THE IMMOBILISATION OF *THERMUS* STRAIN Rt41A PROTEINASE AND ITS USE IN PEPTIDE SYNTHESIS

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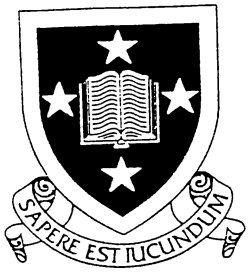
by



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ABSTRACT

A proteinase isolated from *Thermus* strain Rt41A was purified to apparent homogeneity using ion exchange and FPLC chromatography and had a final specific activity of approximately 9000 azocasein units/mg. The substrate specificity of the proteinase against various peptide p-nitroanilide substrates was investigated and compared with the results of Peek (unpublished results) and Cowan *et al.* (1987b) Rt41A proteinase has a preference for aromatic amino acids in the P_1 site but, will also cleave the pNA group from peptides with leucine, arginine, alanine and lysine in this position. When utilising p-nitroanilide substrates the peptide must have a minimum of 3 amino acids for activity to be observed.

Rt41A proteinase was immobilised to controlled pore glass (CPG) and Polymer Carrier Va-Epoxy Biosynth resin. The former of which was compared to the free proteinase. Both large (azocasein) and small (SucAAPFpNA) molecular weight substrates were utilised to measure the activity of the immobilised proteinase. Pore size of the CPG beads had little effect on % recovery of enzyme activity when it was added at a fixed level of activity/g of beads, whereas, it increased with increasing pore size when added at a fixed level/m^2 of support. Saturation of the CPG beads, with 105 nm pore size, was observed at 540 azocasein units/m^2. Lower levels (50 azocasein units/m^2 of 50 nm beads) were used in characterisation experiments. The specific activity of the immobilised proteinase was 5284 Au/mg with azocasein and 144 U/mg for SucAAPFpNA. The pH optimum of the immobilised Rt41A proteinase was 8.0 for azocasein and 9.5 for SucAAPFpNA compared to 10.5 for both substrates for the free enzyme. The immobilised enzyme retained approximately 65% of its maximum activity, against azocasein, at pH 12 whereas the free proteinase retained less than 10%. Stability towards pH increased on immobilisation, the greatest increase in half-life being approximately 12 fold at pH 7.0 at and 80 °C. Temperature-activity profiles for both the free and immobilised enzymes were very similar when using either substrate. The stability of the immobilised proteinase however, was higher than that of the free enzyme in the presence and absence of $CaCl_2$ at various temperatures. Overall the results show that low levels of calcium (10 μM) protect against thermal denaturation, but that a high calcium concentration or immobilisation are required to protect against

autolysis. Immobilisation of the proteinase also increased its stability towards two organic solvents, DMF and heptanol.

Both the free and immobilised proteinase were utilised for peptide synthesis and the observed maximum yields were the same in both cases. A number of dipeptides were produced from amino acid esters and amides. The best acyl components, from those tested, were found to be Ac-Phe-OEt and Bz-Ala-OMe and Tyr-NH_2, Ala-NH_2, Trp-NH_2, Phe-NH_2, Leu-pNA and Val-pNA were all reactive nucleophiles. The substrate specificity for peptide synthesis was therefore very similar to that found for hydrolysis reactions.

The synthesis of Bz-Ala-Tyr-NH2 was optimised by studying the effect of pH, temperature, solvent concentration, ionic strength and nucleophile and acyl donor concentration on the partition constant and the maximum yield. The initial conditions used were 25 mM Bz-Ala-OMe, 25 mM Tyr-NH_2, 70 °C, pH 8.0, and 10% v/v DMF. The optimