results show that there is indeed significant fluorescence detected in the enzyme sample above that of the permeate (free-FMA), indicating the presence of protease-FMA. A comparison of the permeate and RO water scans shows that there is no enzyme present in the permeate.

Protease samples pretreated by the addition (and partial removal) of DTT were incubated with FMA at a concentration (1mM) in excess of the concentration of remaining DTT (~10μM). Samples were analysed by fluorescence spectroscopy, as above. It was found that approximately the same quantity of fluorescent label was present in the enzyme as in the sample not pretreated with DTT. This suggests that there has been no significant increase in the binding of FMA to the protease with the cysteine residues exposed. This is probably because mercuric acetates (e.g. FMA) are able to bind to intact disulphide bonds as well as free cysteine residues (Martin, 1979).

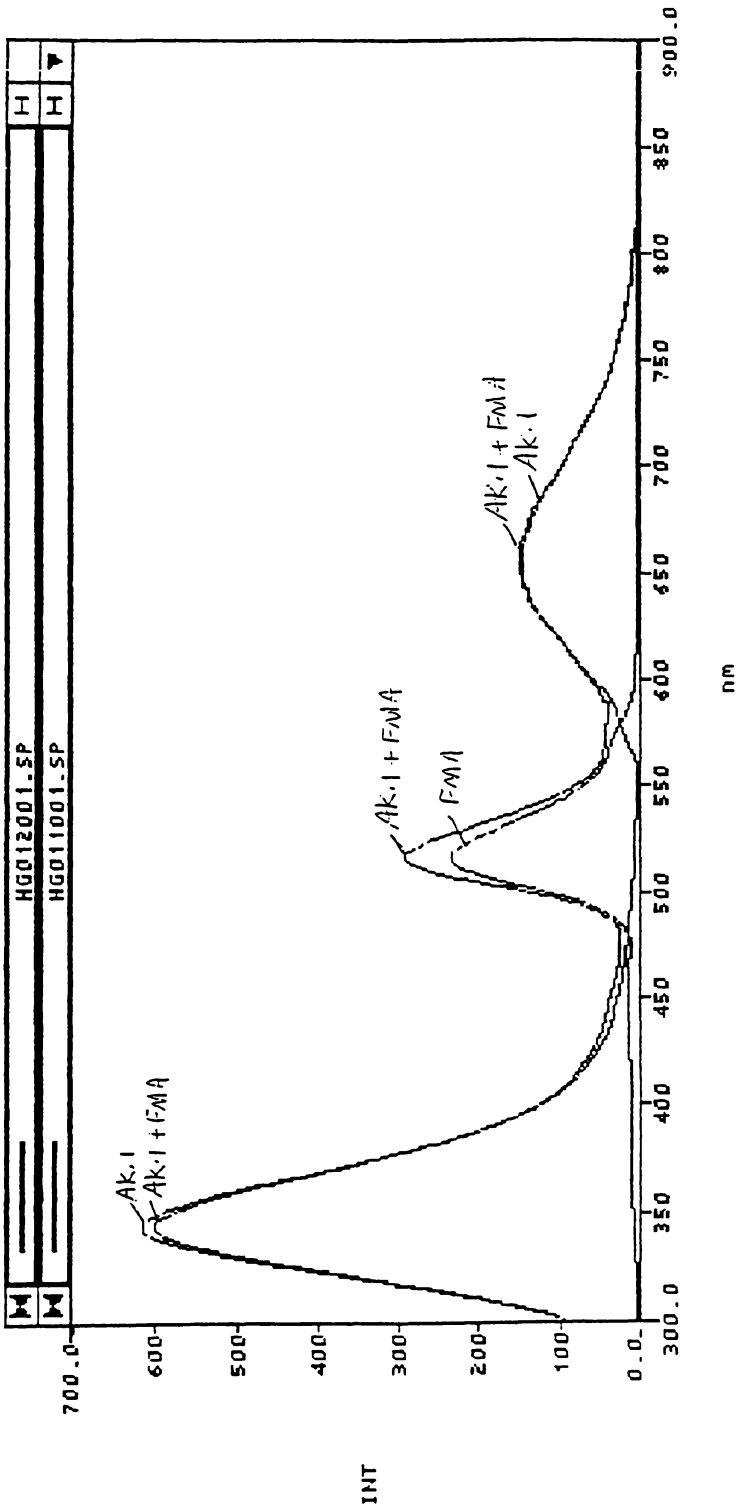


Figure 8.12 Comparison of Emission Scans of FMA and Protease Samples (Excitation λ = 258 nm)

FMA = fluorescein mercuric acetate. Ak.1 = Ak.1 protease

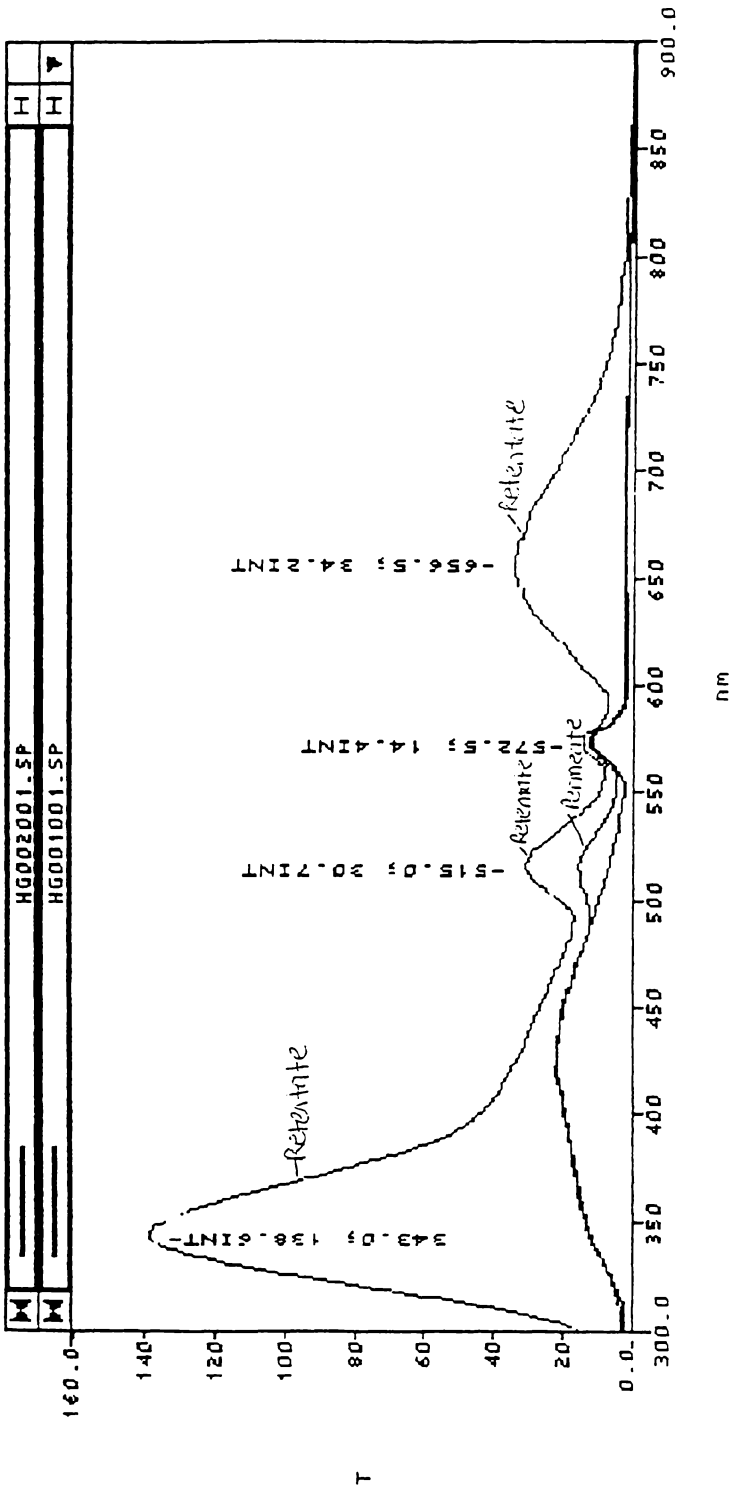


Figure 8.13 Comparison of Emission Scans of FMA and Protease Samples After Ultrafiltration (Excitation λ = 258 nm)

FMA = fluorescein mercuric acetate. Ak.1 = Ak.1 protease. The retentate contains Ak.1 protease-FMA complex + trace free FMA. The permeate contains only the trace FMA.

8.3.4.3 Removal of the FMA Label from Ak.1 Protease

Mercury ions prefer to bind to sulphydryl residues, but will bind to other groups such as amino groups. If mercury has bound to sulphur, the addition of a reductant, such as DTT, will cause the mercury to be removed from the sulphur.

Figure 8.14 shows a comparison of emission scans of protease-FMA (after ultrafiltration) and DTT/protease/FMA (after ultrafiltration). The ultrafiltration steps were conducted to remove the DTT and unbound FMA. These results have been corrected for the presence of free FMA in solution by the subtraction of their respective permeate scans. The protein peaks (343 nm) are approximately the same, indicating the presence of about the same quantity of protease. These results show that DTT has indeed removed the FMA from the protease. This suggests that FMA was bound to the cysteine residue/s of the protease.

This data needs to be interpreted with caution as a comparison of emission scans of FMA and FMA/DTT show that the presence of DTT causes an 18% decrease in the fluorescence of FMA (results not shown). However, as absolutely no fluorescence at 517 nm was detected after DTT treatment/removal, it is likely that very little FMA is present as a peak 18% smaller than the previous protease-FMA peak would be easily detectable.

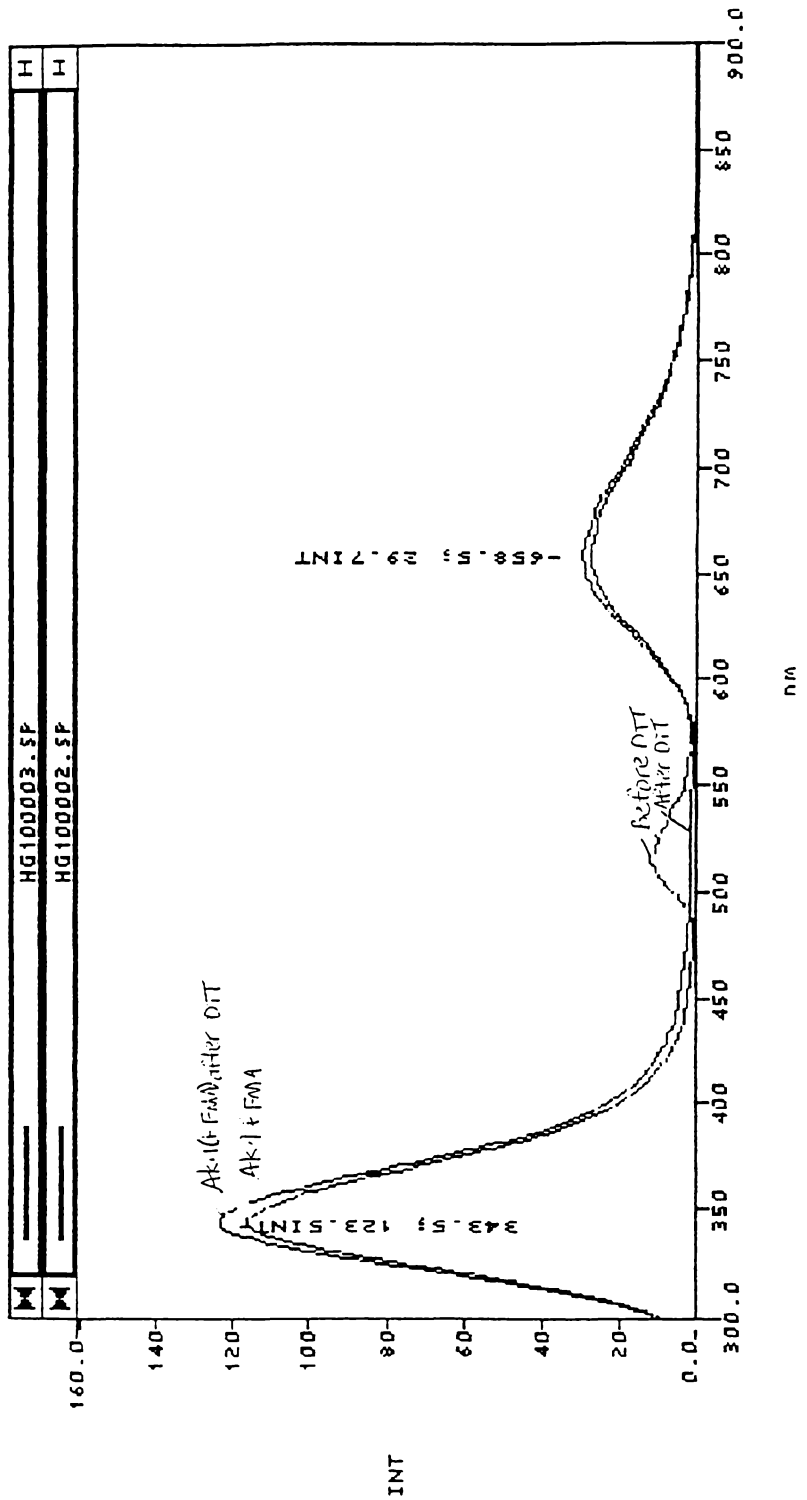


Figure 8.14 Comparison of Emission Scans of Protease-FMA and Protease/FMA/DTT Samples After Ultrafiltration (Excitation λ = 258 nm)

FMA = fluorescein mercuric acetate. Ak.1 = Ak.1 protease. 'Before DTT' is the Ak.1 protease-FMA complex. 'After DTT' is Ak.1 protease-FMA after DTT addition and removal (i.e. the FMA is no longer bound to the enzyme, but has been removed by ultrafiltration). Both the scans have been corrected for the presence of trace free FMA by subtracting their respective ultrafiltration permeate scans.

8.3.4.4 Effect of FMA and DTT on the Activity of Ak.1 Protease

Figure 8.15 shows the effect of different concentrations of DTT and FMA on the activity of Ak.1 protease. This shows that both DTT and FMA (Hg^{2+}) inhibit the activity of the protease, though FMA has a lower inhibitory effect than DTT. This suggests that DTT has a higher affinity for binding to disulphide bonds than Hg^{2+}.

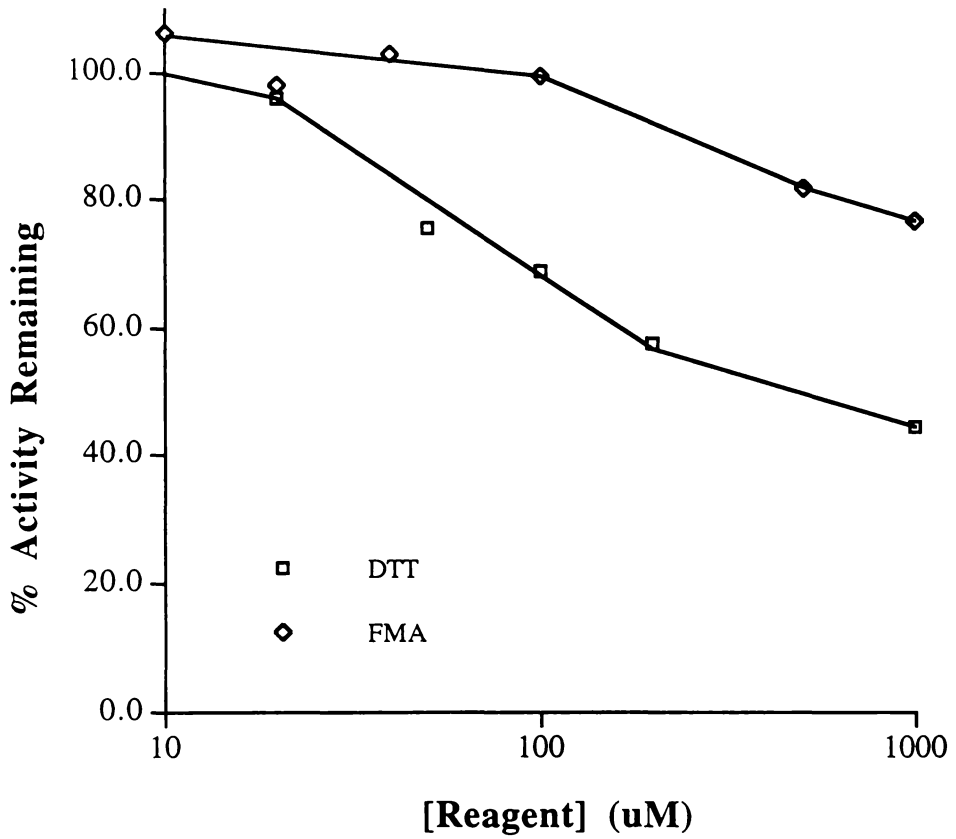


Figure 8.15 Effect of DTT and FMA on the Activity of the Protease.
The protease was preincubated with 0-1mM concentrations of these reagents, then assayed for remaining activity with 1 mM SAAPFpNA as in section 8.2.4.

8.3.5 Effect of the Disulphide Bond on the Thermostability of Ak.1 Protease

A disulphide bond is a covalent linkage between two cysteine residues. These bonds are often between cysteine residues within certain domains of proteins. They can be responsible for linking together different parts of the protein. This helps to decrease the flexibility of the protein and maintain its ideal shape. By decreasing the flexibility of a protein, it decreases the likelihood of denaturation (unfolding). Thus disulphide bonds have been known to increase the thermostability of a protein.

Figure 8.16 shows the % activity remaining of the protease in the presence of the additives after an incubation of 1 hour at 85°C. These results show that the reductants DTT and βME have caused the thermostability of the protease to decrease significantly. The alkylating agent, IAA, has caused a complete loss of activity of the protease. This is surprising as IAA acts on free sulphydryl groups, and the native protein contains a disulphide bond. IAA in the presence of the reductants also caused a complete loss of activity at 85°C for 1 hour. Thus while IAA has only a slight effect on activity at 20°C (Table 8.3), it has a significant effect on the thermostability of the protease at 85°C.

Figure 8.16 also shows the thermostability of the protease after treatment (and removal) of IAA, and compared it to the thermostability in the presence of IAA. These results show a significant difference between the thermostability of the two protease samples. After the removal of IAA, the % activity remaining of the protease has decreased to only 79%, compared to 0% activity in its presence. This suggests that at high temperatures, IAA is irreversibly non-specifically binding to other sites in the protease, forming a species of a much lower thermostability. IAA is known to bind to other functional groups such as amino- and carboxyl- groups, though with a lower affinity than to thiols (Imoto & Yamada, 1990).

Daniel *et al* (1996) described common degradative processess of proteins occuring at temperatures above 80°C. Cysteine residues can undergo reactions such as oxidation and β-elimination. These residues are also susceptible to disulphide exchange, where disulphide bonds are reduced, and later reformed between different cysteine residues. At 85°C, there could be an equilibrium between the free sulphydryl group form of Ak.1 protease and the disulphide bond form of the protease existing in solution. IAA could be alkylating the protease when it is in the reduced form, forming a species with a much lower

thermostability. Alternatively, it may be binding to other functional groups instead of thiol groups.

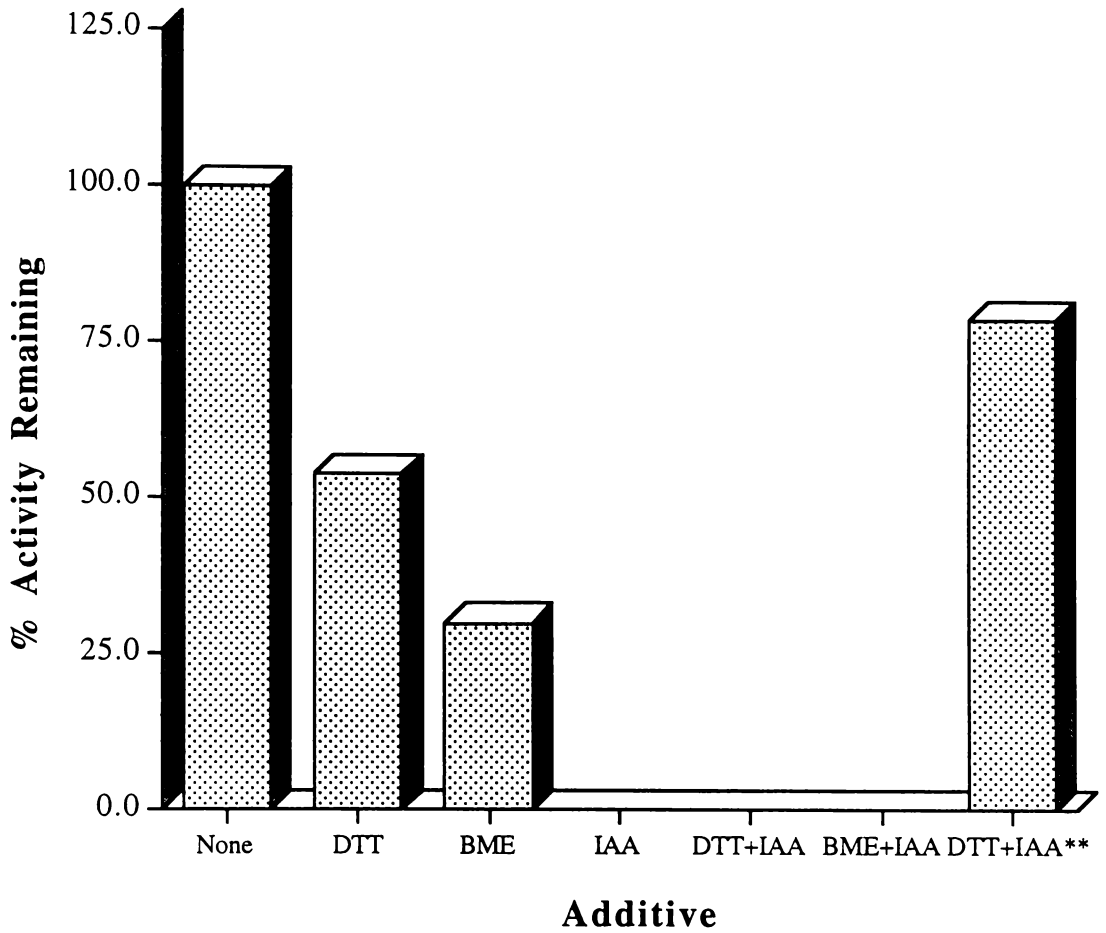


Figure 8.16 % Activity Remaining After an Incubation of 1 Hour at 85°C in the Presence and Absence of Reducing and Modifying Agents.

Ak.1 protease was preincubated with the reagents for 1 hour at room temperature. For DTT+IAA**, the reagents were subsequently removed from the enzyme by passing it through Sephadex G25, followed by concentration by ultrafiltration (YM-10). The samples were incubated for 1 hour at 85°C, then assayed for remaining activity with 1 mM SAAPFpNA as described in section 8.2.7.

Figure 8.4 (earlier) shows the Log V_{max} vs 1/Temperature (K). As mentioned previously, the slope of the DTT samples is lower than that of the control at higher temperatures. These results show that the increasing difference in the V_{max} between the control enzyme and the DTT-enzyme is possibly due to a decrease in stability of the protease when the disulphide bond is open. If so, then the V_{max} at the higher temperatures (75°C) is probably an underestimation of the V_{max} possible as a significant proportion of the enzyme has been denatured.

Figure 8.17 shows the thermostability data of the protease at 85°C in the presence and absence of DTT. Table 8.6 shows the half lives of the protease derived from this data. This shows that the protease is 9.3 fold more thermostable at 85°C with a disulphide bond than with free sulphydryl residues. This is not suprising as disulphide bonds are known to stabilise enzymes against heat-denaturation (e.g. Ahern & Klibanov, 1986).

	Half Life	
Sample	Control	DTT-Enzyme
Ak.1 Protease	317min	34 min

Table 8.6 Half Life of Ak.1 Protease at 85°C With and Without DTT.
Ak.1 protease was incubated for the appropriate time periods at 85°C, cooled on ice then then assayed for remaining activity with 1 mM SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 at 20°C as in section 8.2.7.

An analysis of the first and second order plots (Figure 8.17 B & C) is inconclusive. It is not clear whether the loss of activity is most significant due to denaturation or autolysis.

(A)

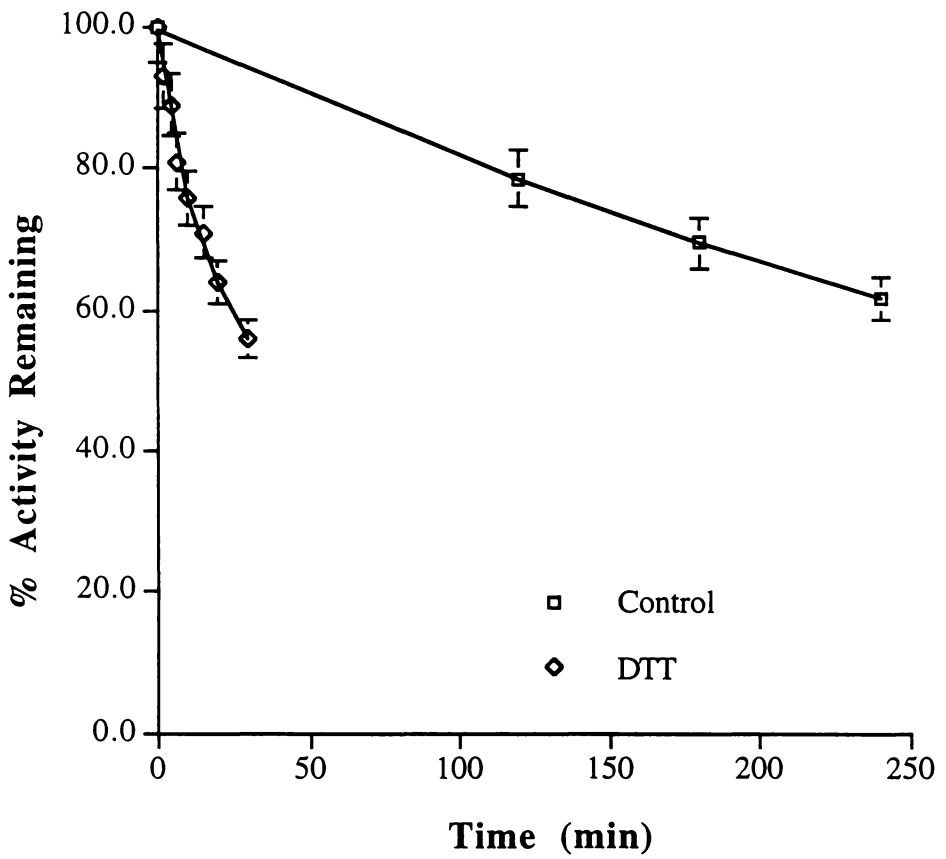
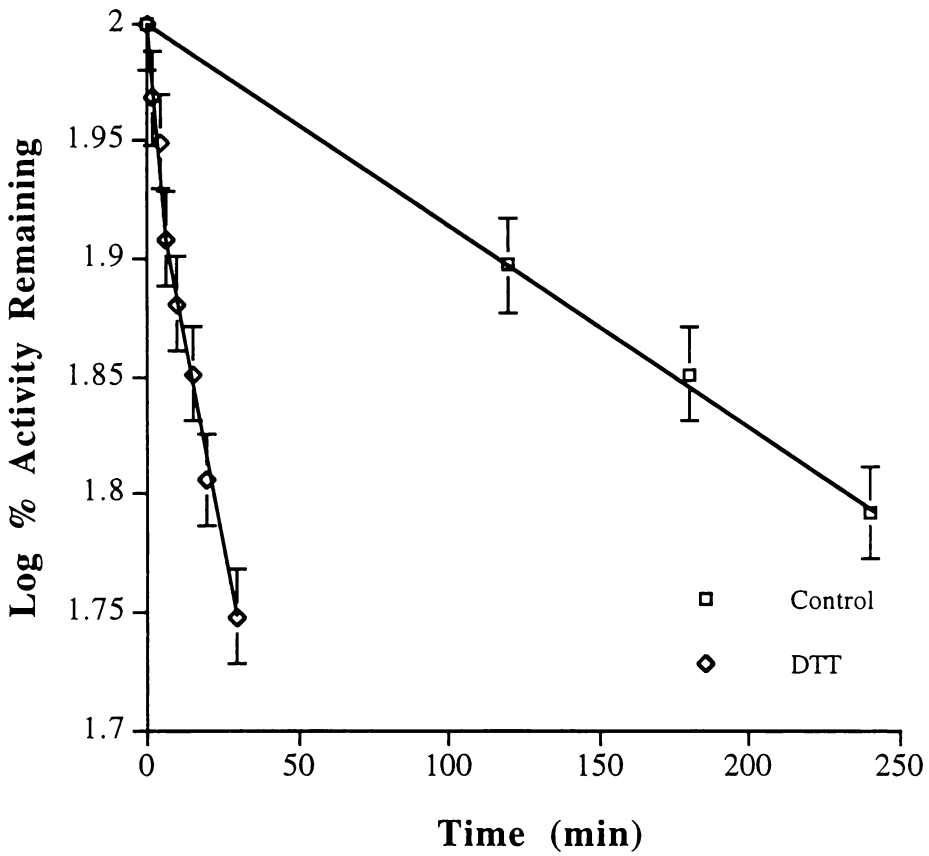


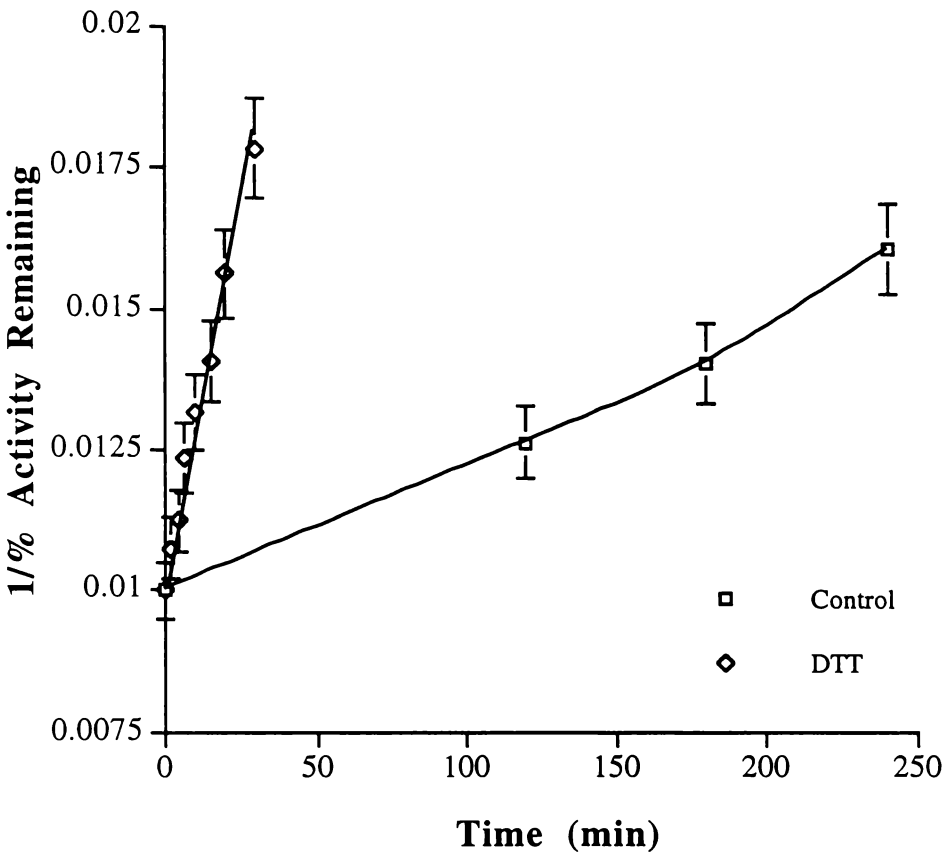
Figure 8.17 Thermostability of Ak.1 Protease with and without DTT at 85°C. (A) % Activity Remaining Vs Time; (B) Log% Activity Vs Time (First Order Plot); (C) 1/% Activity Remaining Vs Time (Second Order Plot).

Ak.1 protease was incubated for the appropriate time periods at 85°C, cooled on ice then then assayed for remaining activity with 1 mM SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100 at 20°C as in section 8.2.7.

(B)


Log % Activity Remaining
2
1.95
1.9
1.85
1.8
1.75
1.7
0
50
100
150
200
250
Time (min)
Control
DTT

(C)


0.02
0.0175
0.015
0.0125
0.01
0.0075
1/% Activity Remaining
0
50
100
150
200
250
Time (min)
Control
DTT

8.4 CONCLUSIONS

The sequence of Ak.1 protease indicates the presence of two cysteine residues, separated by only one amino acid. The 3D structure showed that these cysteine residues exist as a disulphide bond. The results (e.g. Ellman assay) confirmed the presence of not only two cysteine residues, but that in native conditions (i.e. in solution) these residues exist as a disulphide bond.

Reductants, such as dithiothreitol (DTT) typically reduce disulphide bonds into their constituent cysteine residues. It needed to be confirmed that this was the effect of DTT in Ak.1 protease. The Ellman assay proved that the two cysteine were exposed upon the addition of DTT. The lower thermostability in the presence of DTT indicates that it appears to have opened a disulphide bond as disulphide bonds are known to increase the thermostability of proteins. It is possible that the DTT was interacting with the calcium binding site/s, which could account for the decrease in the thermostability of the protease. However, tests showing the effect of DTT on the protease were conducted in the temperature range of 5-75°C. DTT still affected the K_m even at the lower temperatures where calcium binding is not important for stability due to the intrinsic thermostability of Ak.1 protease at temperatures below at least 40°C. So DTT appears to be affecting the enzyme by opening the disulphide bond at the active site.

It was proposed that the reduction of the disulphide bond caused a localised opening of the substrate binding cleft at the P_4 site, due to the location of the disulphide bond in this position. This was supported by the results that showed that the larger the substrate, the greater the effect of DTT on the K_m of the protease with that substrate. For example, the K_m was unchanged with DTT with a substrate occupying only sites P_2-P_1', while a substrate which occupies sites P_5-P_1' showed a significant increase of the K_m, suggesting it binds less significantly to the binding cleft.

Heavy metals such as Hg^{2+} and Pb^{2+} bind to Ak.1 protease, causing a decrease in the specific activity of the protease. Heavy metals tend to bind to proteins at sulphur atoms (e.g. cysteine residues). The effects of the heavy metals on activity is much smaller than with DTT. In general, the K_m and V_{max} were only changed to a small degree.

Fluorescein mercuric acetate (FMA) binds to Ak.1 protease, causing the fluorescence of FMA to increase. This is a way of determining that Hg^{2+} ions are affecting the protease by binding to it.

The presence of DTT caused a decrease in the thermostability of Ak.1 protease of 9.3 fold at 85°C. This is not surprising as disulphide bonds are known to increase the thermostability of proteins.

Therefore, the disulphide bond has a dual role, that of maintaining the integrity of the substrate-binding cleft and increasing the thermostability of the protease.

APPENDIX 1

STANDARD CURVES

A1.1 STANDARD CURVES OF AK.1 PROTEASE ASSAYS WITH PROTEIN SUBSTRATES

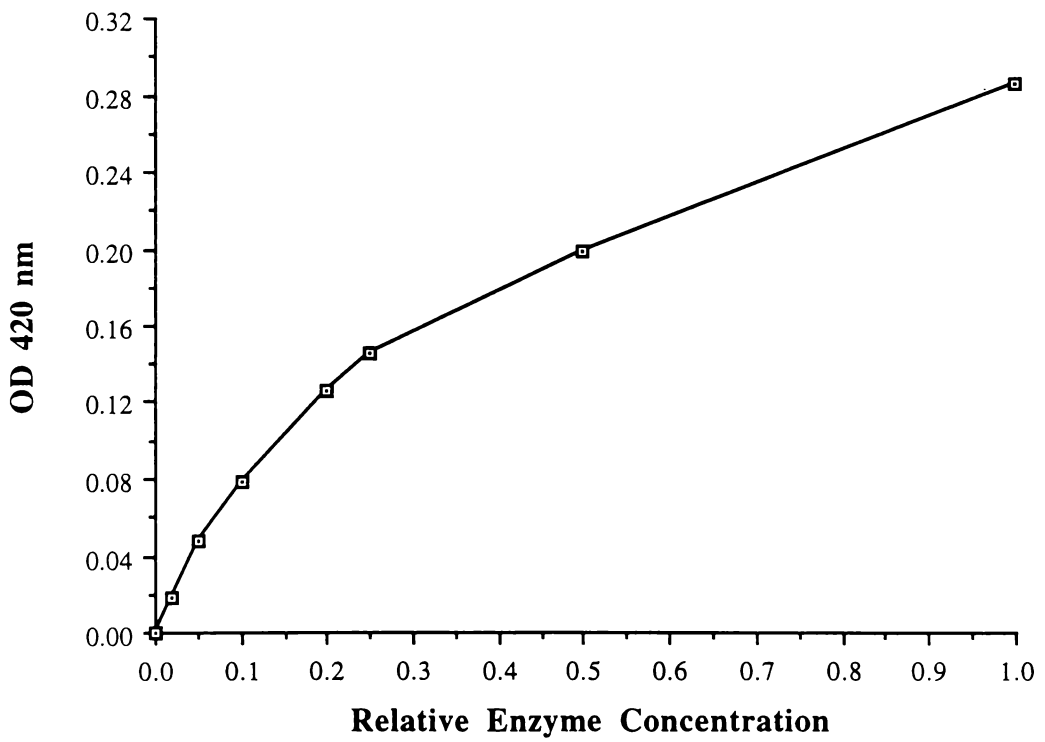


Figure A1.1.1 Standard Curve of the Azocasein Assay with Ak.1 Protease
Ak.1 protease was assayed with 0.2% azocasein in 50 mM HEPES/NaOH pH75 7.5 containing 5 mM $CaCl_2$ for 10 minutes at 75°C.

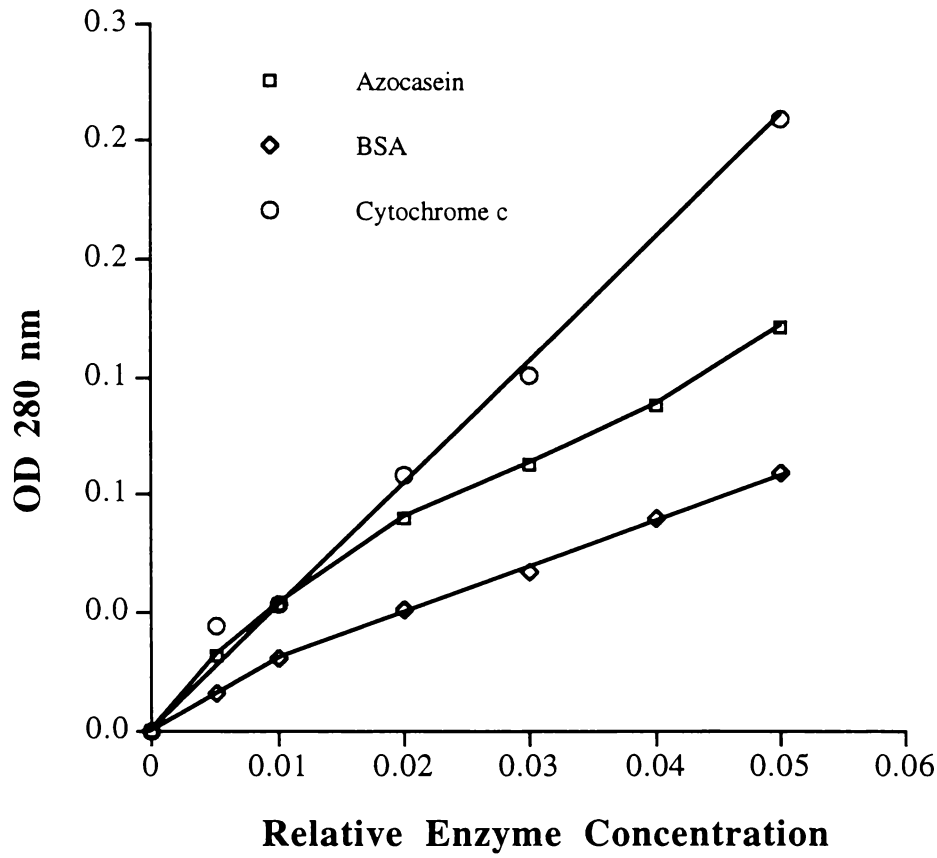


Figure A1.1.2 Standard Curve of Protease Assays with Protein Substrates With Ak.1 Protease.

Ak.1 protease was assayed with 0.2% protein solution in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ for 30 minutes at 75°C.

A1.2 STANDARD CURVE FOR PNA-LINKED PEPTIDE SUBSTRATES

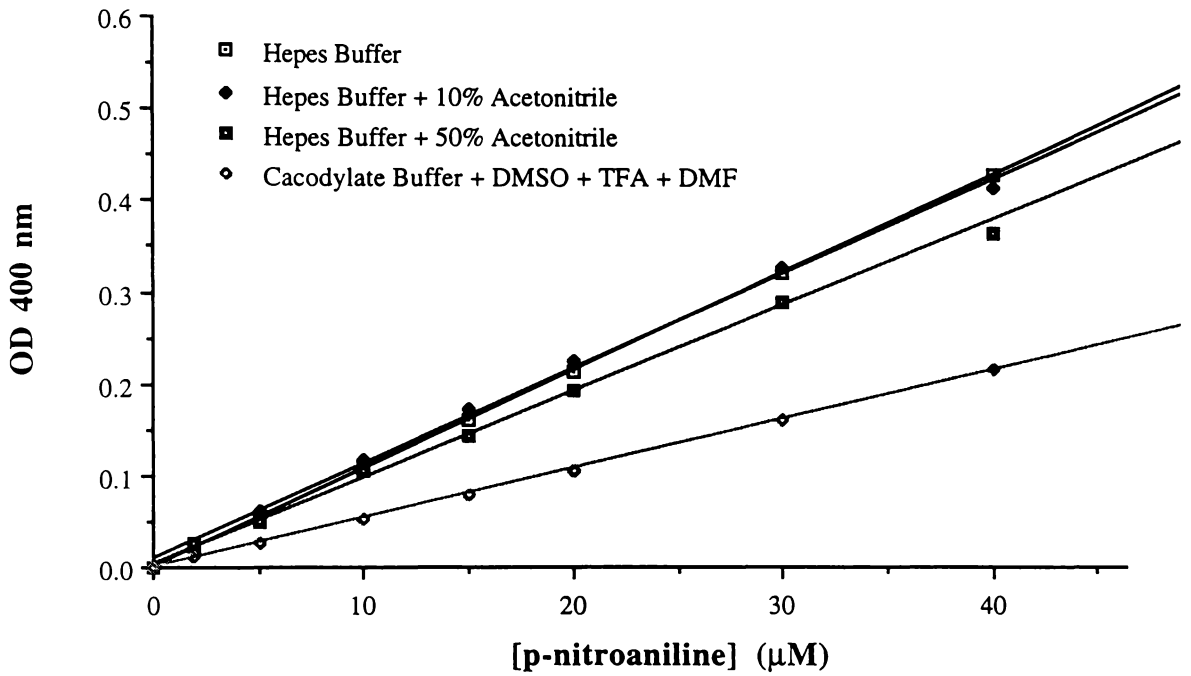


Figure A1.2.1 Standard Curve of *p*-Nitroaniline in Several Buffer Systems.
Standard solutions of p-nitroaniline were dissolved in the buffers: (A) 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$ and 0.01% Triton X-100; (B) 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 10% acetonitrile; (C) 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 50% acetonitrile; (D) 50 mM cacodylate pH 7.5 containing 50% DMSO, TFA and DMF.

A1.3 STANDARD CURVE FOR PNP-ESTER-LINKED SUBSTRATES

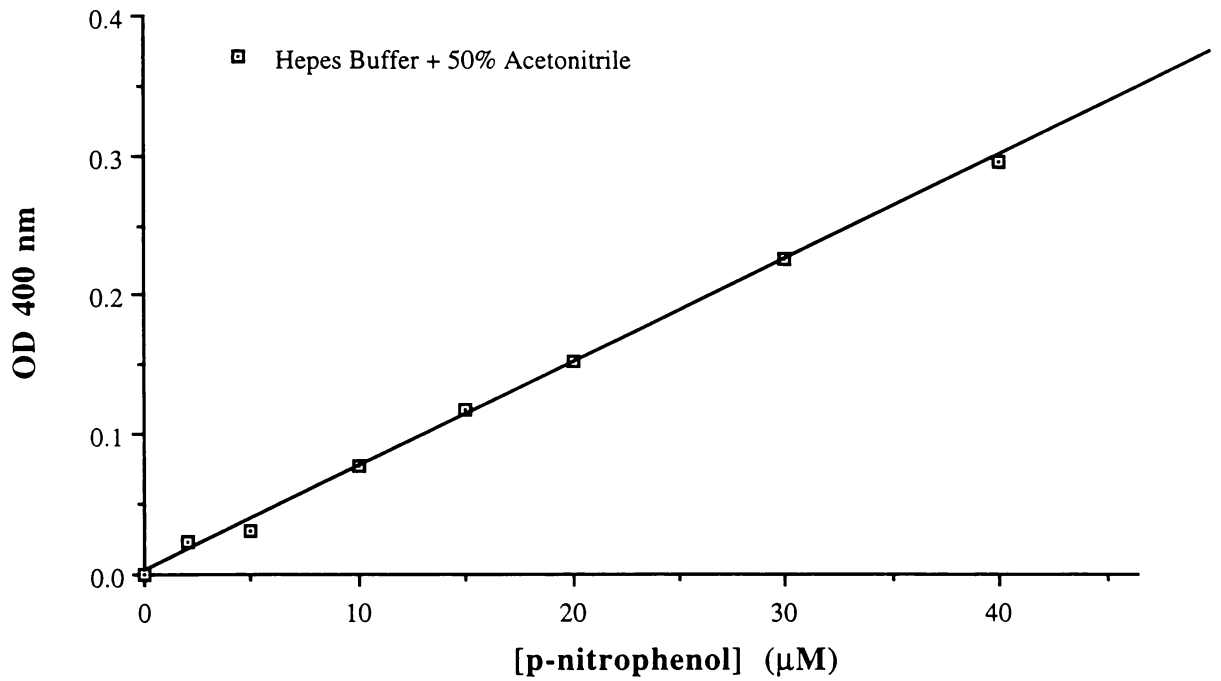


Figure A1.3.1 Standard Curve of p-Nitrophenol in 50% Acetonitrile.
Standard solutions of p-nitrophenol were dissolved in 50 mM Hepes/NaOH pH 7.5 containing 5 mM $CaCl_2$, 0.01% Triton X-100 and 50% acetonitrile.

APPENDIX 2

PUBLICATIONS

A2.1 Thermostable Proteases

DANIEL, R.M., TOOGOOD, H.S. & BERGQUIST, P.L. (1995). *Biotechnology and Genetic Engineering Reviews* **13**, 51-100.

Another publication of Ak.1 protease is a brief summary paper prepared for a handbook of proteases.

TOOGOOD, H.S. & DANIEL, R.M. (1998) *Bacillus* Ak.1 Protease. in. *Handbook of Proteolytic Enzymes* (ed. A.J. Barrett), In Press.

A2.2 Sequence of the Gene Encoding a Highly Thermostable Neutral Proteinase from *Bacillus* sp. Strain EA1: Expression in *Escherichia coli* and Characterisation.

SAUL, D.J., WILLIAMS, L.C., TOOGOOD, H.S., DANIEL, R.M. & BERGQUIST, P.L. (1996). *Biochimica et Biophysica Acta* **1308**, 74-80.

APPENDIX 3

THERMOSTABILITY GRAPHS

A3.1 EA1 PROTEASE

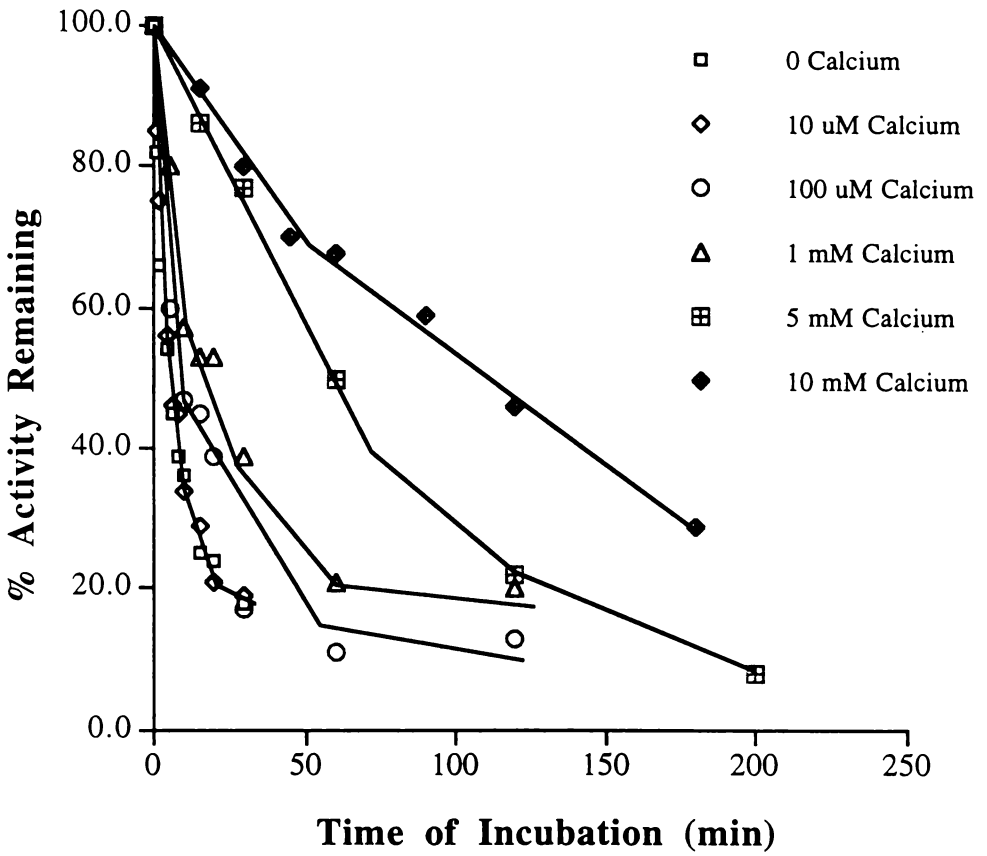


Figure A3.1.1 % Activity Remaining Vs Time of Native EA1 Protease at 85°C with 0-10 mM $CaCl_2$.

EA1 protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

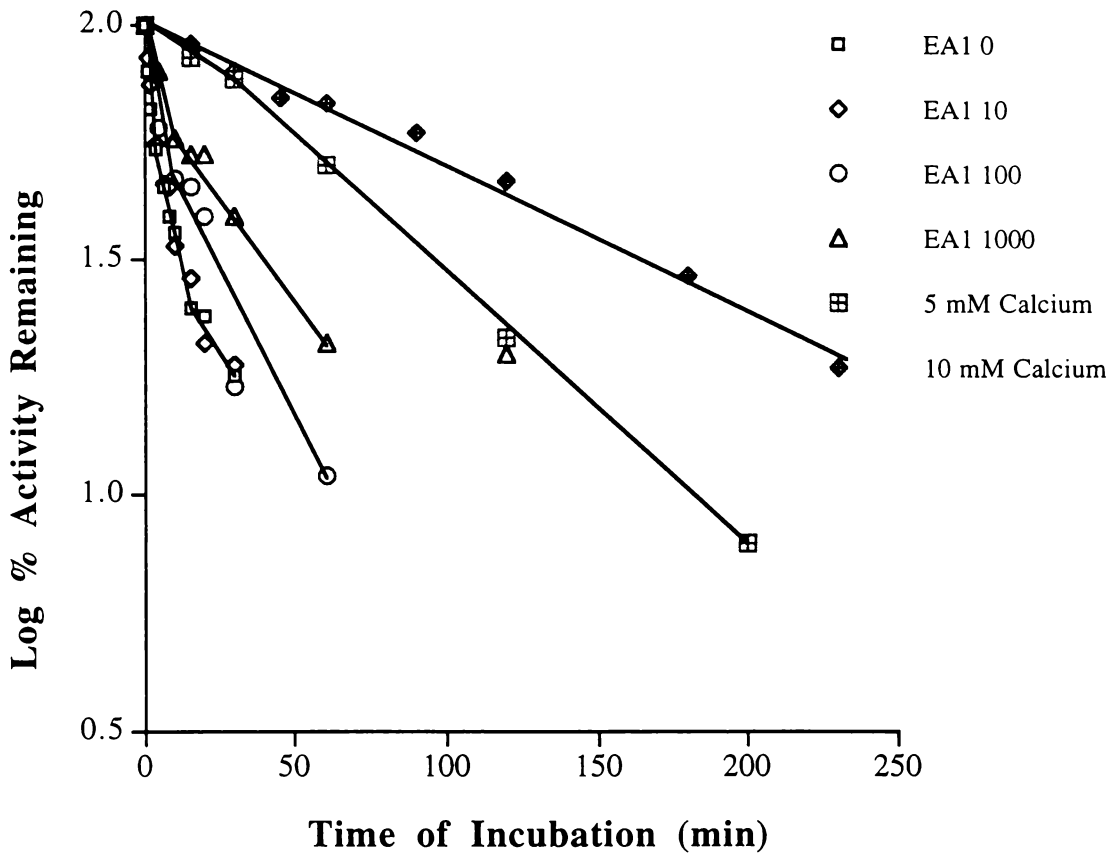


Figure A3.1.2 First Order Plots of Native EA1 Protease at 85°C with 0-10 mM $CaCl_2$.

EA1 protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

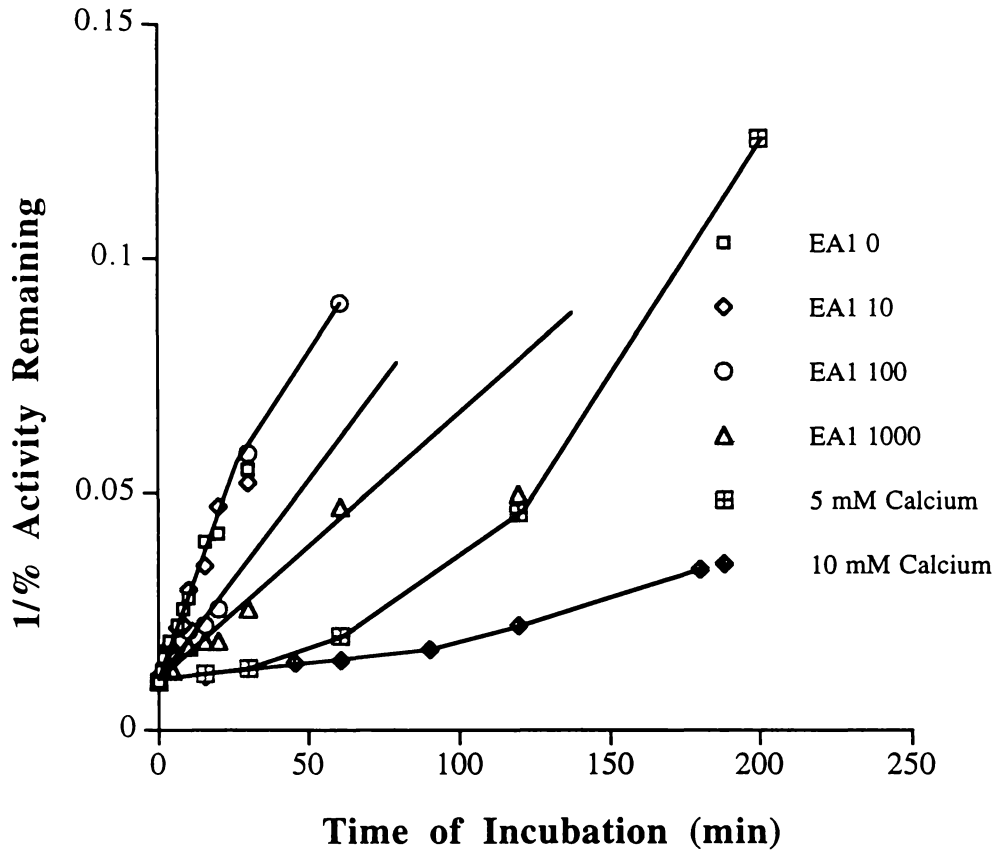


Figure A3.1.3 Second Order Plots of Native EA1 Protease at 85°C with 0-10 mM $CaCl_2$.

EA1 protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

A3.2 YP-T PROTEASE

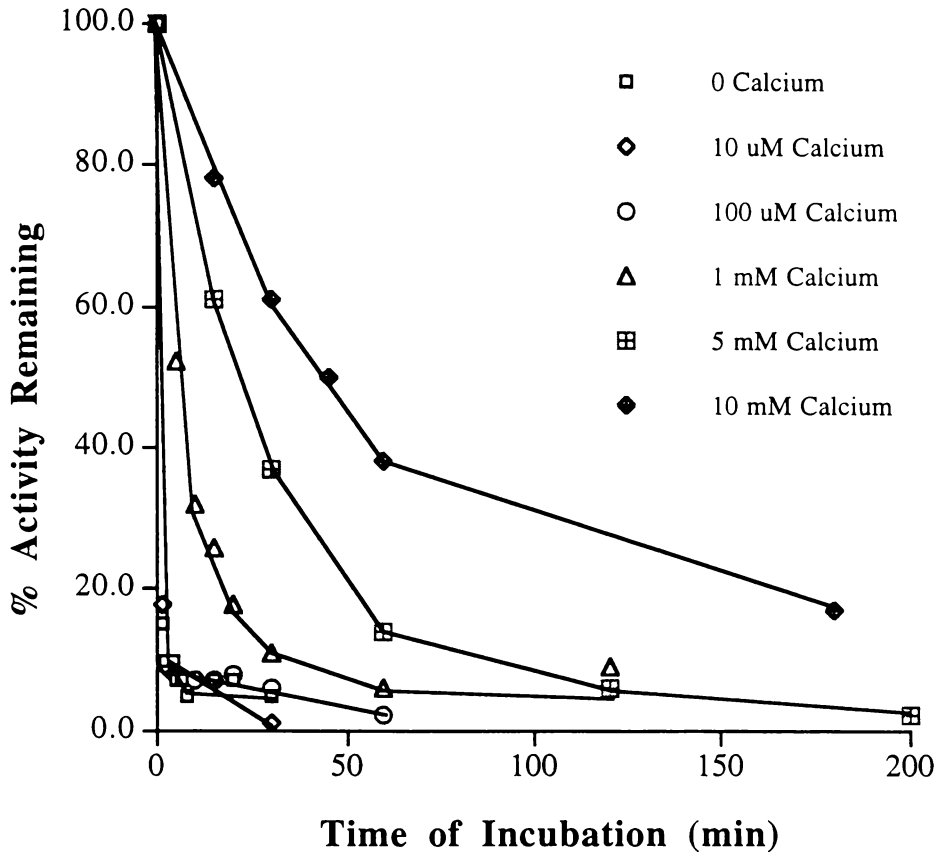


Figure A3.2.1 % Activity Remaining Vs Time of Native EA1 Protease at 85°C with 0-10 mM $CaCl_2$.

YP-T protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

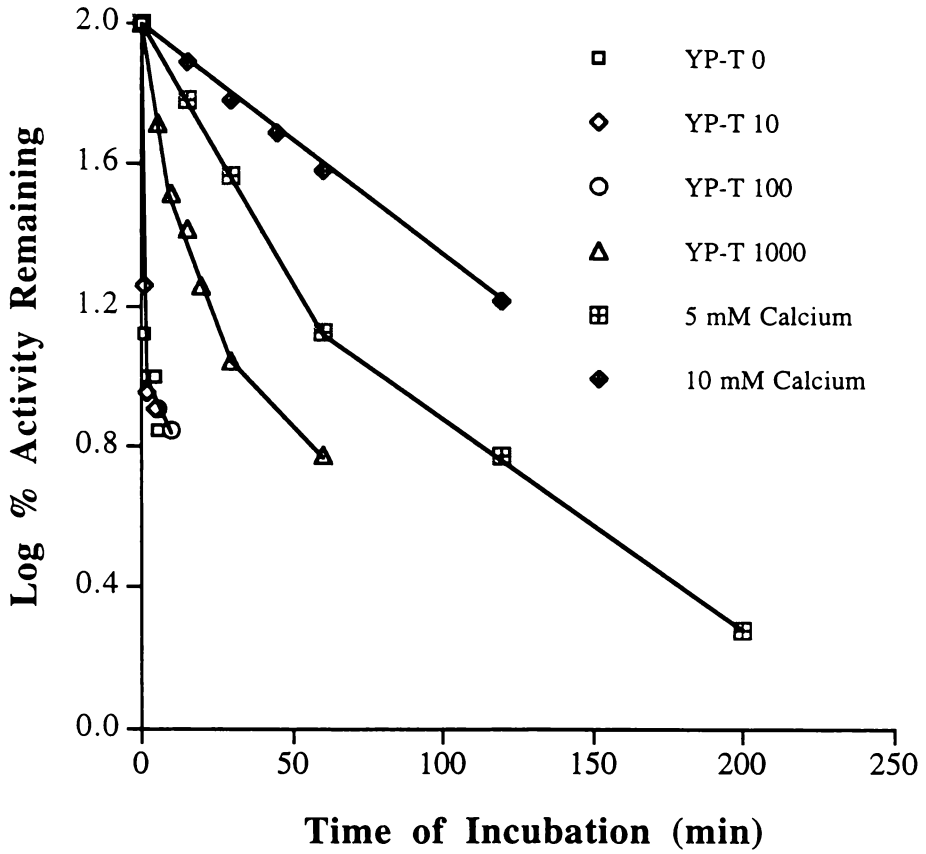


Figure A3.2.2 First Order Plots of Native YP-T Protease at 85°C with 0-10 mM $CaCl_2$.

YP-T protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

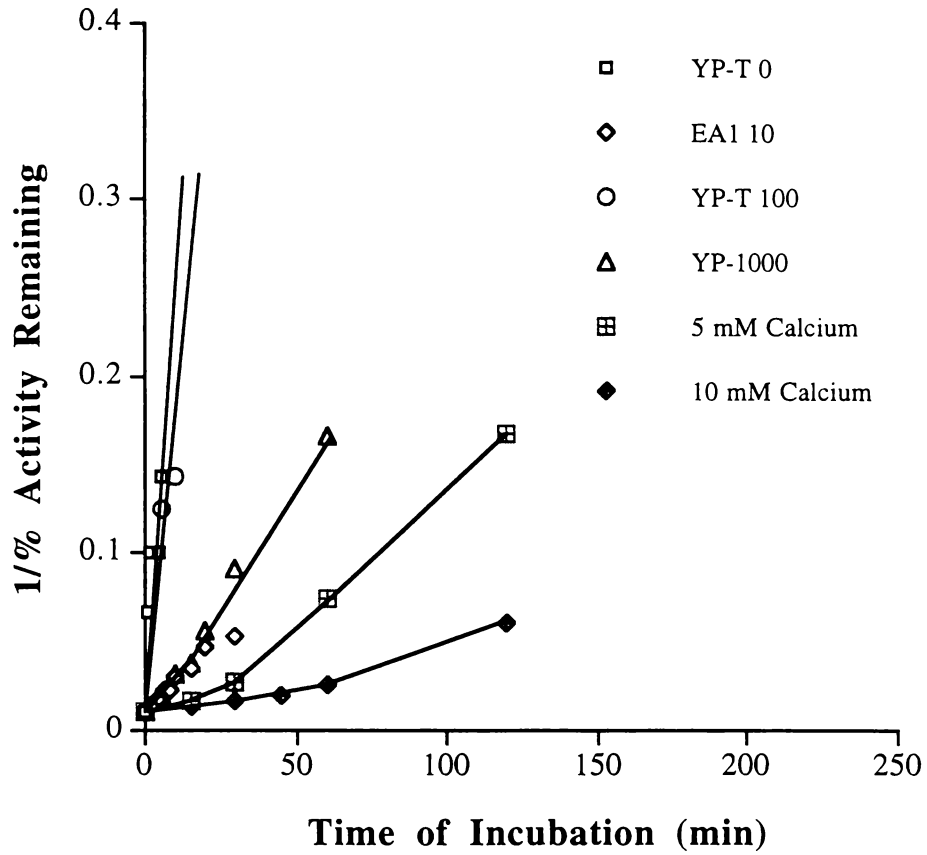


Figure A3.2.3 Second Order Plots of Native YP-T Protease at 85°C with 0-10 mM $CaCl_2$.

YP-T protease, pre-equilibrated in 0-10 mM $CaCl_2$, was incubated at 85°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 6.5 containing 5 mM $CaCl_2$ at 75°C.

A3.3 AK.1 PROTEASE

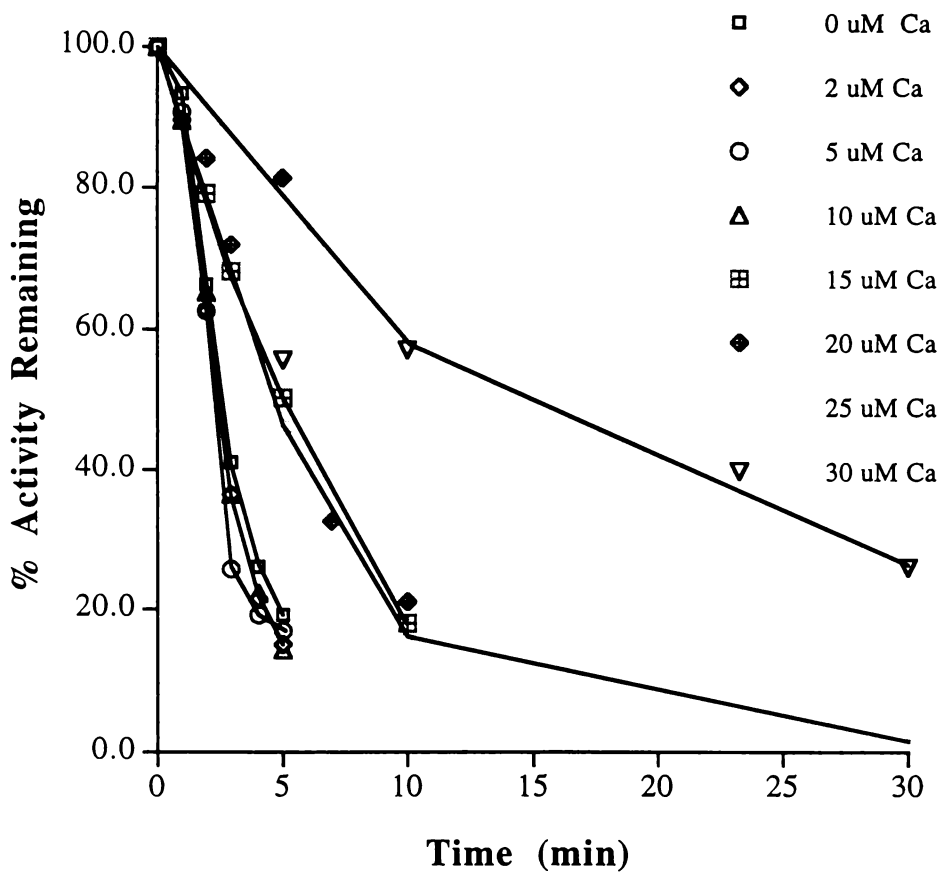


Figure A3.3.1 % Activity Remaining Vs Time of Ak.1 Protease at 70°C with 0-30 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 0-30 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

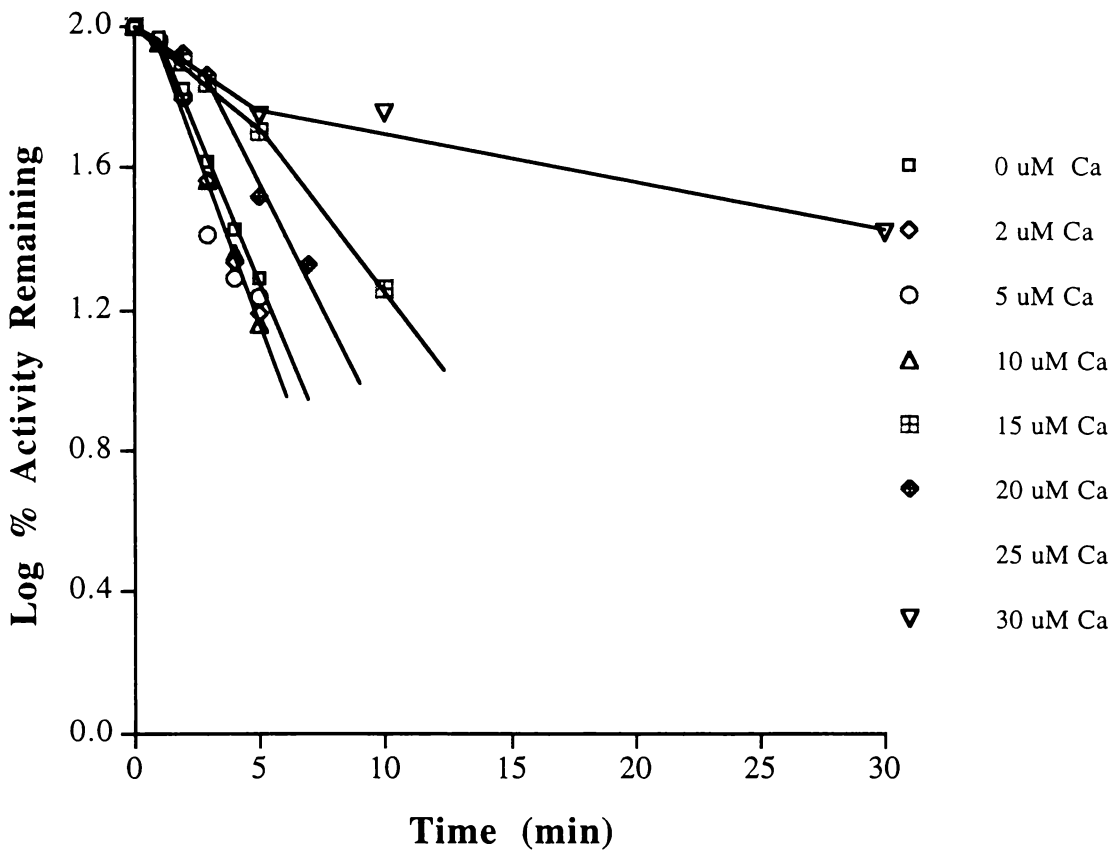


Figure A3.3.2 First Order Plots of Ak.1 Protease at 70°C with 0-30 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 0-30 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

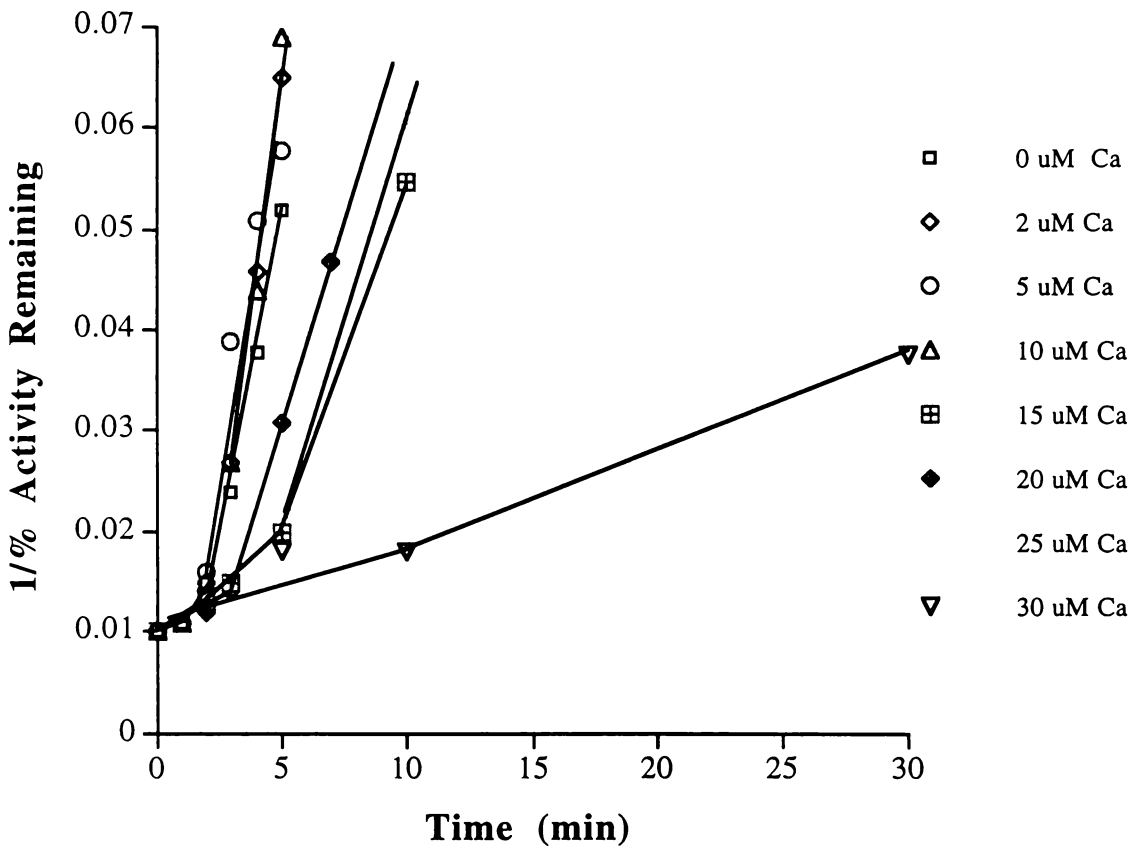


Figure A3.3.3 Second Order Plots of Ak.1 Protease at 70°C with 0-30 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 0-30 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

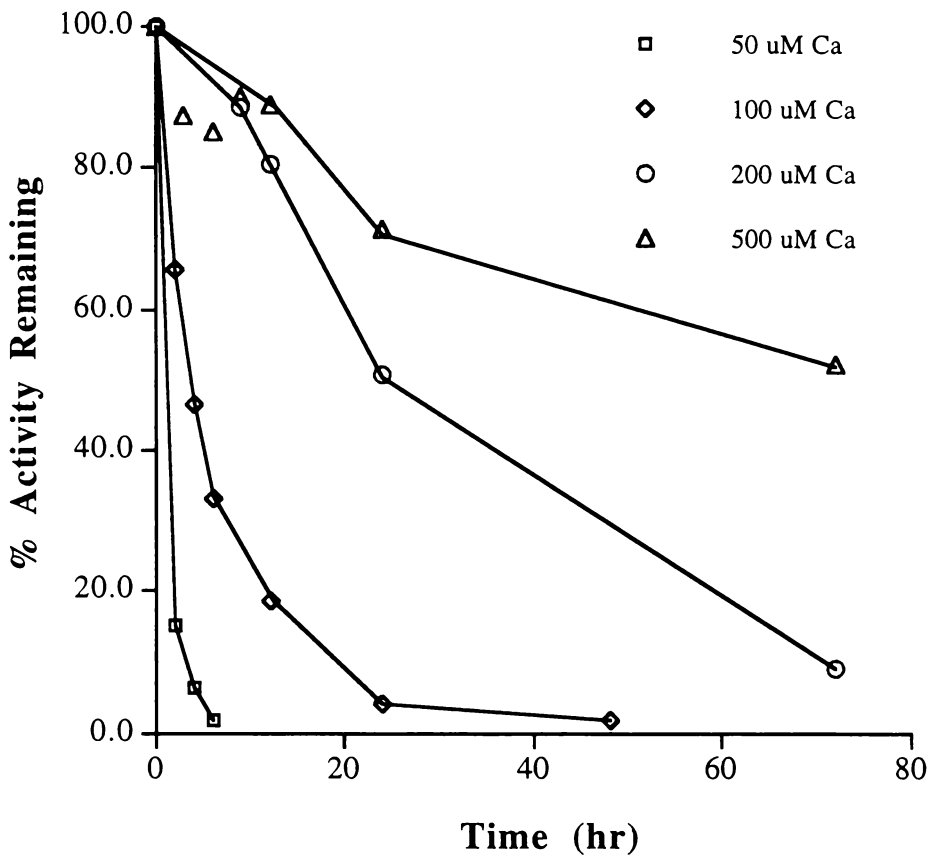


Figure A3.3.4 % Activity Remaining Vs Time of Ak.1 Protease at 70°C with 50-500 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 50-500 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

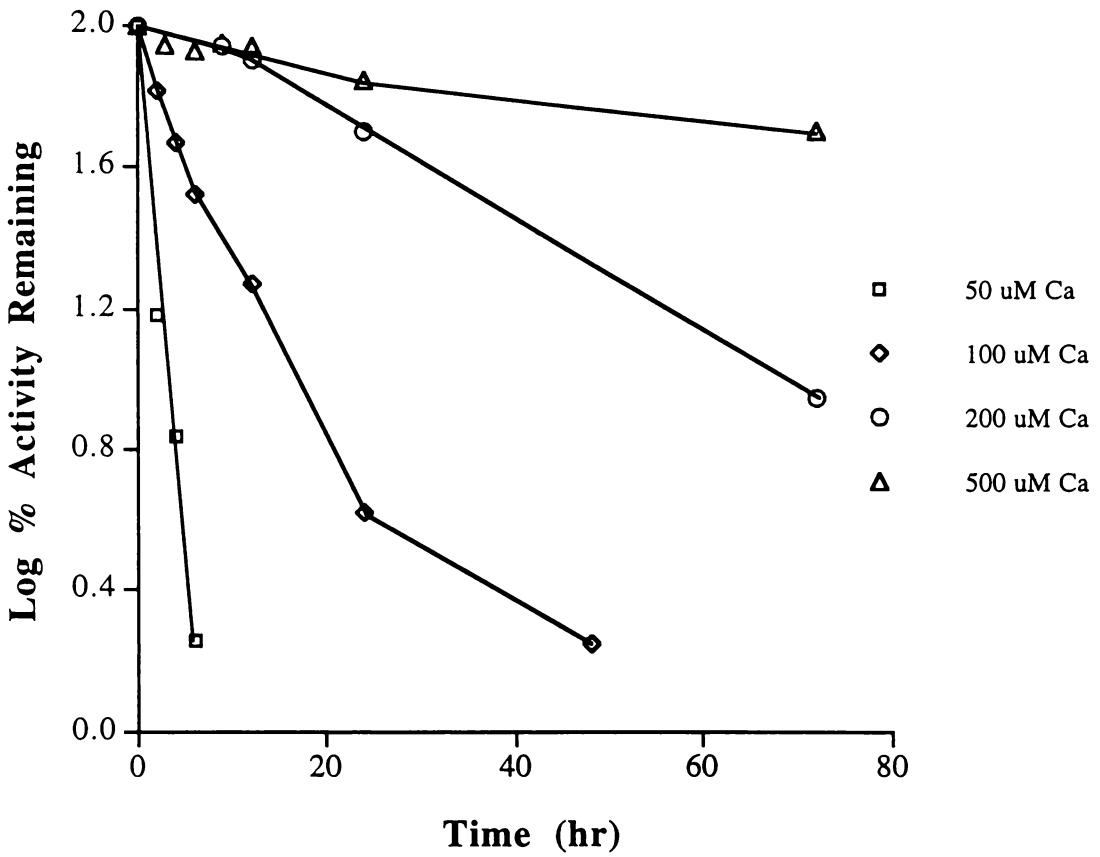


Figure A3.3.5 First Order Plots of Ak.1 Protease at 70°C with 50-500 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 50-500 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

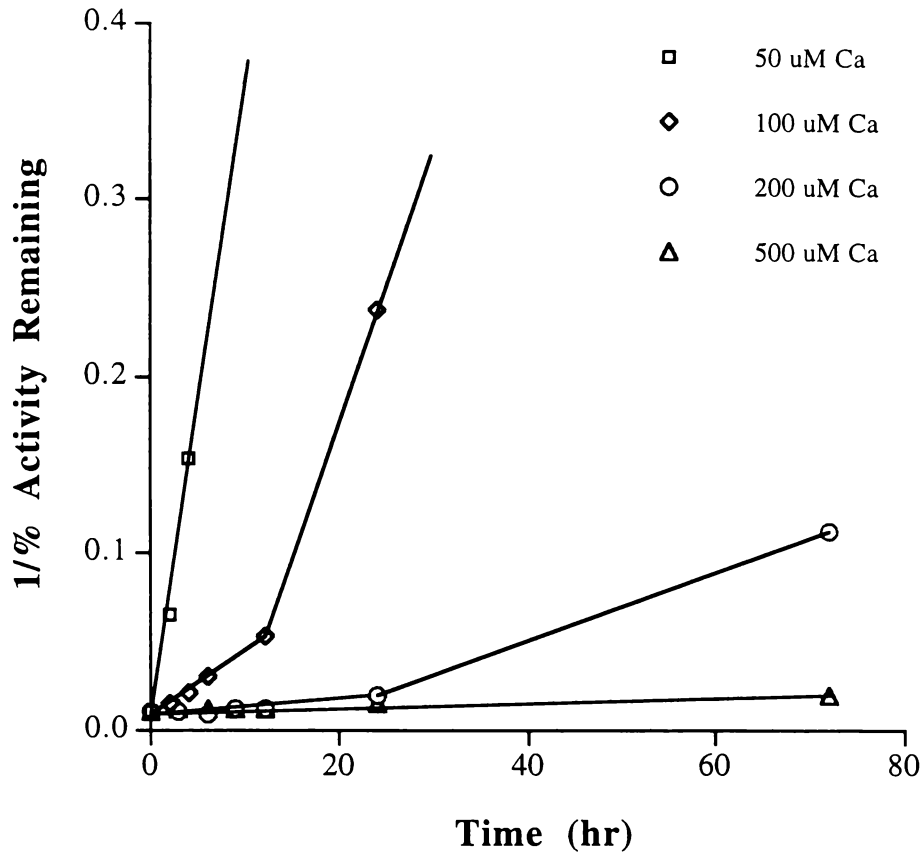


Figure A3.3.6 Second Order Plots of Ak.1 Protease at 70°C with 50-500 μM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 50-500 μM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

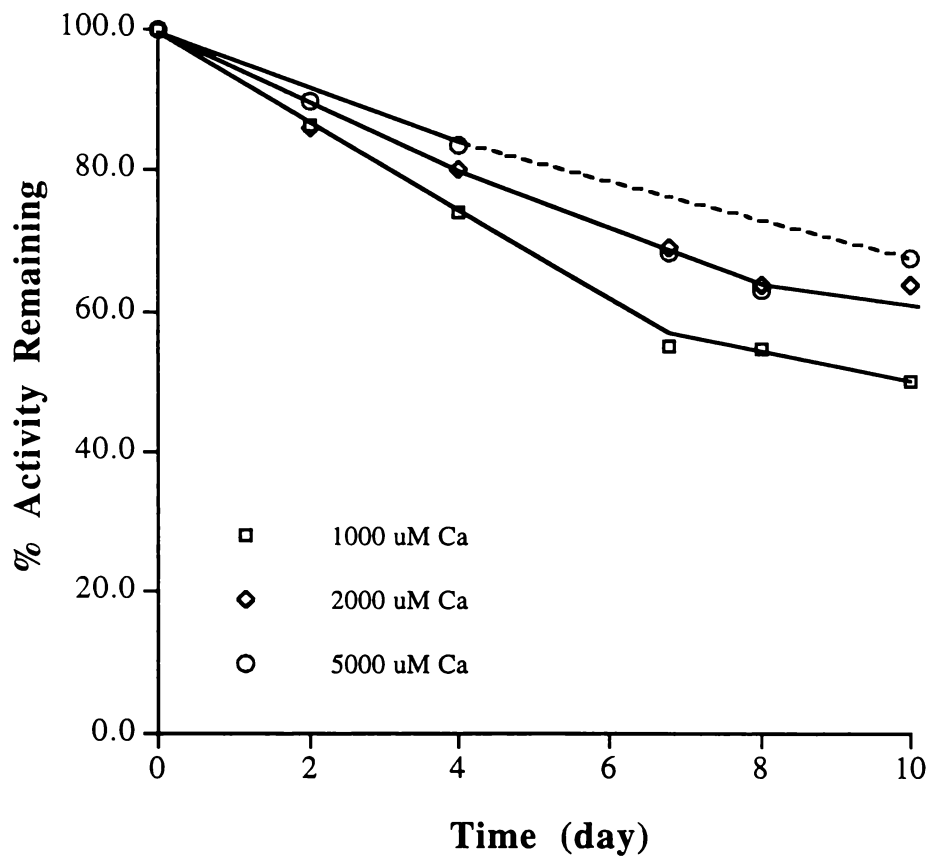


Figure A3.3.7 % Activity Remaining Vs Time of Ak.1 Protease at 70°C with 1-5 mM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 1-5 mM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

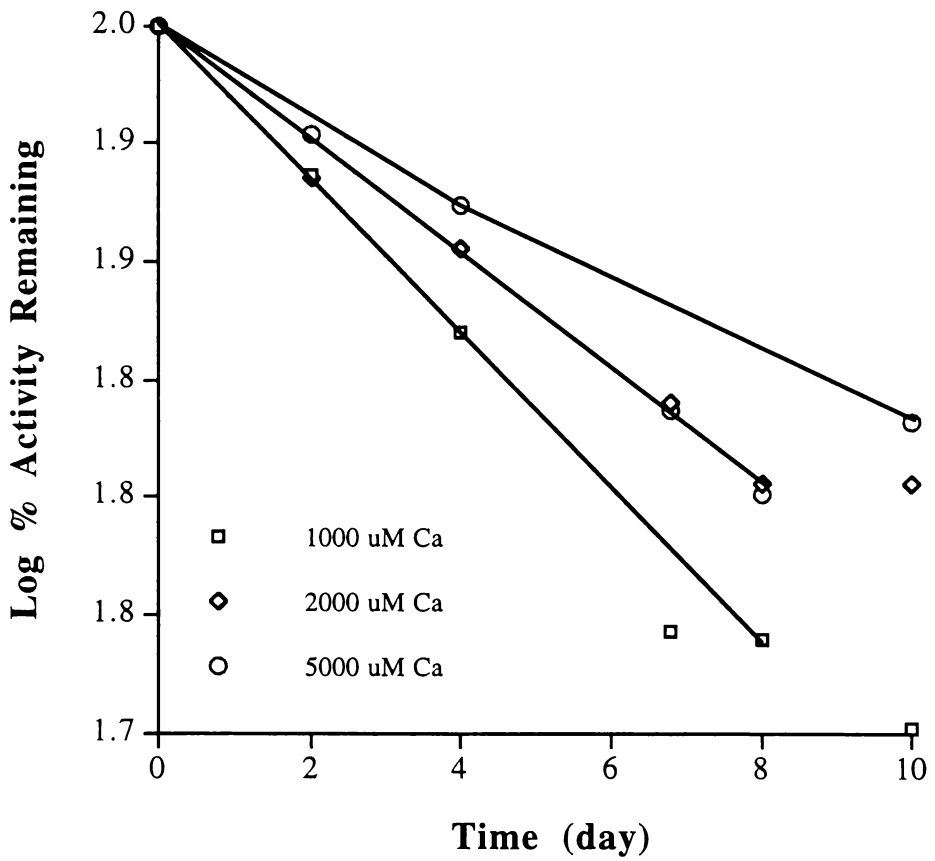


Figure A3.3.8 First Order Plots of Ak.1 Protease at 70°C with 1-5 mM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 1-5 mM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

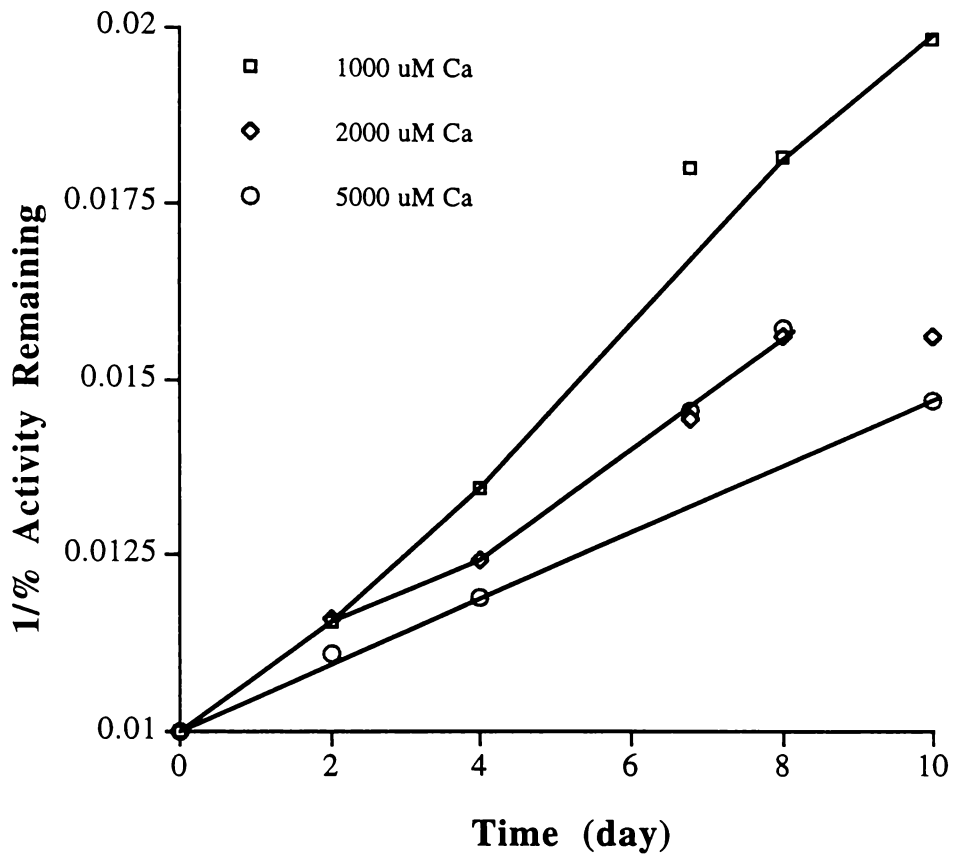


Figure A3.3.9 Second Order Plots of Ak.1 Protease at 70°C with 1-5 mM $CaCl_2$.

Ak.1 protease, pre-equilibrated in 1-5 mM $CaCl_2$, was incubated at 70°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

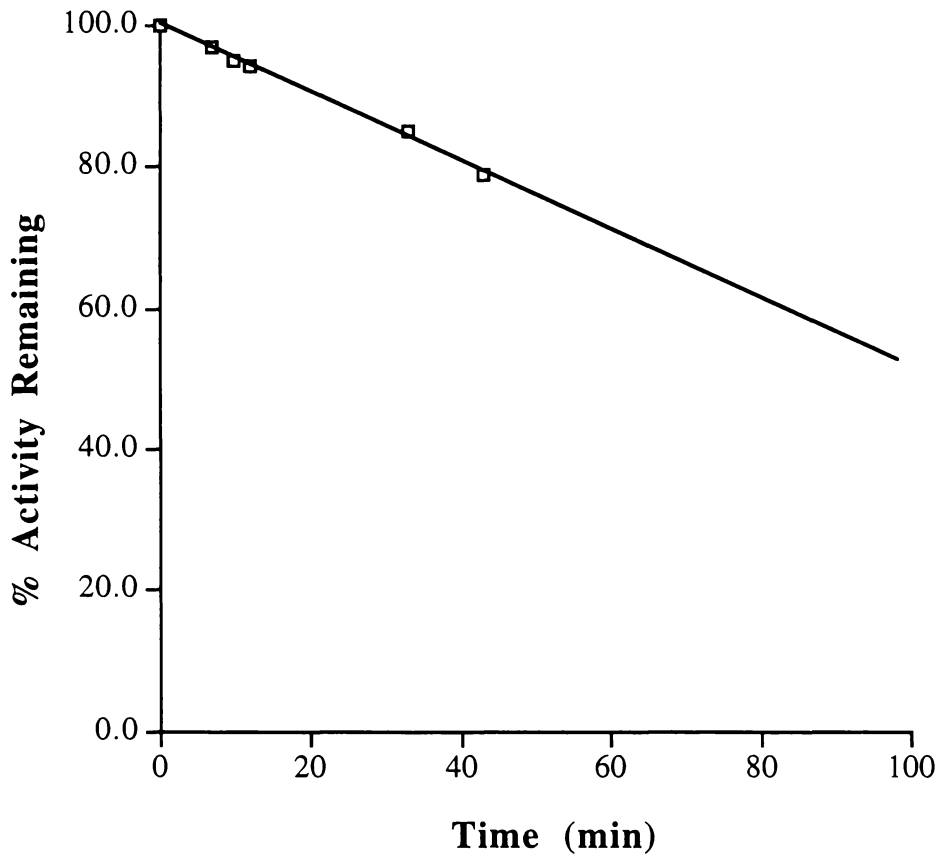


Figure A3.3.10 % Activity Remaining Vs Time of Ak.1 Protease at 105°C Containing 5 mM $CaCl_2$ and 90% Sorbitol.

Ak.1 protease, pre-equilibrated in 50 mM Tricine pH_{90} 7.5 containing 5 mM $CaCl_2$ and 90% sorbitol, was incubated at 105°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

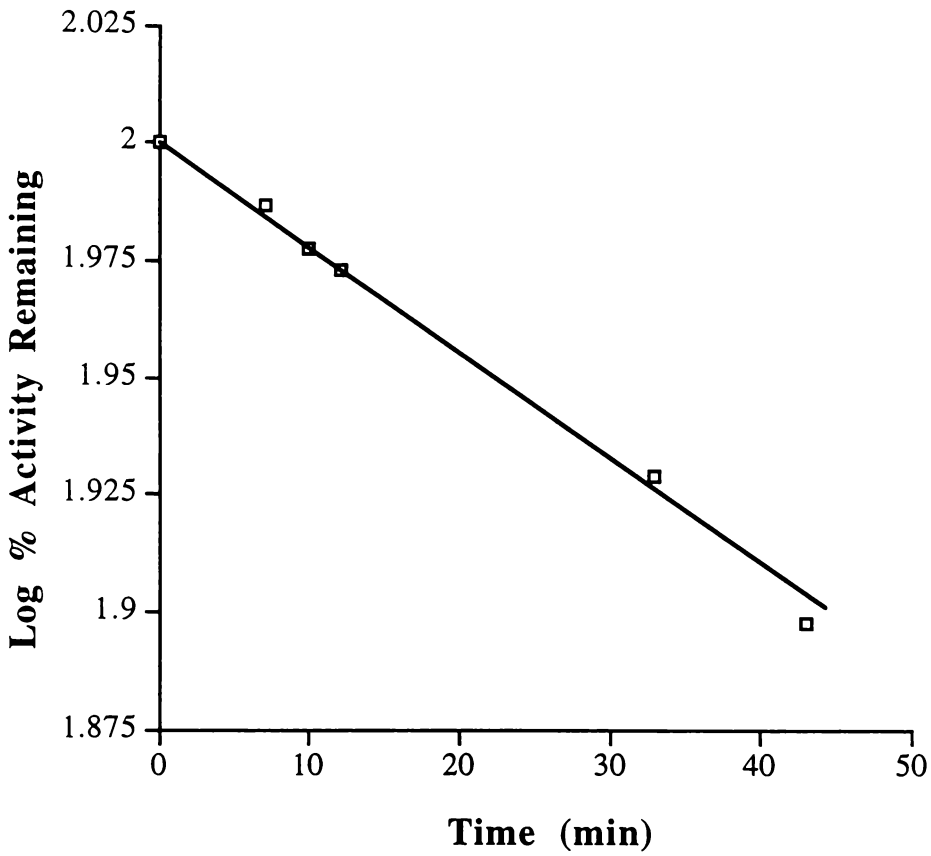


Figure A3.3.11 First Order Plot of Ak.1 Protease at 105°C Containing 5 mM $CaCl_2$ and 90% Sorbitol.

Ak.1 protease, pre-equilibrated in 50 mM Tricine pH_{90} 7.5 containing 5 mM $CaCl_2$ and 90% sorbitol, was incubated at 105°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

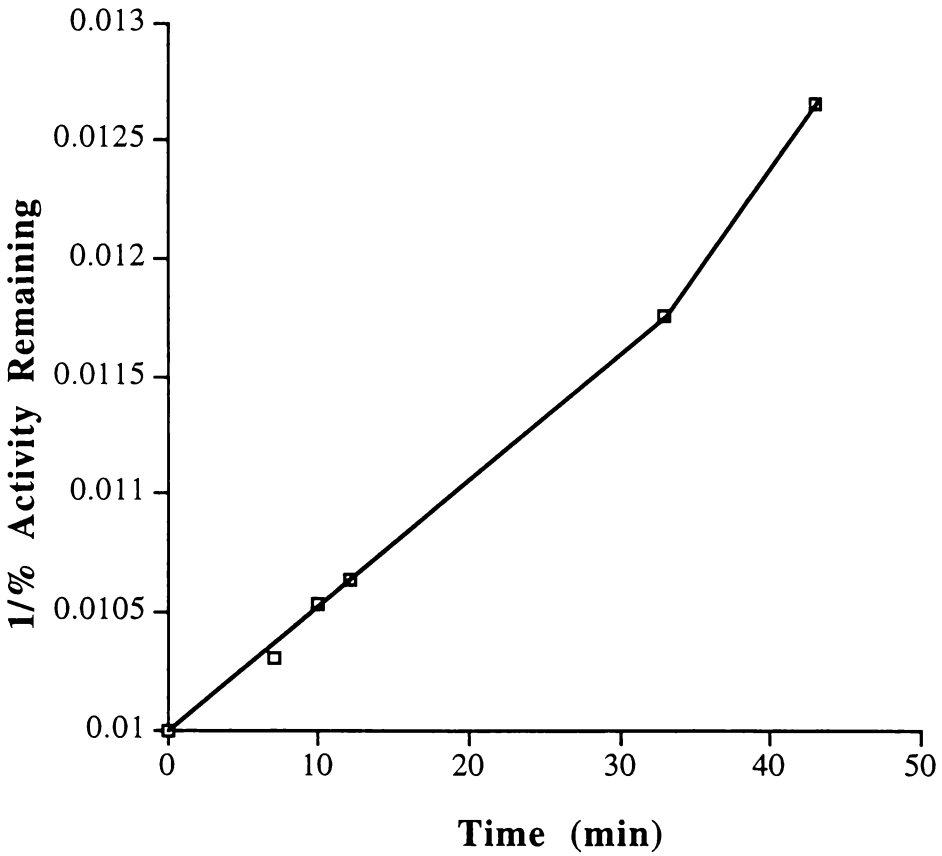


Figure A3.3.9 Second Order Plot of Ak.1 Protease at 105°C Containing 5 mM $CaCl_2$ and 90% Sorbitol.

Ak.1 protease, pre-equilibrated in 50 mM Tricine pH_{90} 7.5 containing 5 mM $CaCl_2$ and 90% sorbitol, was incubated at 105°C for the appropriate time periods, then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ at 75°C.

APPENDIX 4

MICHAELIS MENTEN AND LINEWEAVER-BURK PLOTS

A4.1 SUC-ALA-ALA-PRO-PHE-PNA SUBSTRATE

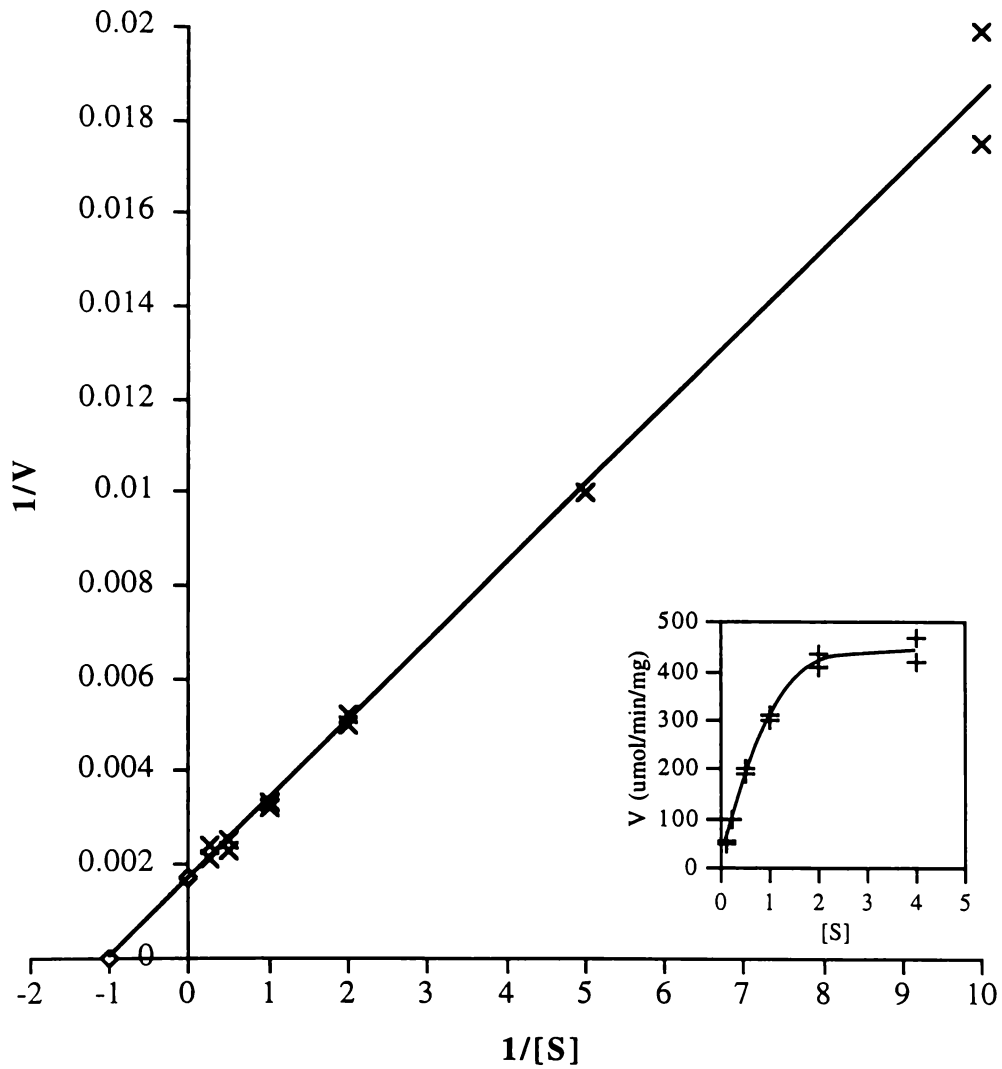


Figure A4.1.1 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 75°C (inset is the Michaelis Menten Plot.

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH75 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 75°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

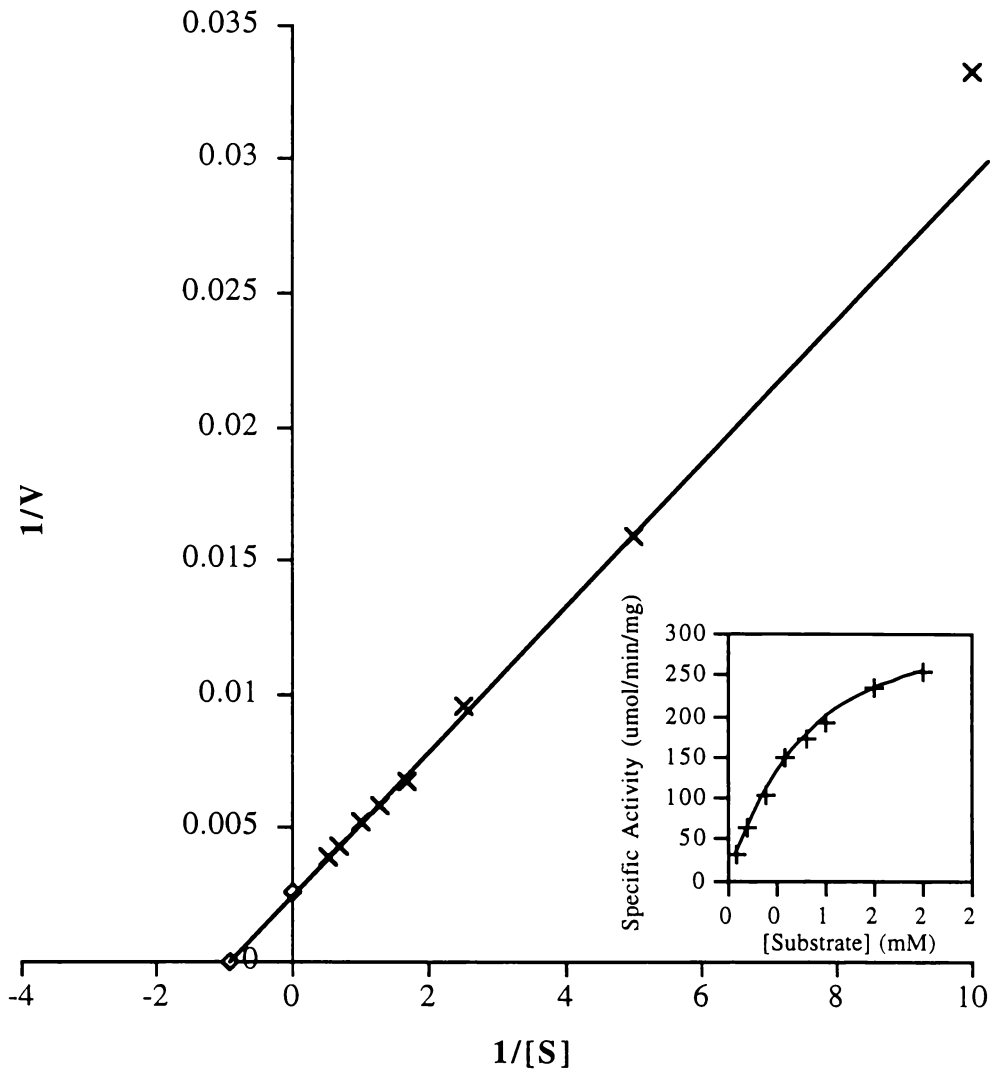


Figure A4.1.2 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 65°C (inset is the Michaelis Menten Plot.

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{65} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 65°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

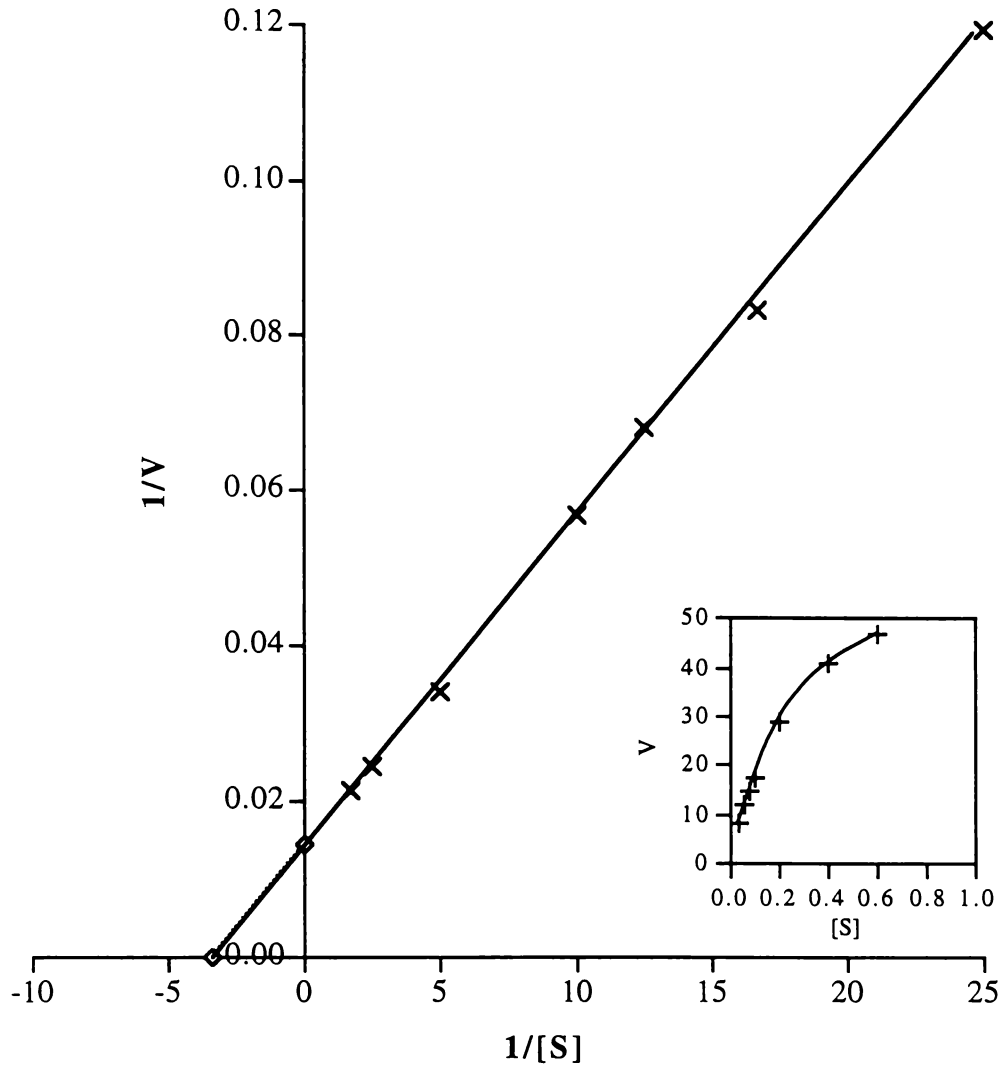


Figure A4.1.3 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 40°C (inset is the Michaelis Menten Plot.

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH40 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 40°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

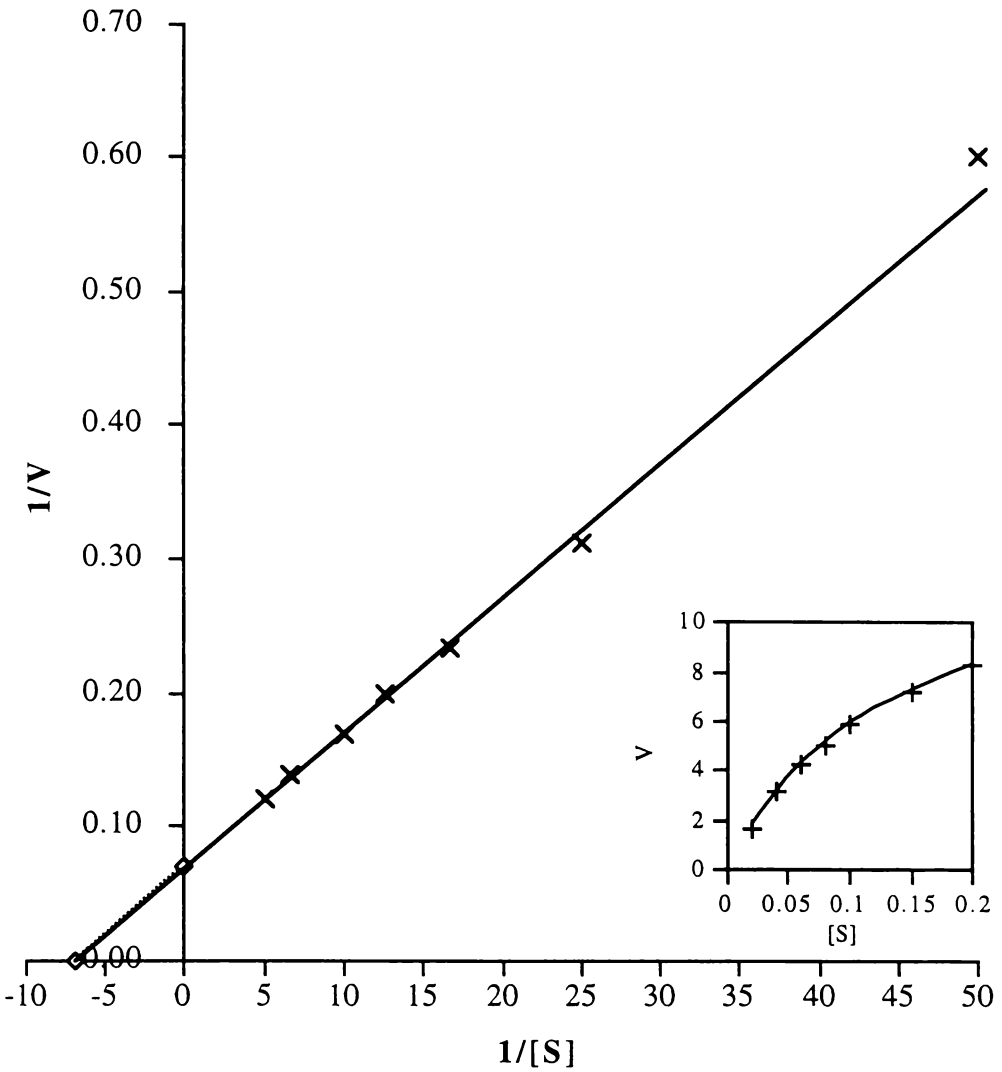


Figure A4.1.4 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 20°C (inset is the Michaelis Menten Plot.

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 20°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

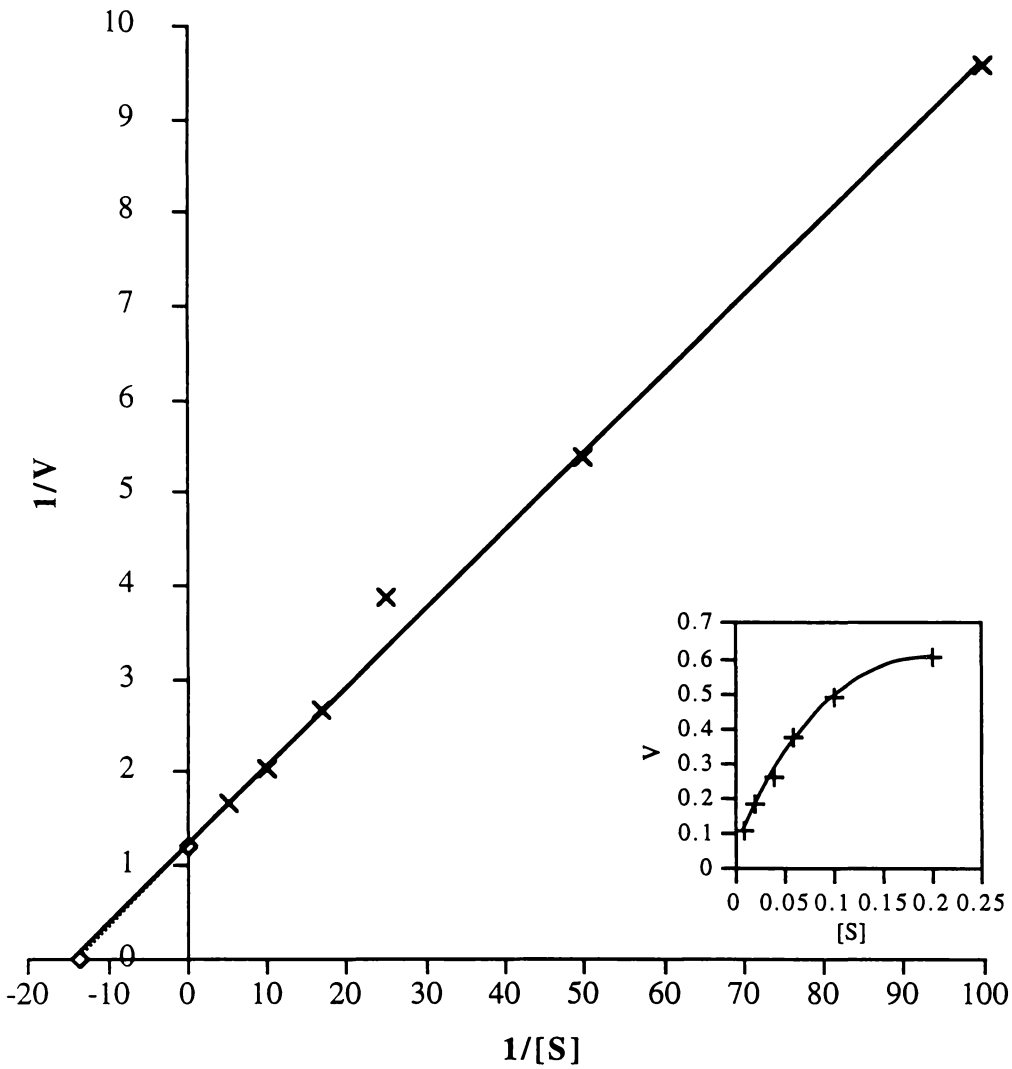


Figure A4.1.5 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 5°C (inset is the Michaelis Menten Plot.

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_5 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 5°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

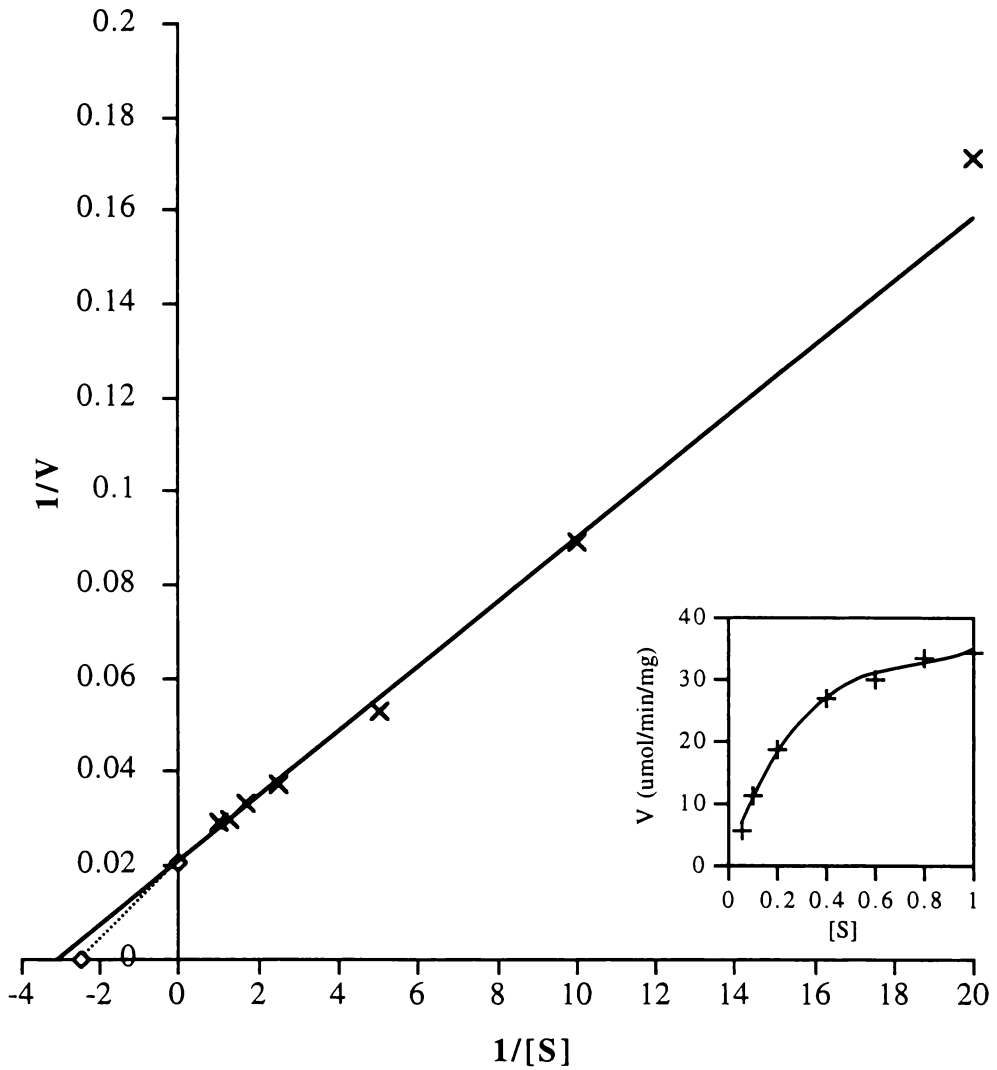


Figure A4.1.6 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 40°C Containing 100 mM NaCl (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 + 100 mM NaCl at 75°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

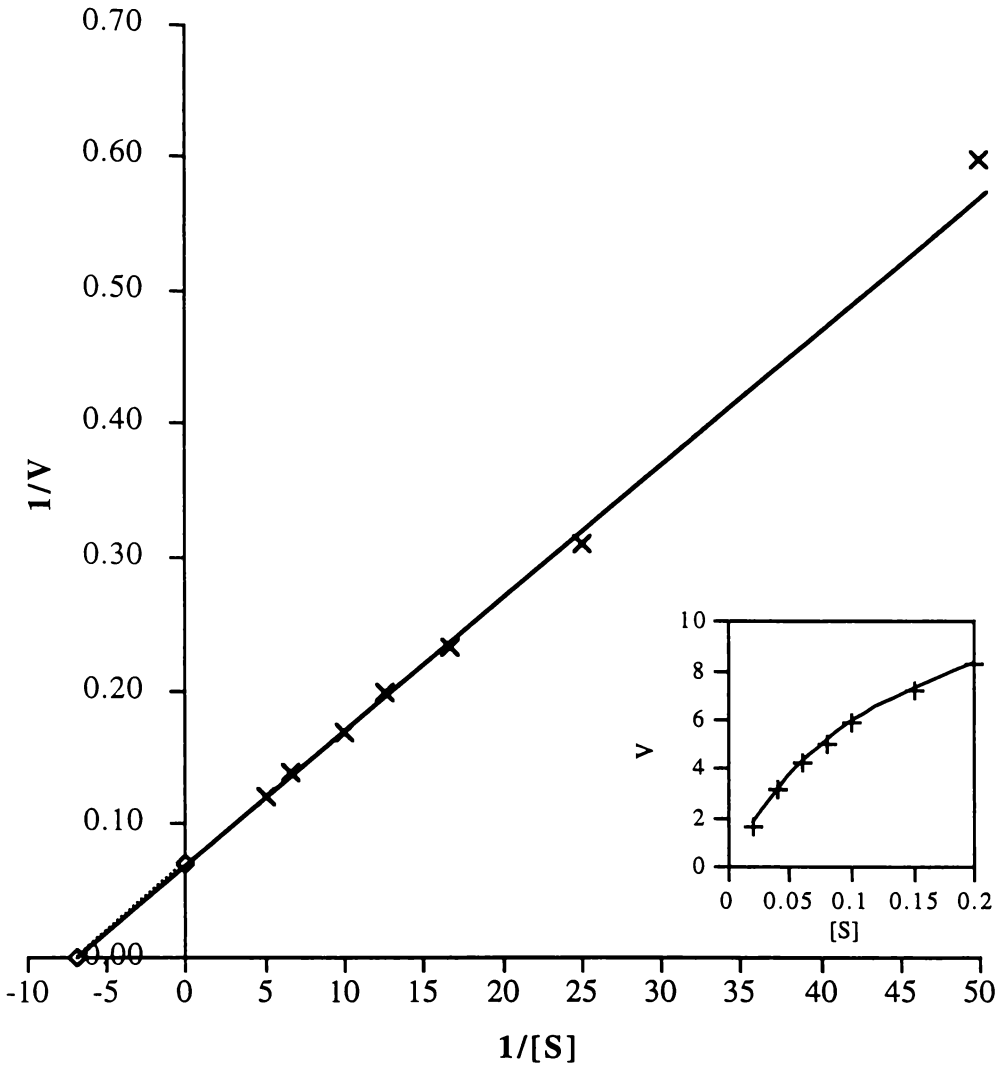


Figure A4.1.7 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 40°C Containing 50% Sorbitol (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 containing 50% sorbitol at 40°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

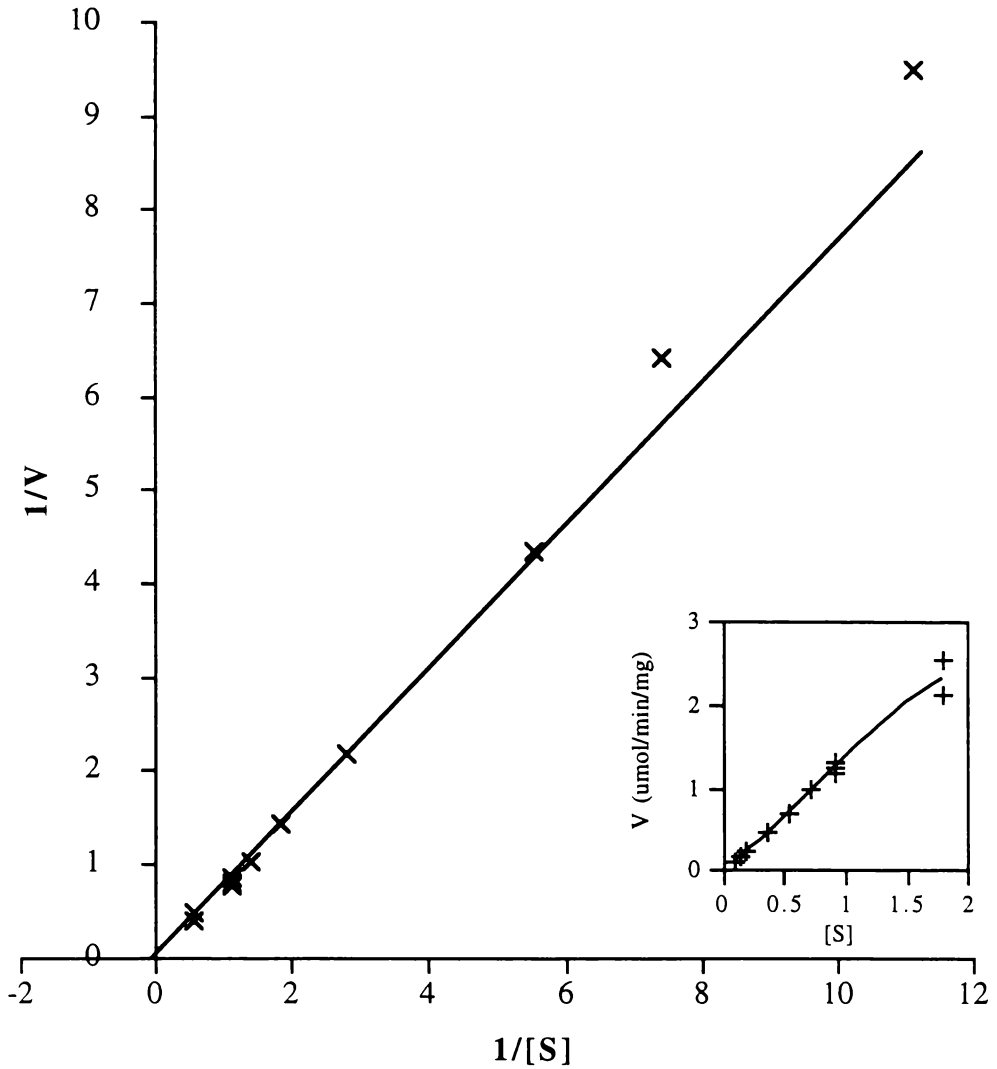


Figure A4.1.8 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA at 40°C Containing 50% Methanol (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 50% methanol at 40°C. **The data points on the X and Y axis were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

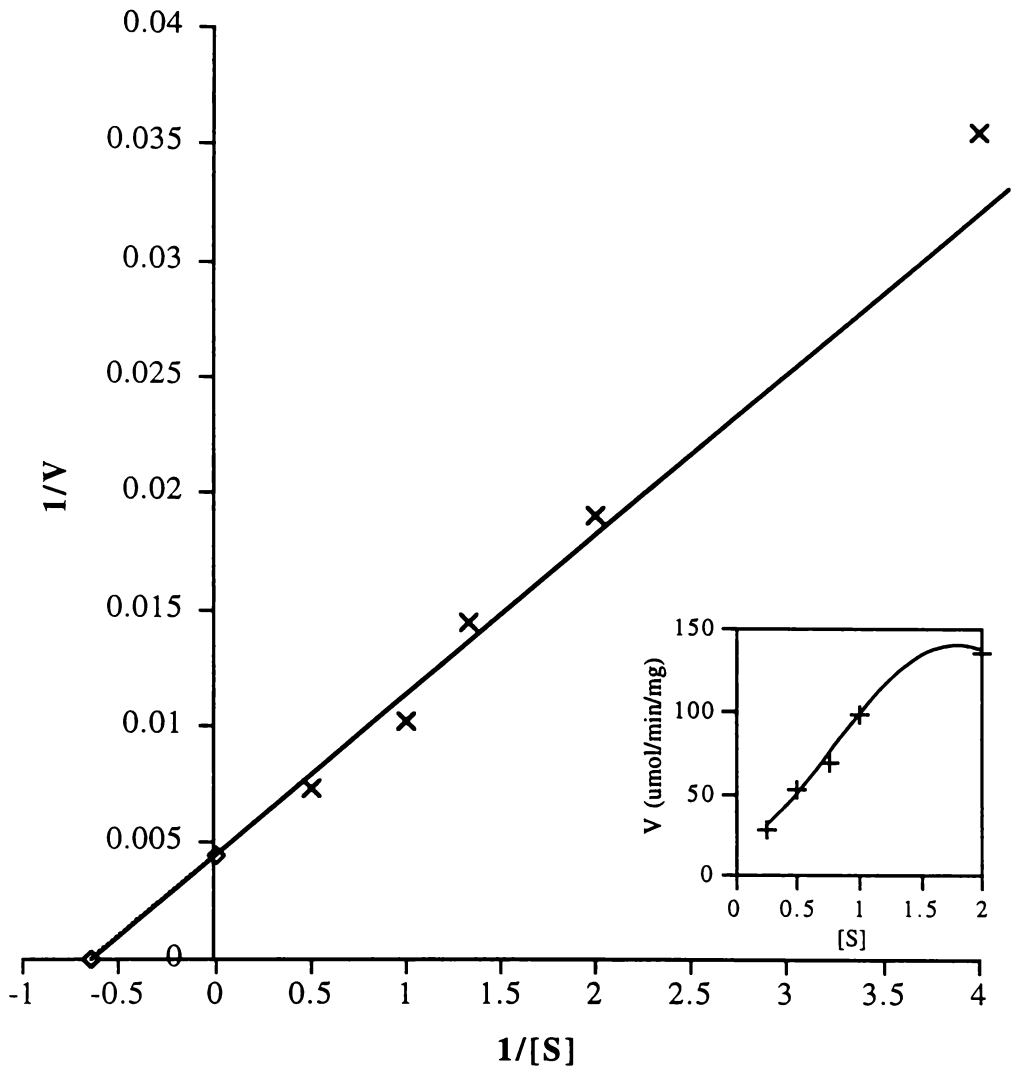


Figure A4.1.9 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 10 mM DTT at 75°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 75°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

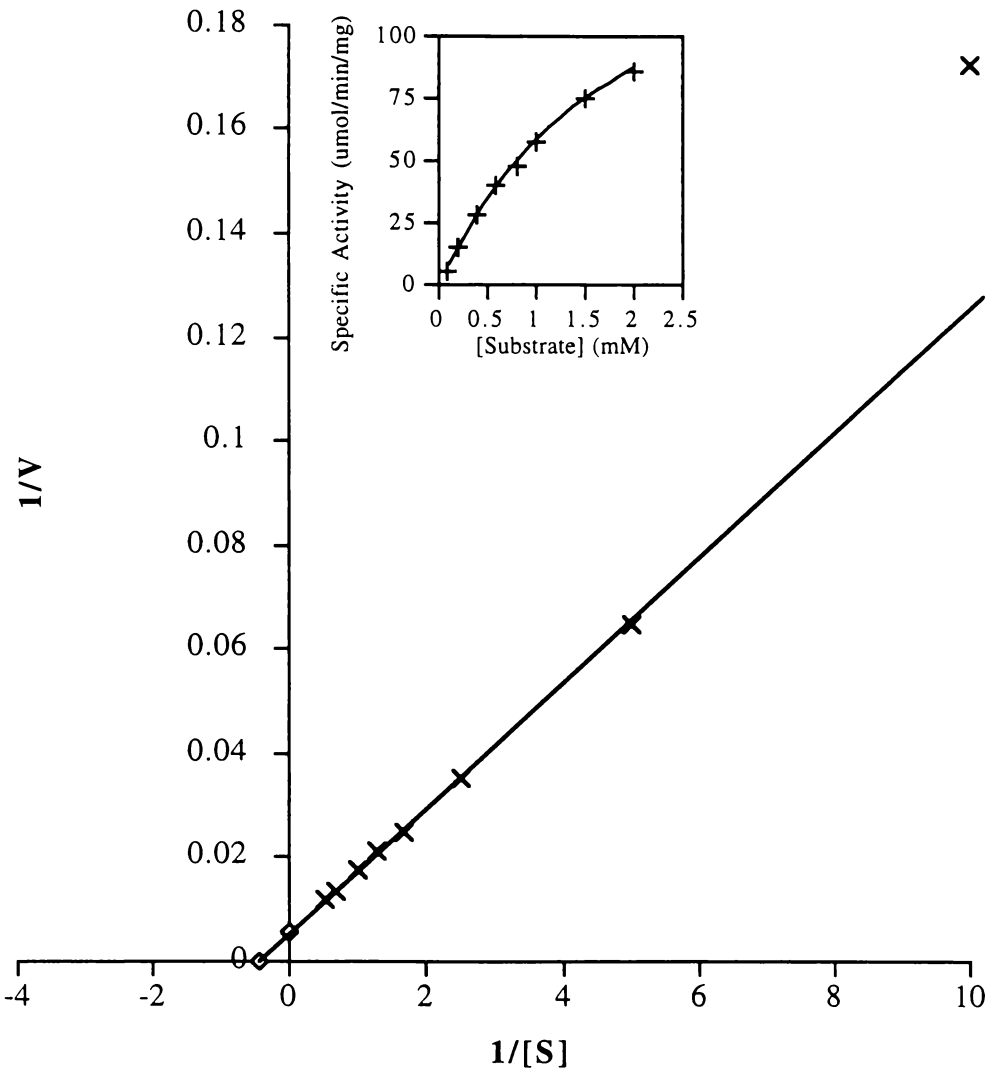


Figure A4.1.10 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 10 mM DTT at 65°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{65} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 65°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

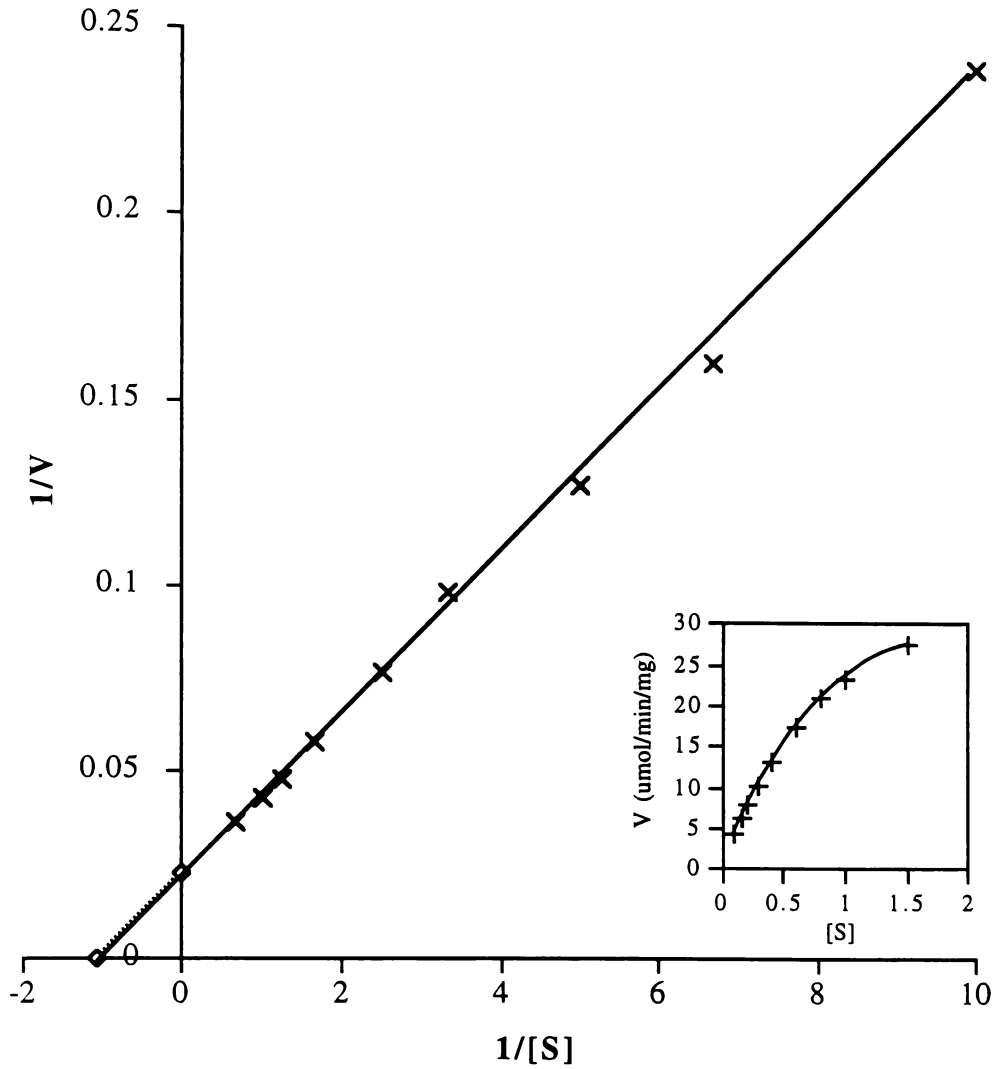


Figure A4.1.11 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 10 mM DTT at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

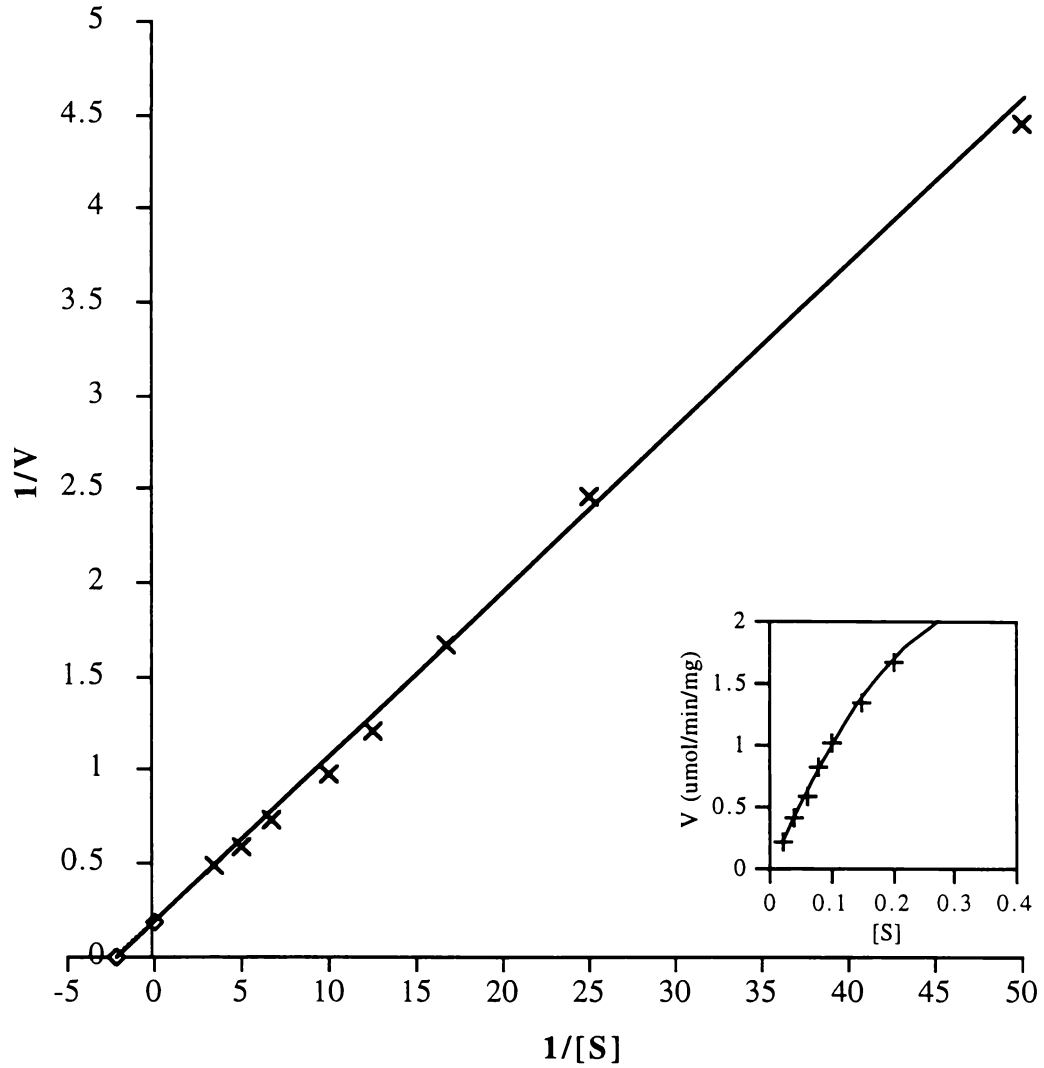


Figure A4.1.12 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 10 mM DTT at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

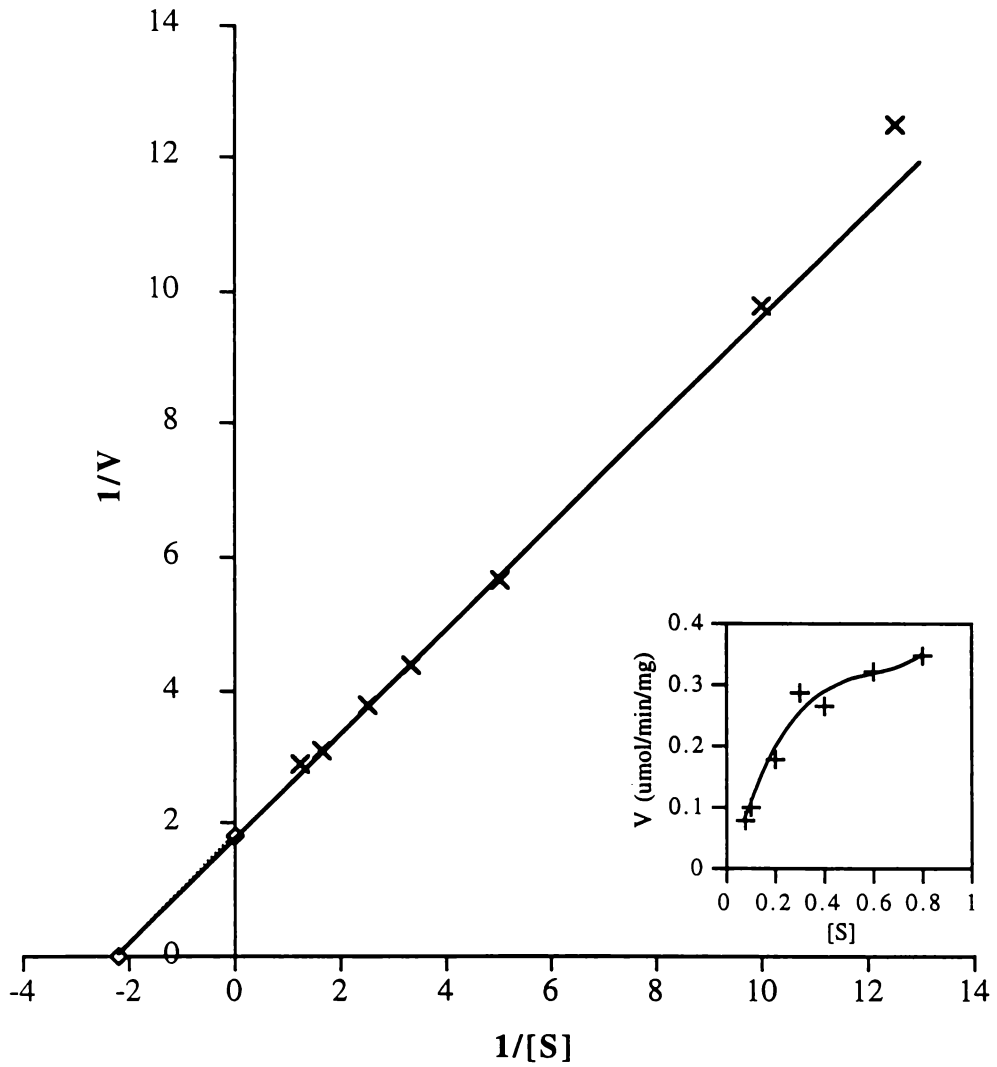


Figure A4.1.13 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 10 mM DTT at 5°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_5 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 5°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

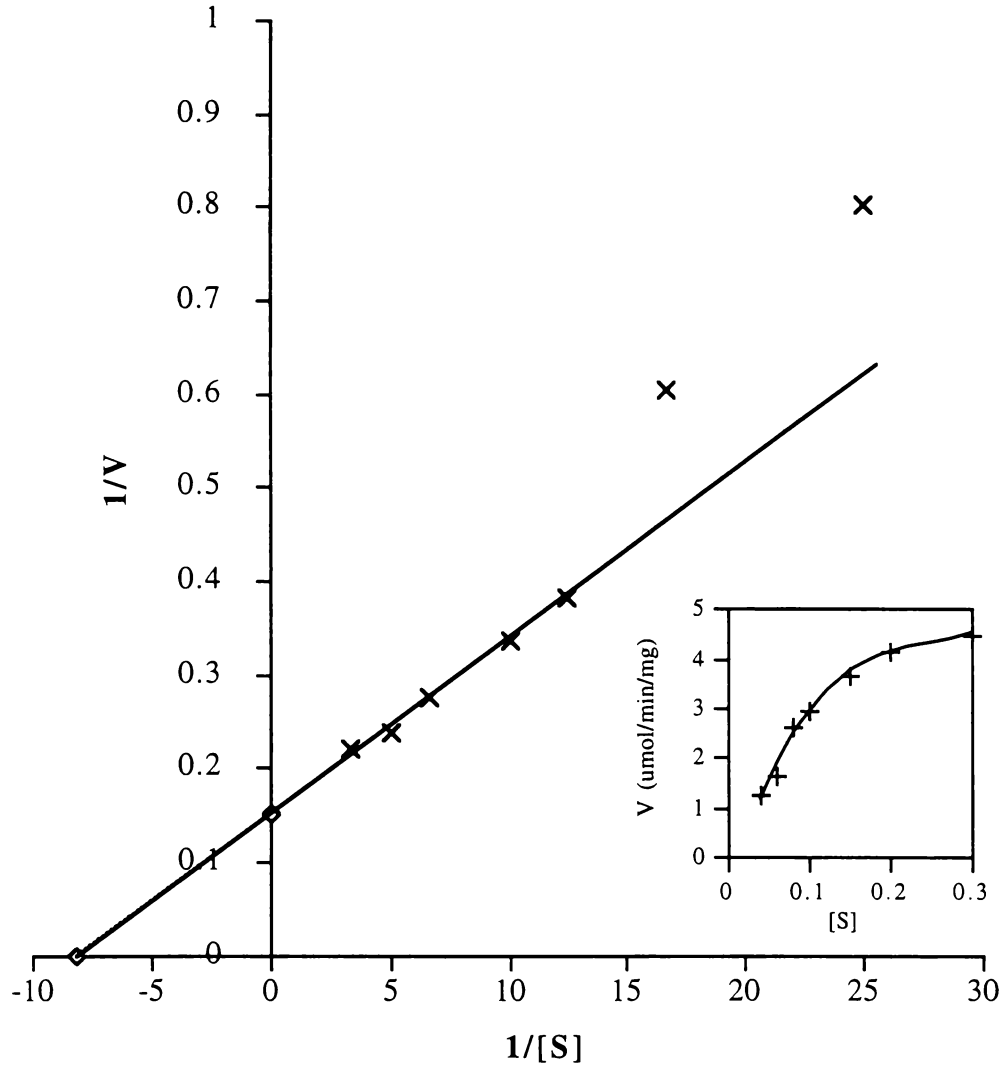


Figure A4.1.14 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 100 μM Hg^{2+} at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 100 μM Hg^{2+} at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

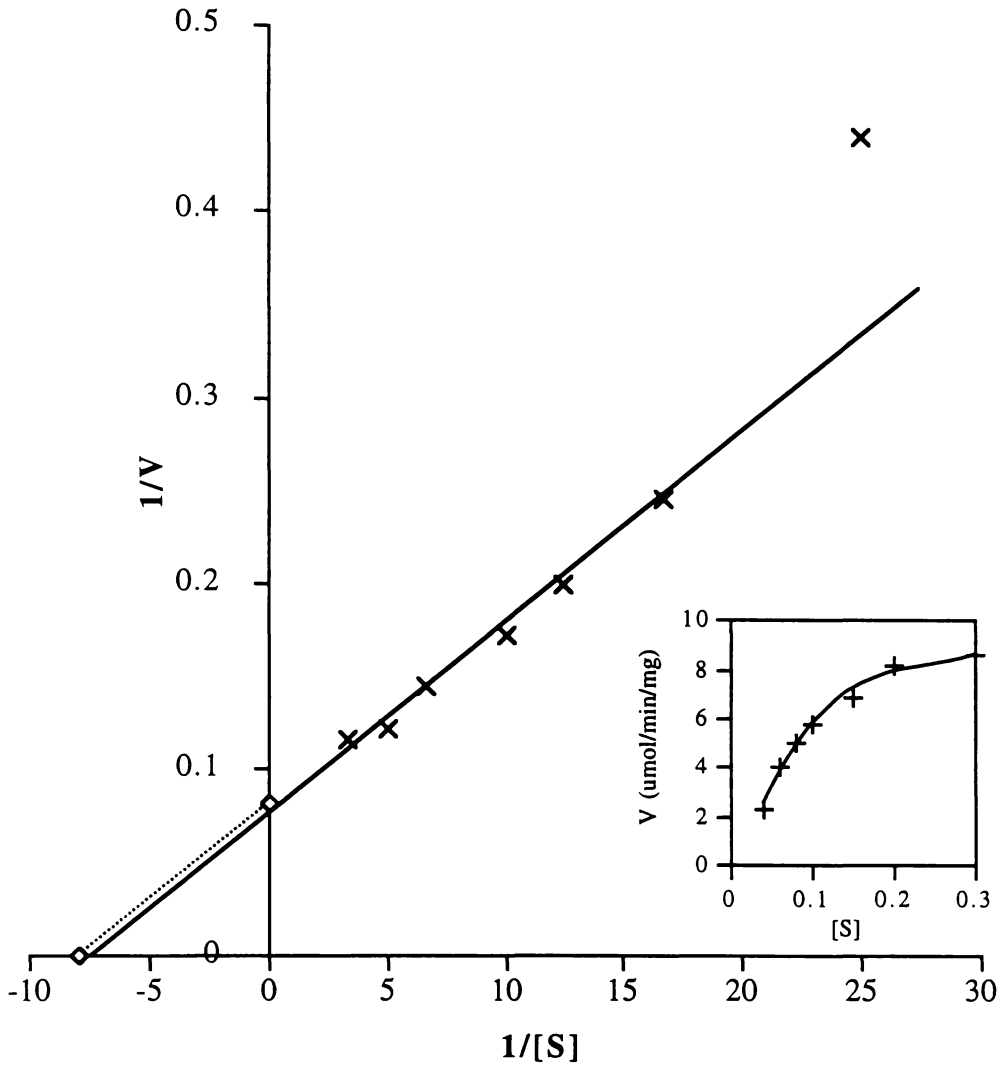


Figure A4.1.15 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 100 μM Pb^{2+} at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 100 μM Pb^{2+} at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

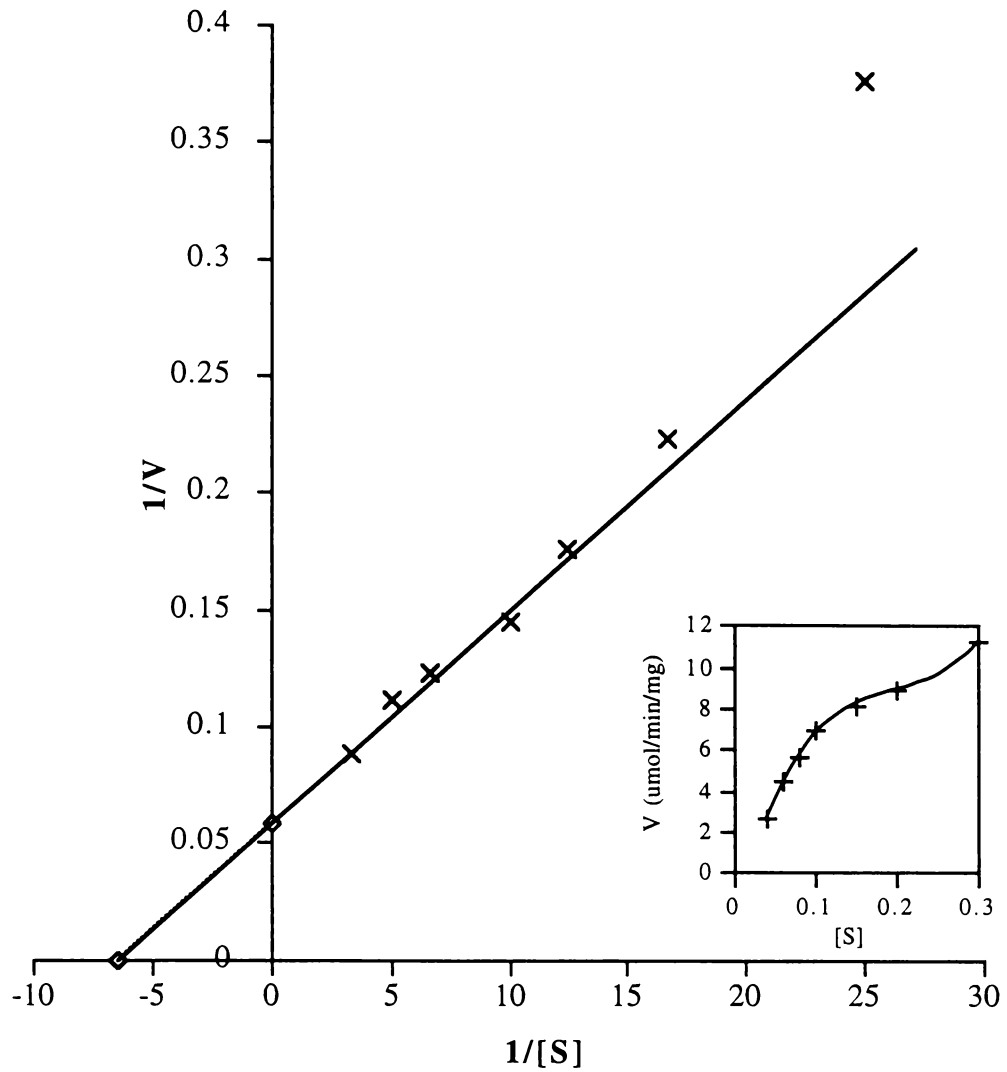


Figure A4.1.16 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 100 μM Cd^{2+} at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 100 μM Cd^{2+} at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

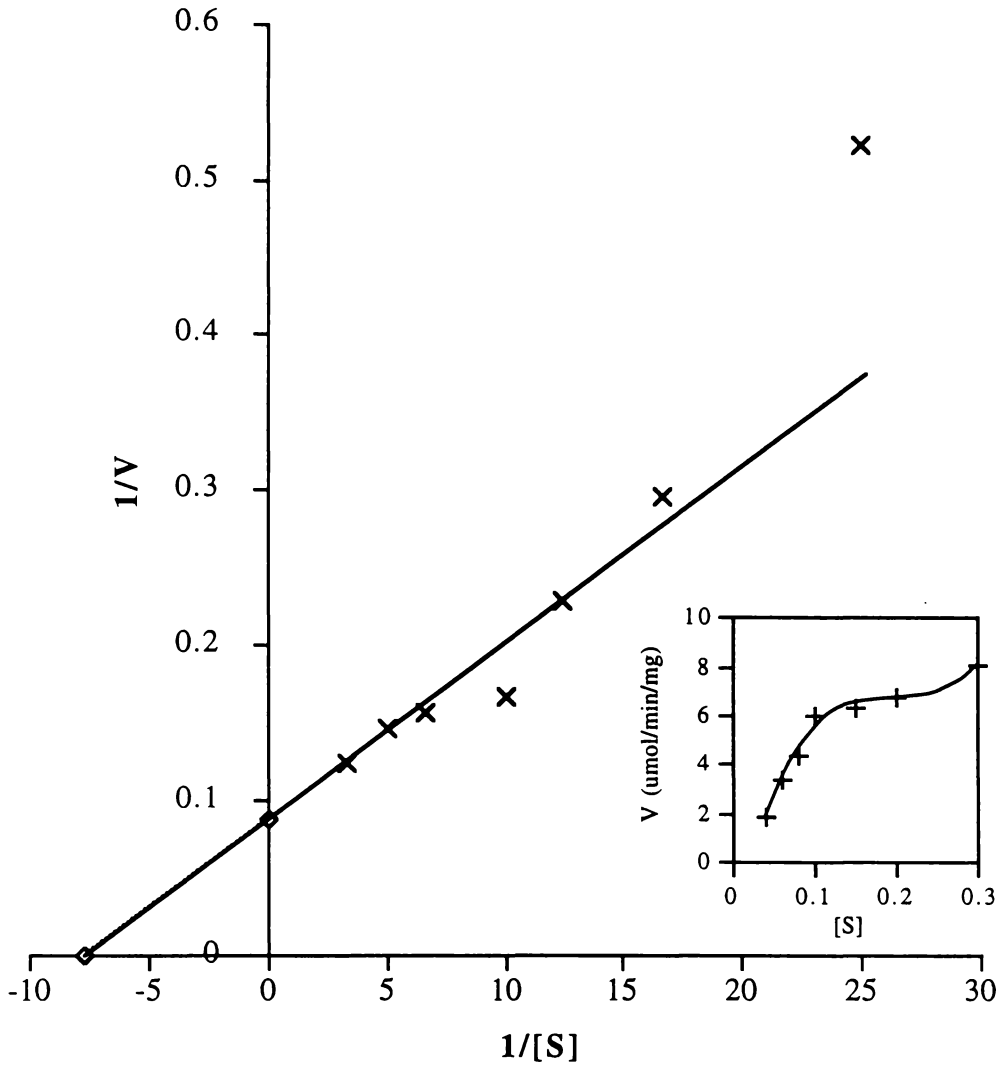


Figure A4.1.17 Lineweaver Burk Plot of Ak.1 Protease with SAAPFpNA Containing 100 μM Zn^{2+} at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPFpNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 100 μM Zn^{2+} at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

A4.2 SUC-ALA-ALA-PRO-LEU-PNA SUBSTRATE

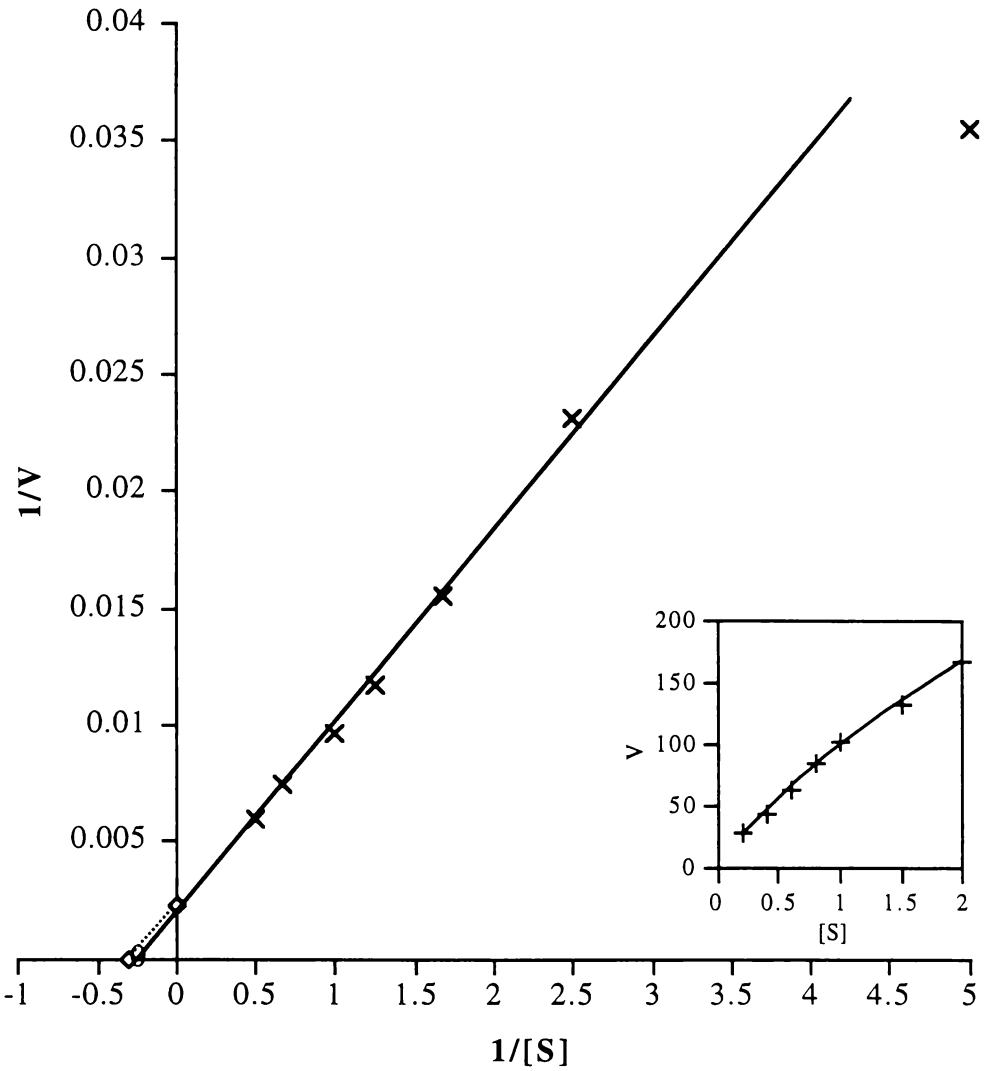


Figure A4.2.1 Lineweaver Burk Plot of Ak.1 Protease with SAAPLpNA at 75°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPLpNA in 50 mM HEPES/NaOH pH75 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 75°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

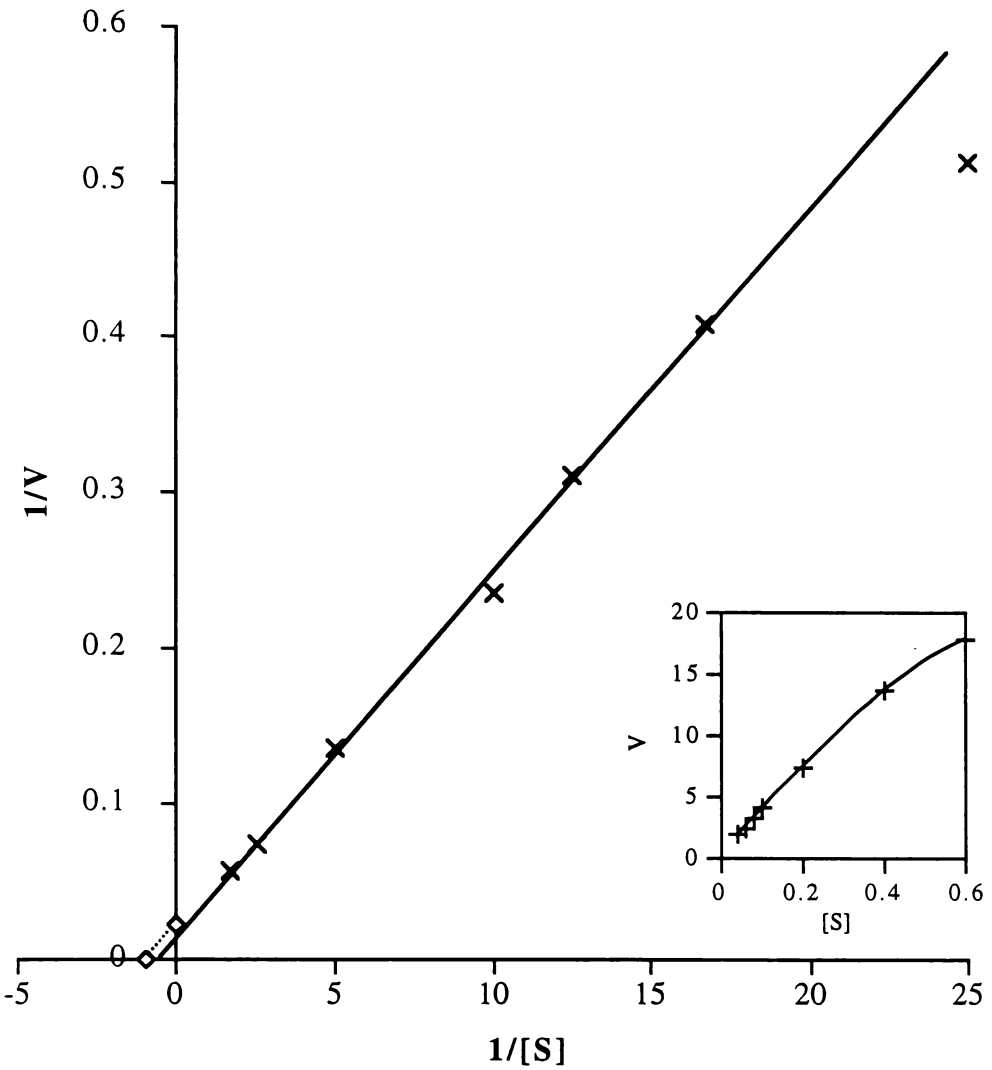


Figure A4.2.2 Lineweaver Burk Plot of Ak.1 Protease with SAAPLpNA at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPLpNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

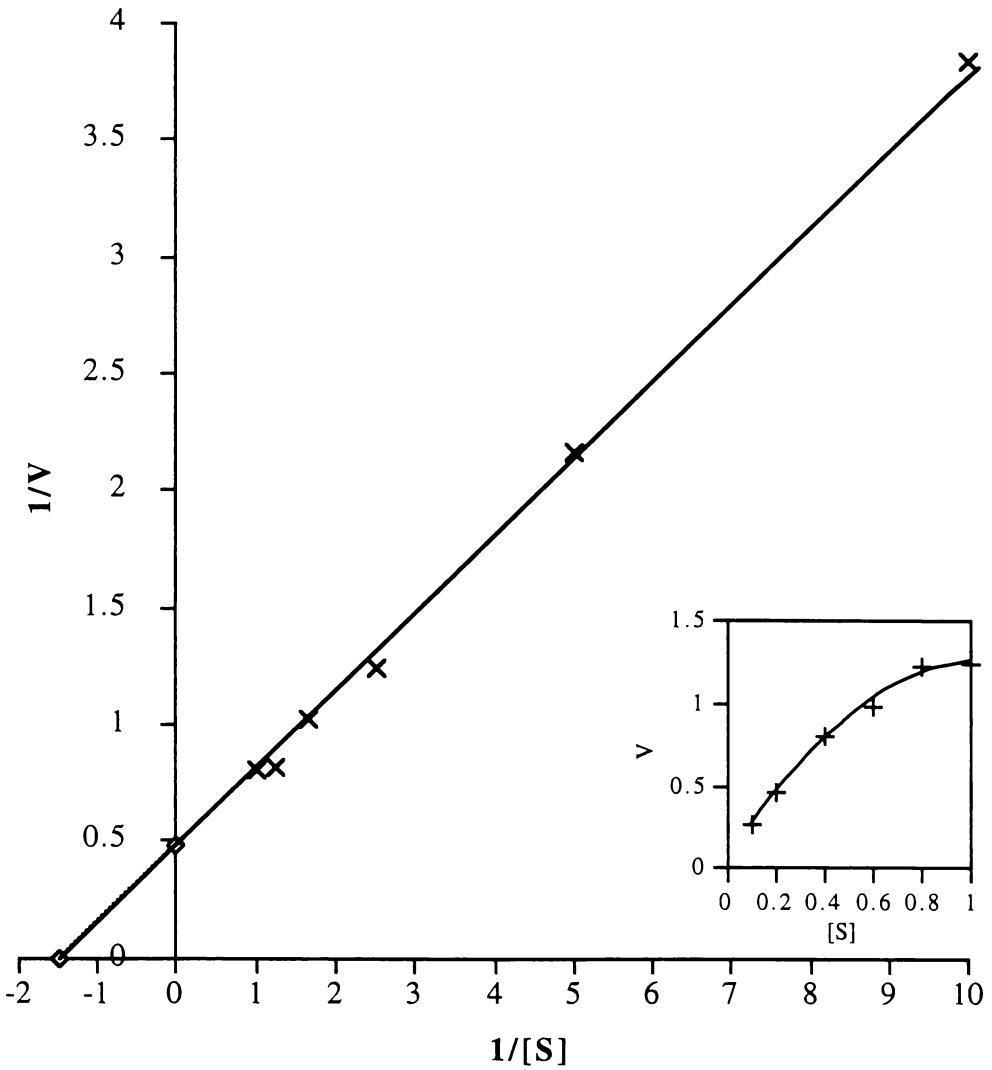


Figure A4.2.3 Lineweaver Burk Plot of Ak.1 Protease with SAAPLpNA at 5°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPLpNA in 50 mM HEPES/NaOH pH_5 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 5°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

A4.3 SUC-ALA-ALA-PRO-ALA-PNA SUBSTRATE

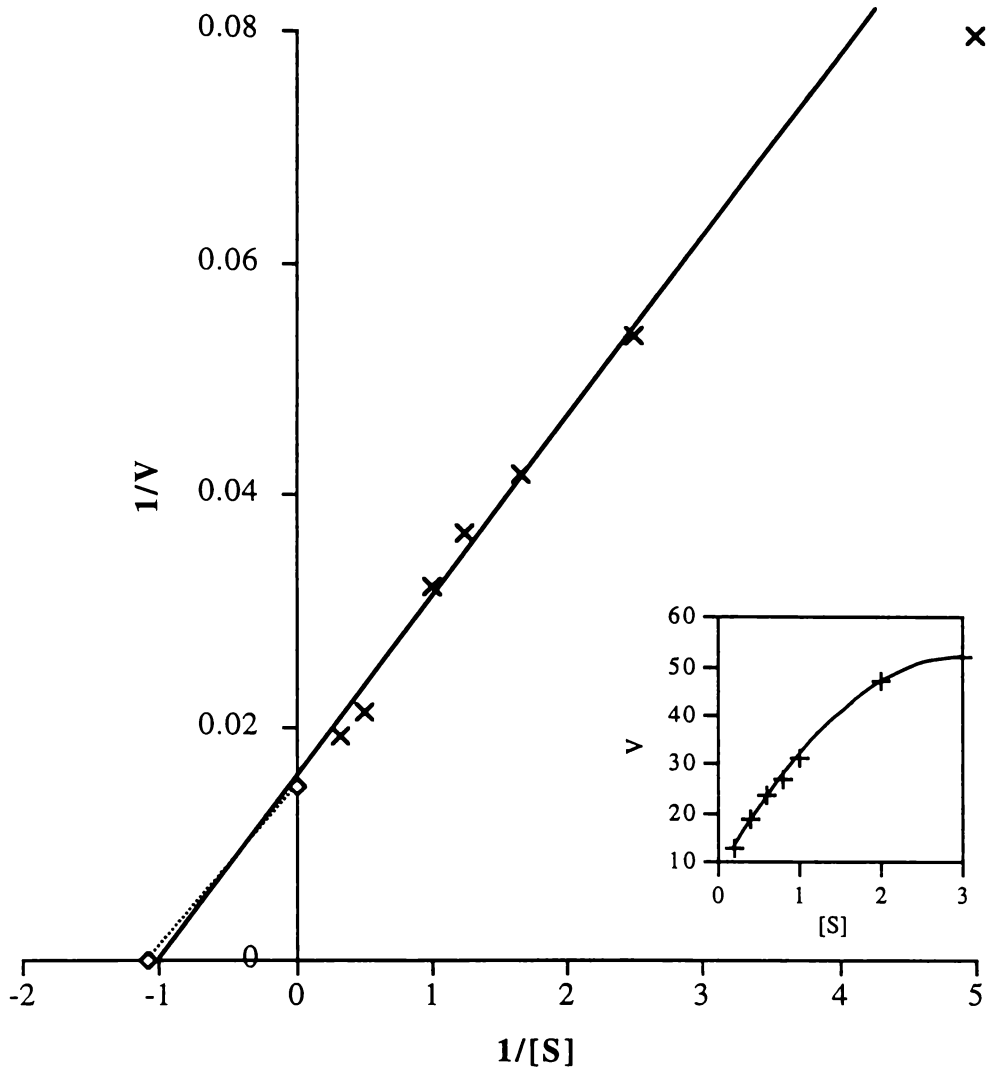


Figure A4.3.1 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA at 75°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH75 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 75°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

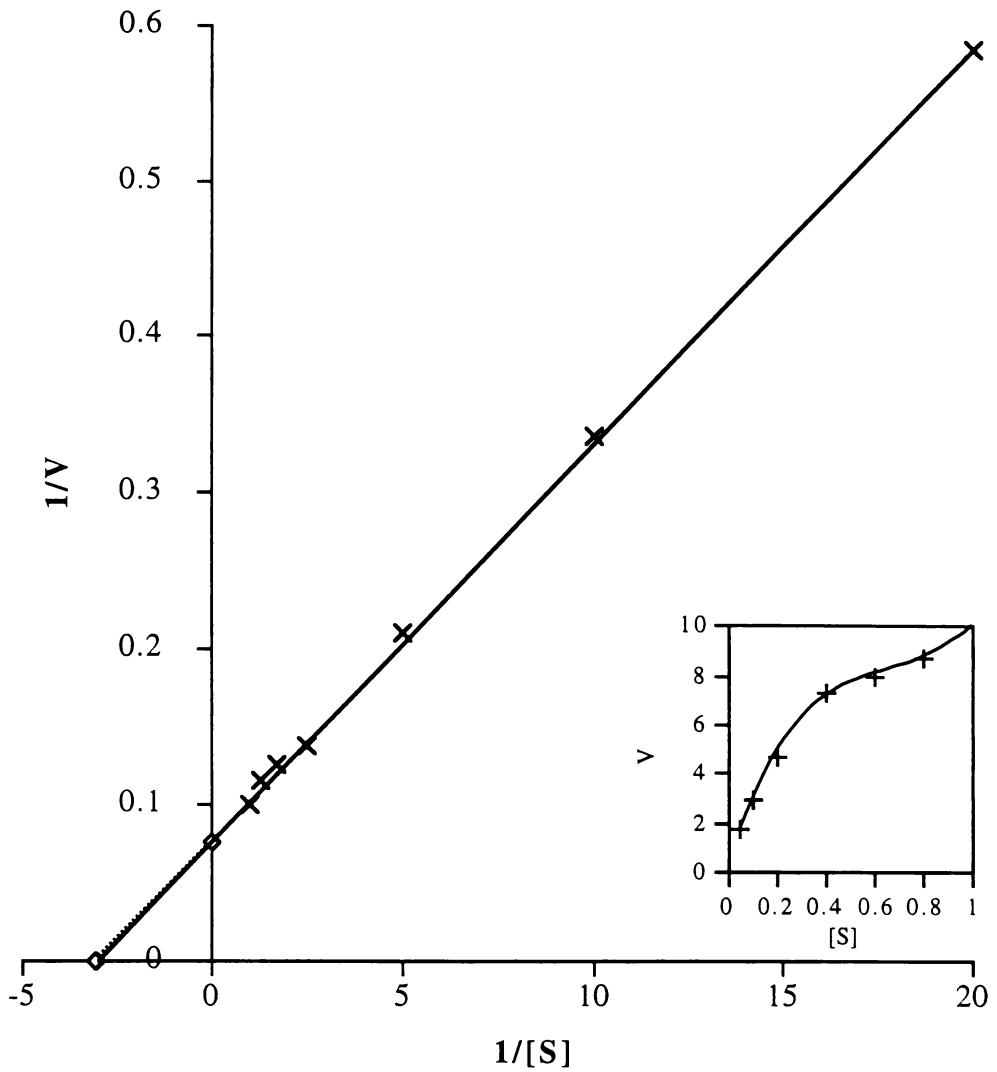


Figure A4.3.2 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH40 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

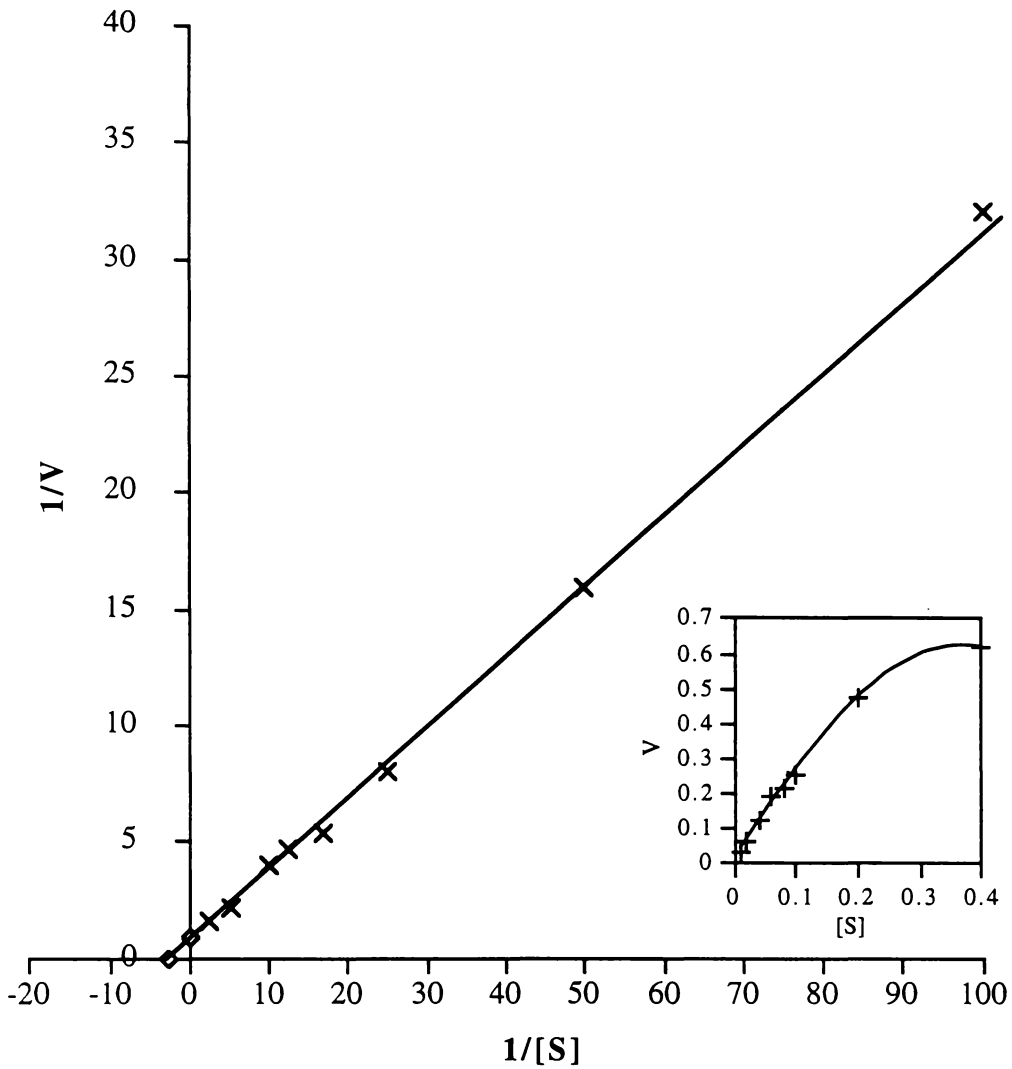


Figure A4.3.3 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA at 5°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH_5 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 5°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

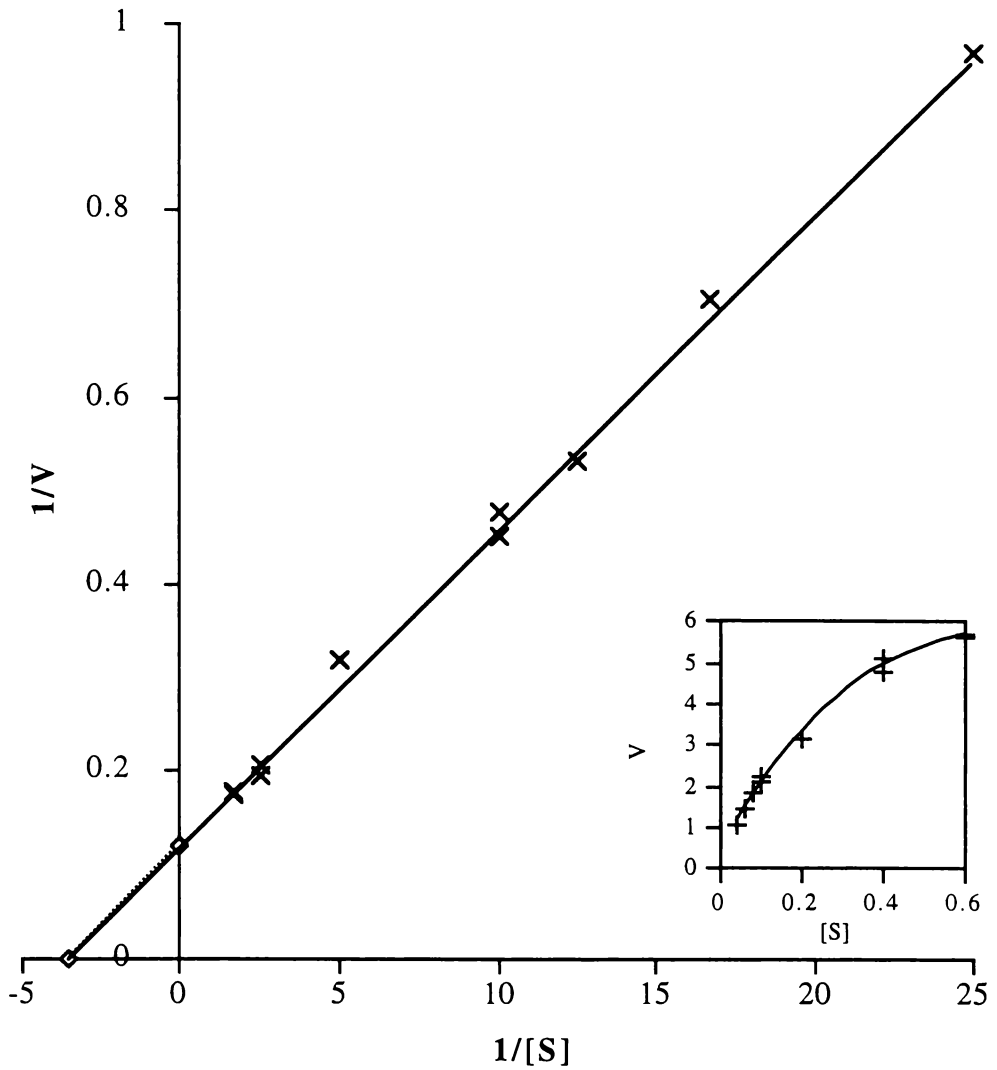


Figure A4.3.4 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA Containing 100 mM NaCl at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH40 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 100 mM NaCl at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

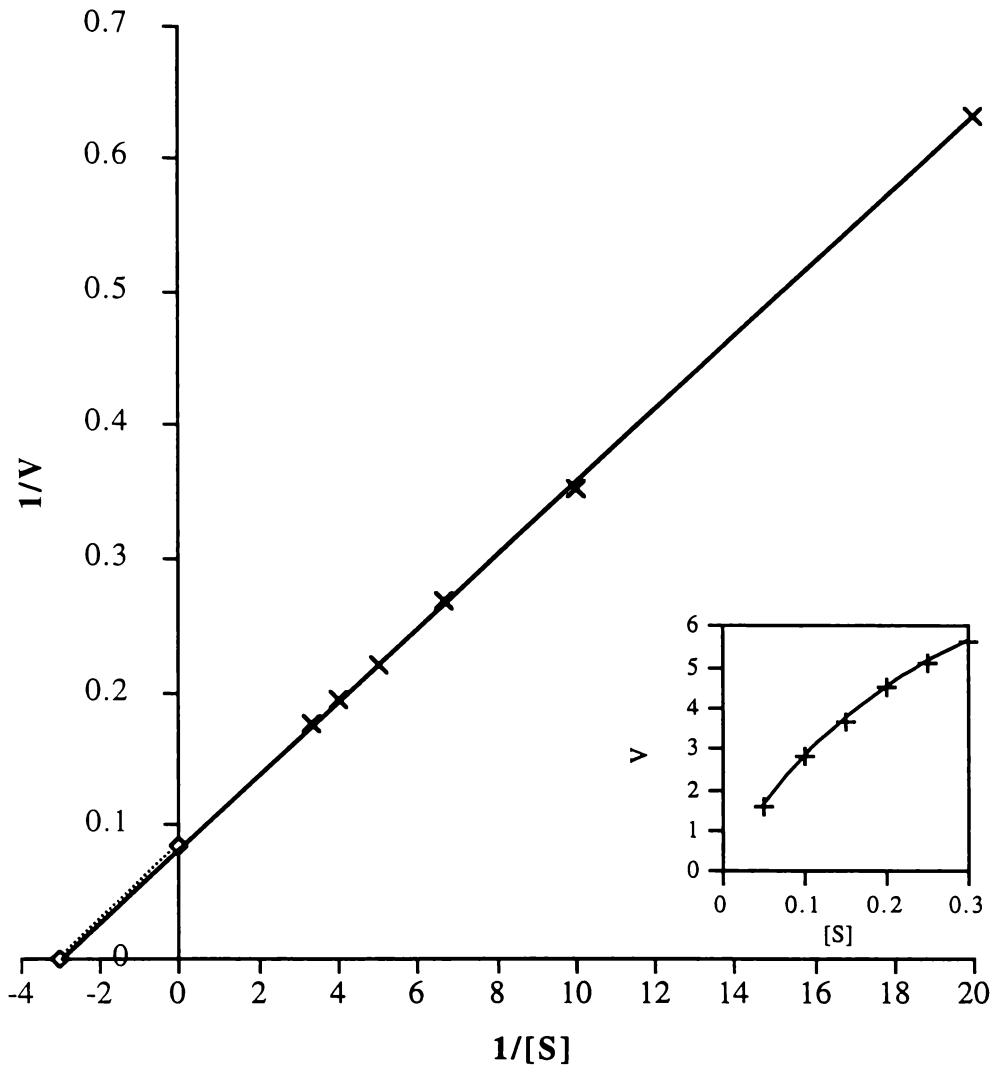


Figure A4.3.5 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA Containing 50% Sorbitol at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 50% sorbitol at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

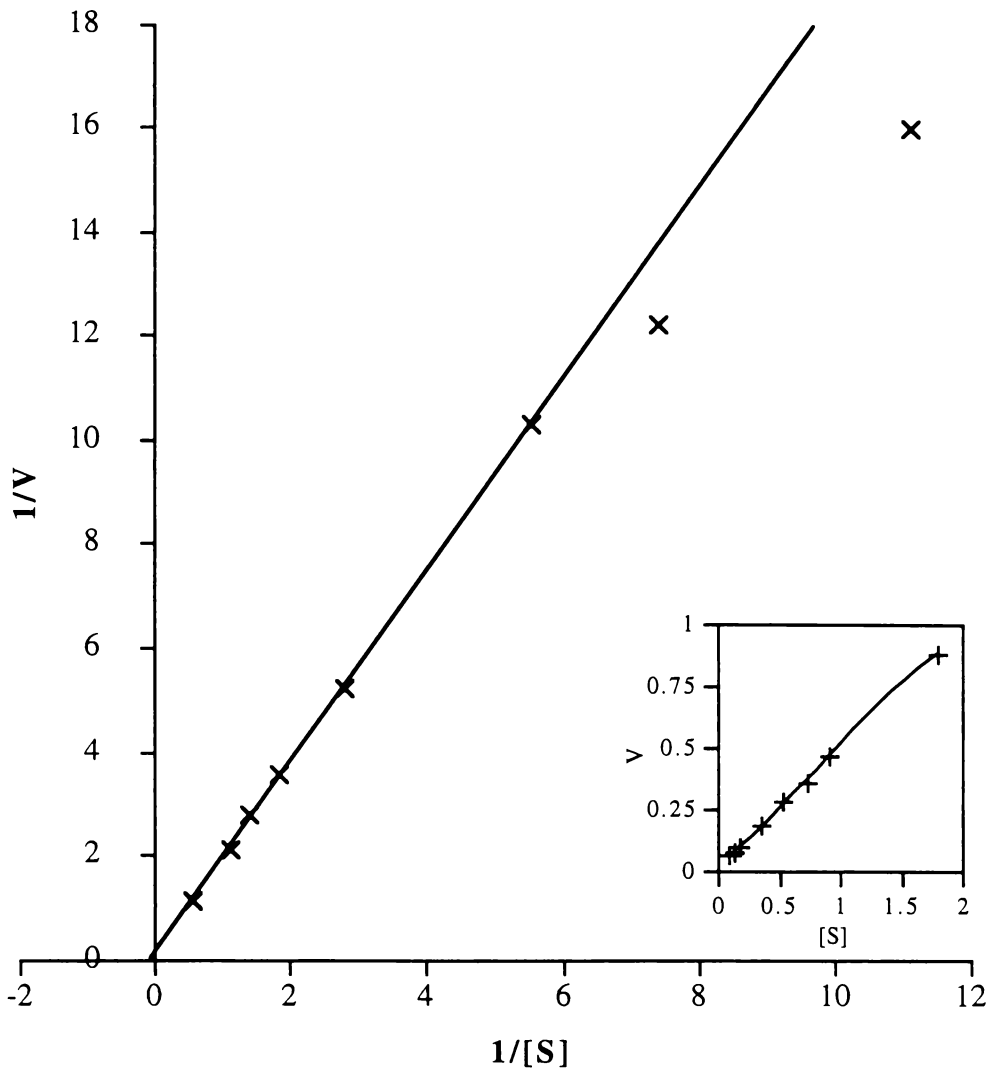


Figure A4.3.6 Lineweaver Burk Plot of Ak.1 Protease with SAAPApNA Containing 50% Methanol at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAPApNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 50% methanol at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

A4.4 SUC-ALA-ALA-ALA-PNA SUBSTRATE

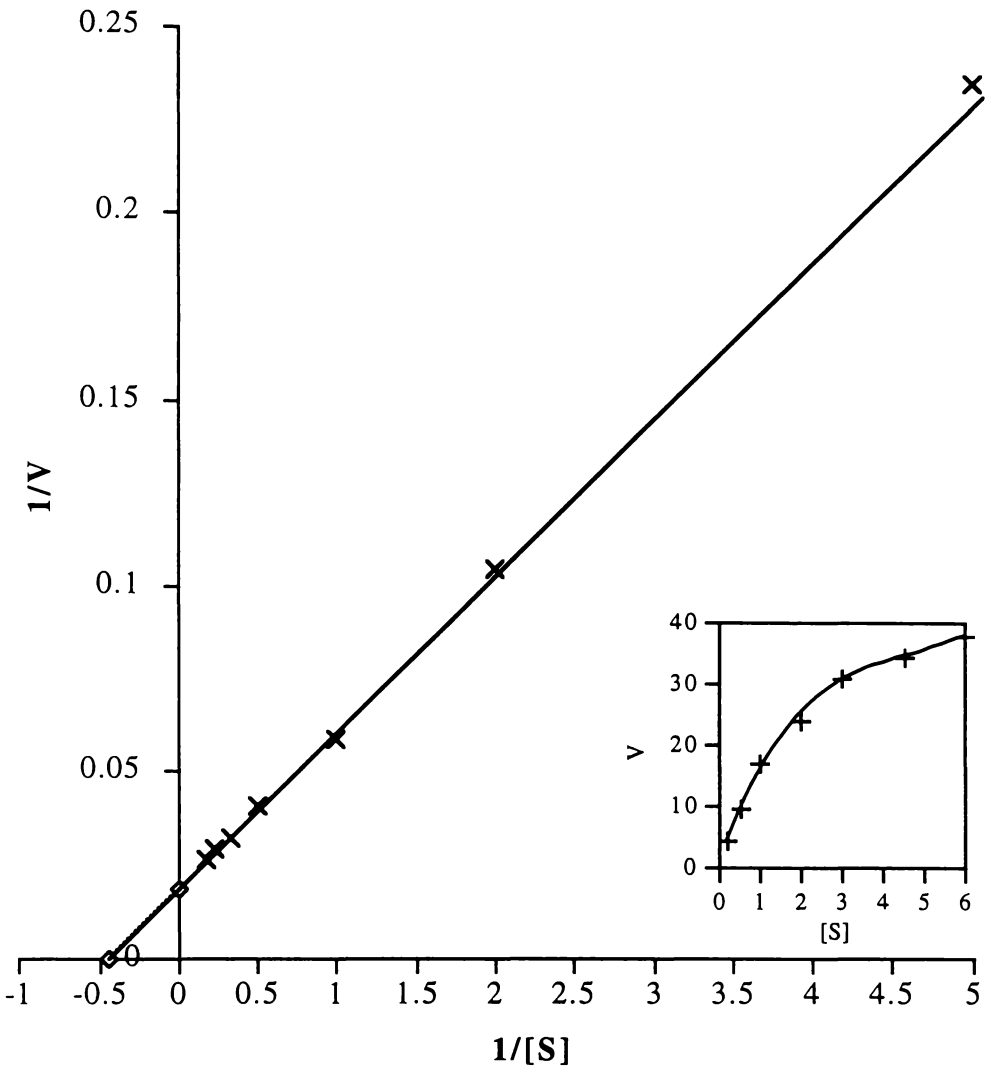


Figure A4.4.1 Lineweaver Burk Plot of Ak.1 Protease with SAAApNA at 75°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAApNA in 50 mM HEPES/NaOH pH_{75} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 75°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

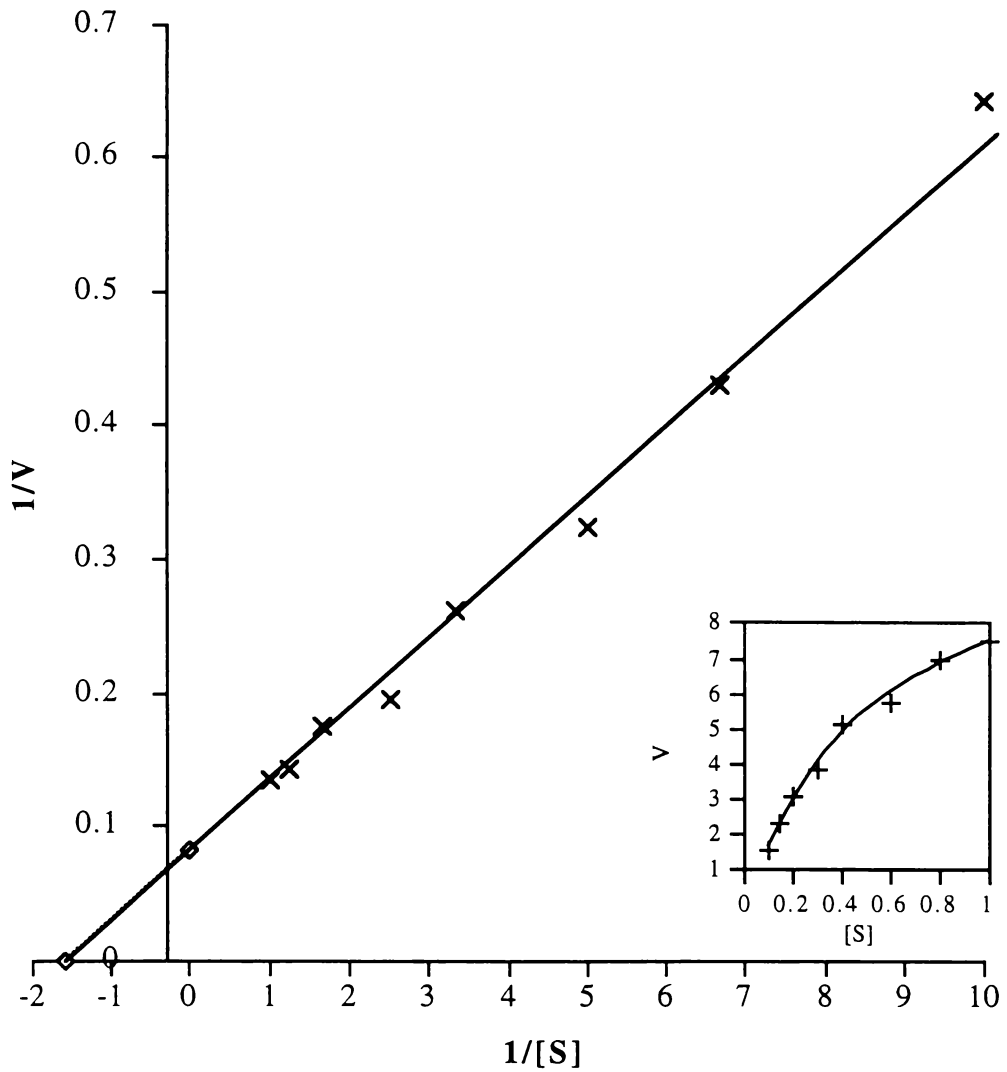


Figure A4.4.2 Lineweaver Burk Plot of Ak.1 Protease with SAAApNA at 40°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAApNA in 50 mM HEPES/NaOH pH_{40} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 40°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

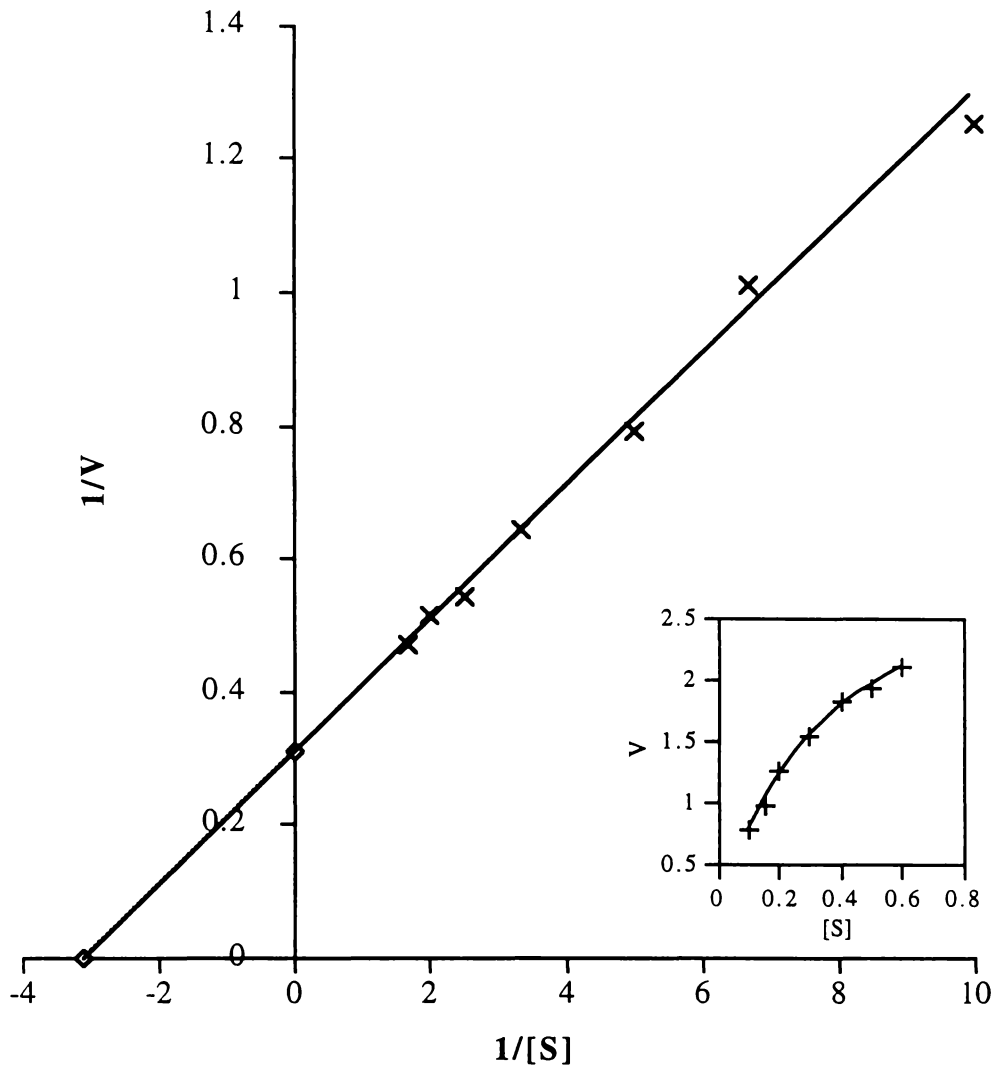


Figure A4.4.3 Lineweaver Burk Plot of Ak.1 Protease with SAAApNA at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAApNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

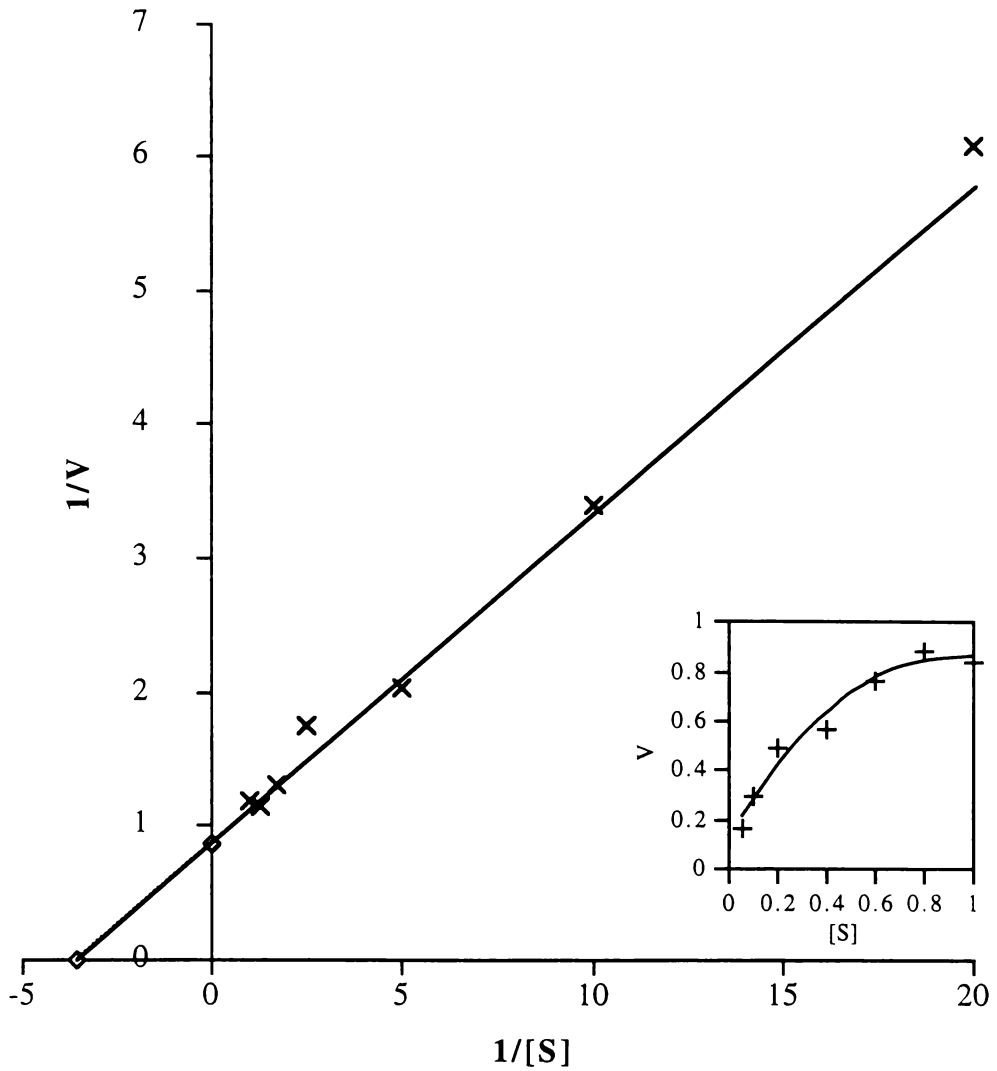


Figure A4.4.4 Lineweaver Burk Plot of Ak.1 Protease with SAAApNA at 5°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAApNA in 50 mM HEPES/NaOH pH5 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 5°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

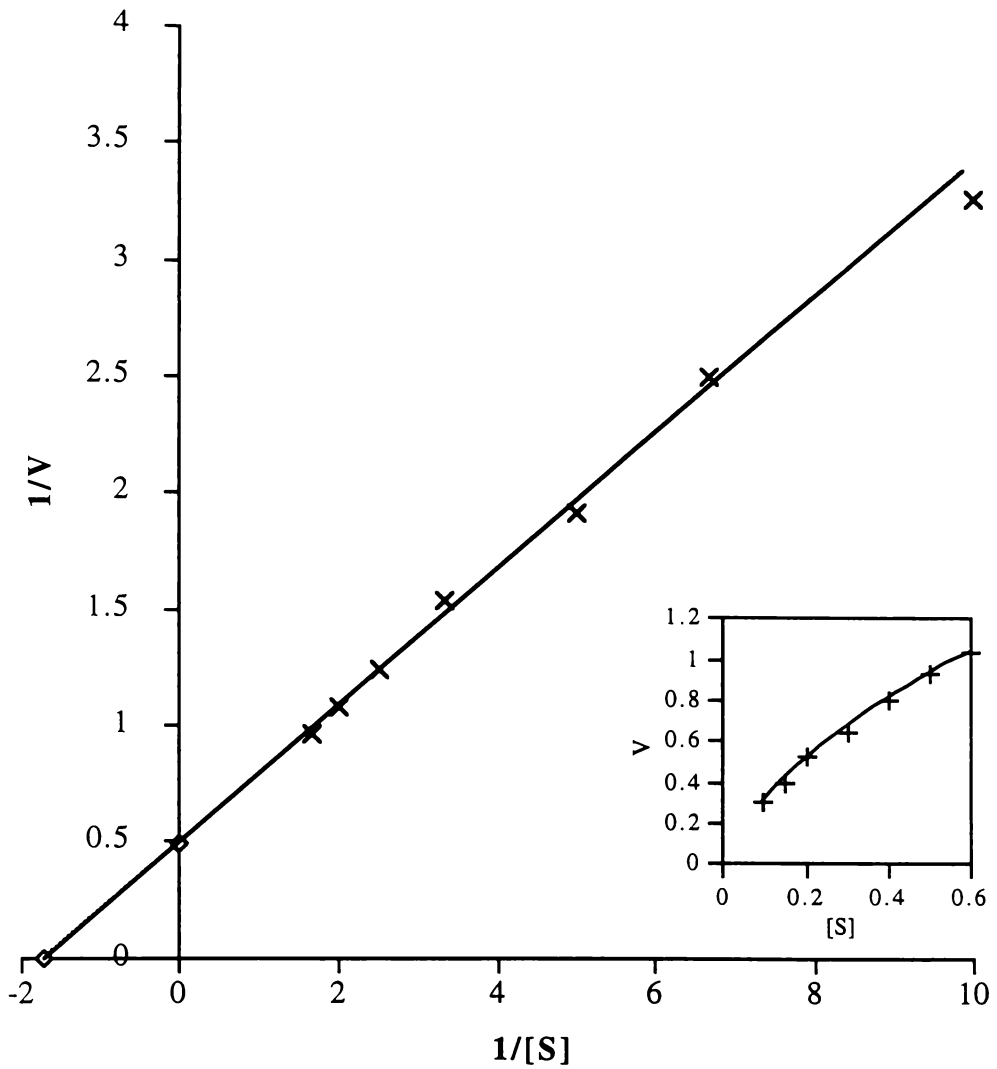


Figure A4.4.5 Lineweaver Burk Plot of Ak.1 Protease with SAAApNA Containing 10 mM DTT at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAAApNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

A4.5 SUC-ALA-ALA-PNA SUBSTRATE

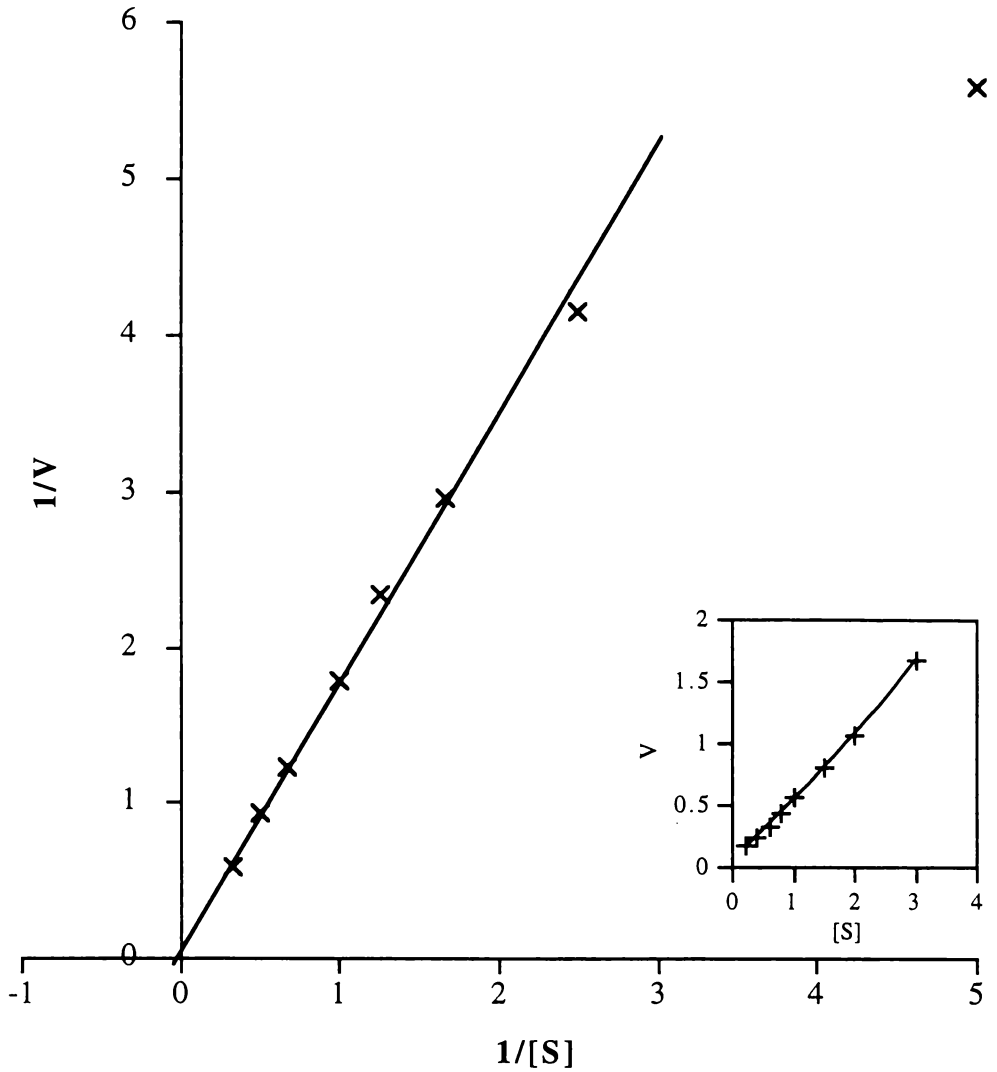


Figure A4.5.1 Lineweaver Burk Plot of Ak.1 Protease with SAApNA at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAApNA in 50 mM HEPES/NaOH pH75 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 75°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

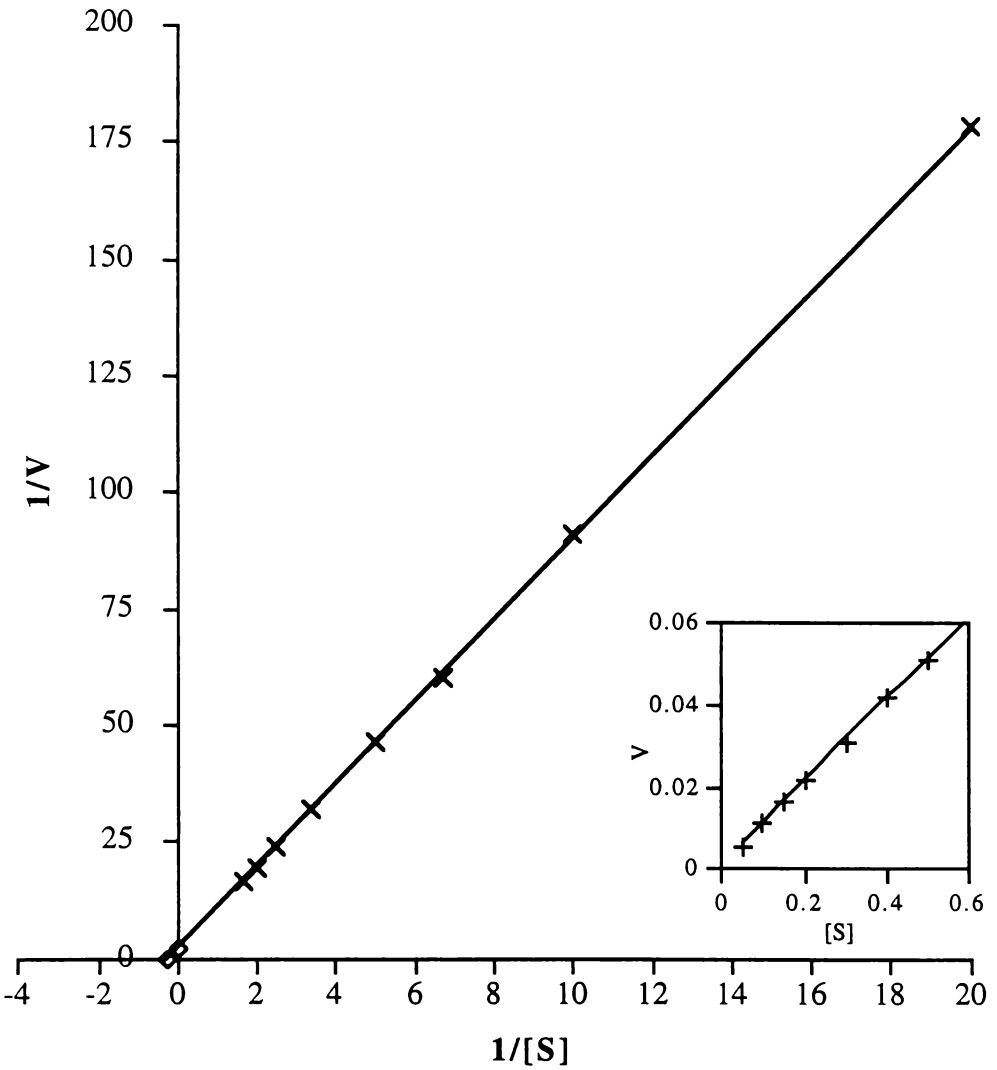


Figure A4.5.2 Lineweaver Burk Plot of Ak.1 Protease with SAApNA at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAApNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

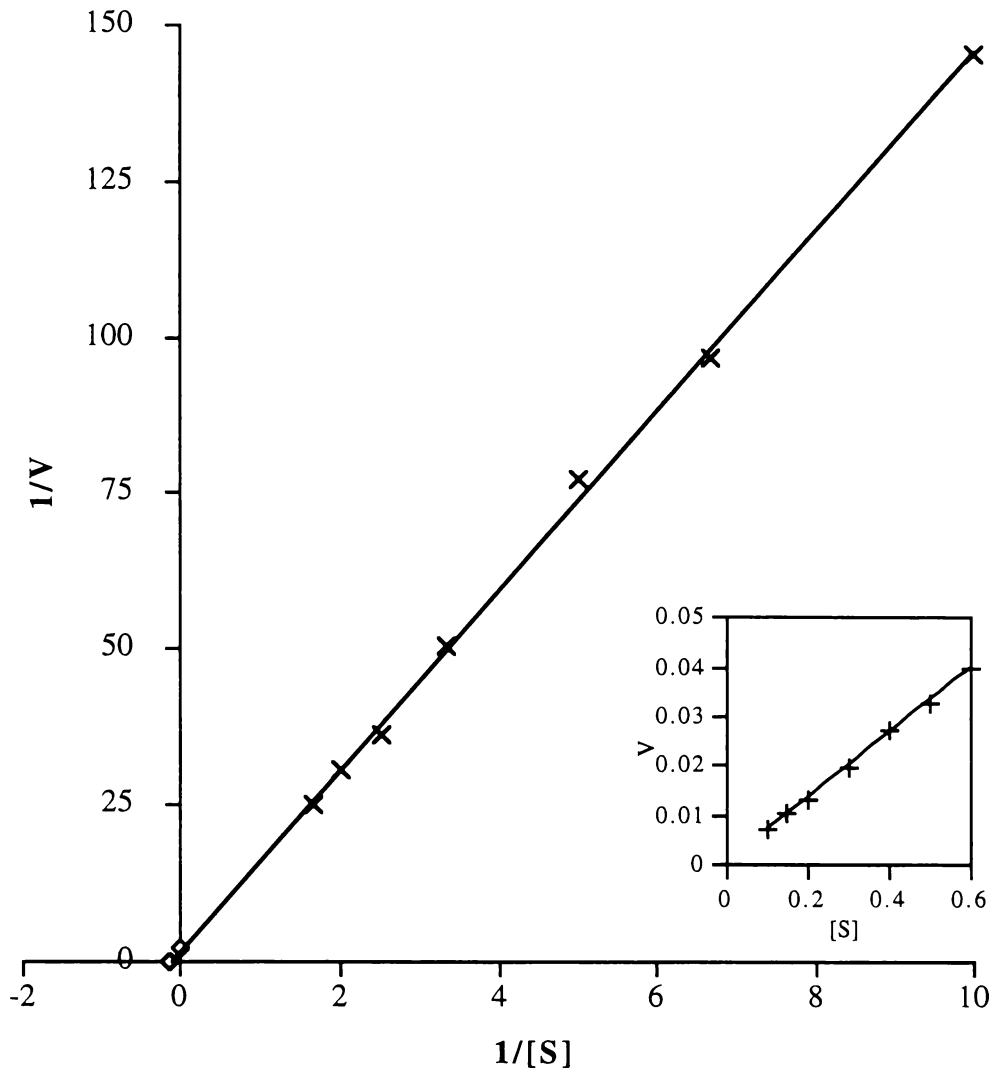


Figure A4.5.3 Lineweaver Burk Plot of Ak.1 Protease with SAApNA containing 10 mM DTT at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of SAApNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 10 mM DTT at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

A4.6 CBZ-GLY-PNP SUBSTRATE

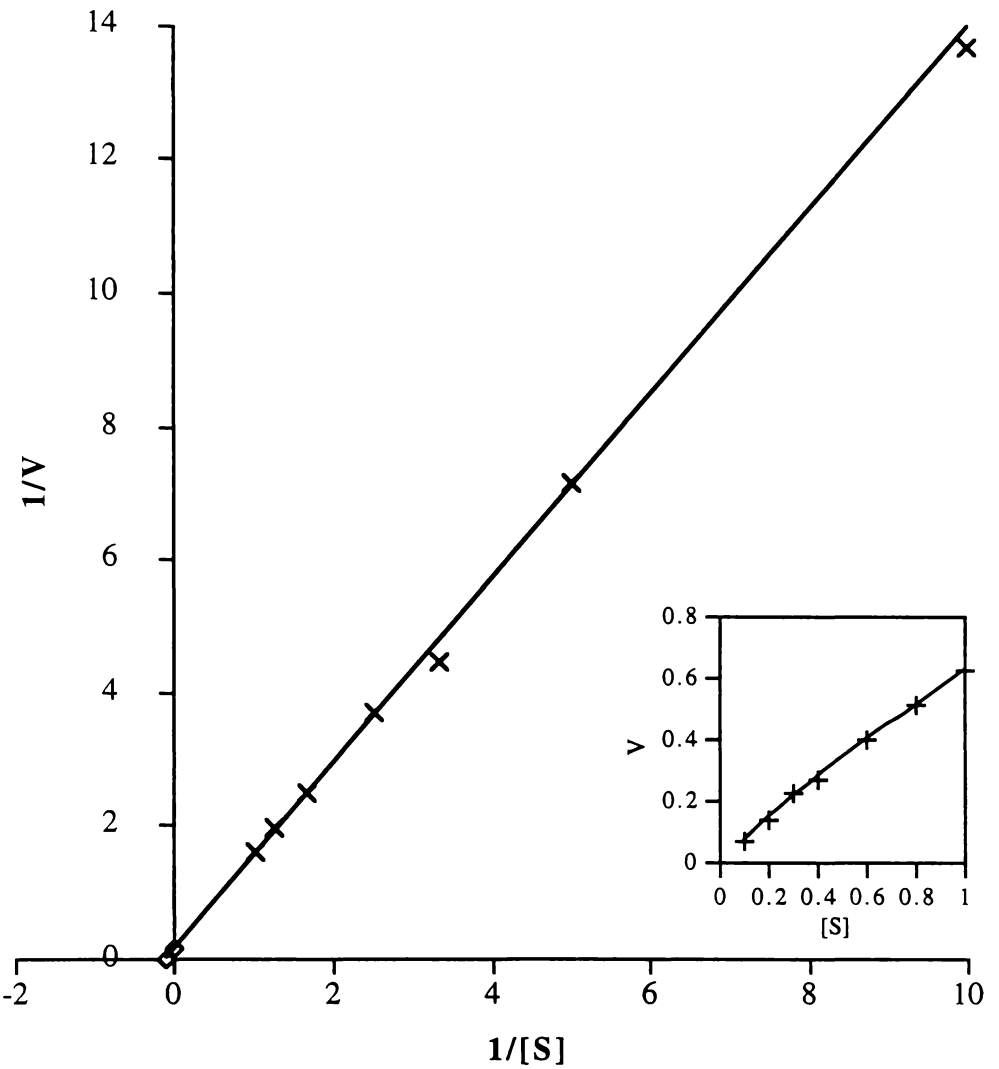


Figure A4.6.1 Lineweaver Burk Plot of Ak.1 Protease with CBZ-Gly-pNP at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of CBZ-Gly-pNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 and 50% acetonitrile at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plots (not shown). They were included to increase the accuracy of the line of best fit.

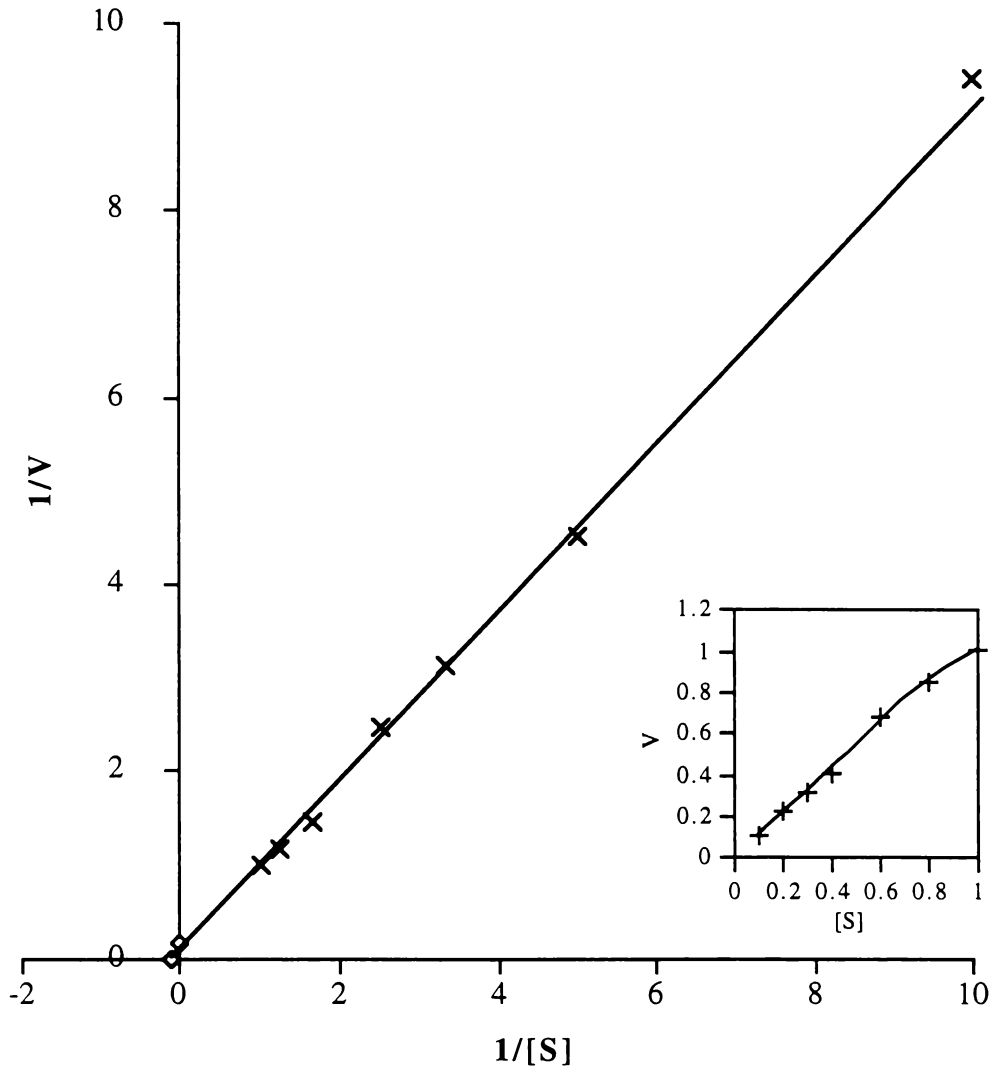


Figure A4.6.2 Lineweaver Burk Plot of Ak.1 Protease with CBZ-Gly-pNP Containing 10 mM DTT at 20°C (inset is the Michaelis Menten Plot).

Ak.1 protease was assayed with several concentrations of CBZ-Gly-pNA in 50 mM HEPES/NaOH pH_{20} 7.5 containing 5 mM $CaCl_2$ + 0.01% Triton X-100 + 10 mM DTT and 50% acetonitrile at 20°C. **The data points on the X and Y axes were obtained from the Direct Linear Plot (not shown). They were included to increase the accuracy of the line of best fit.

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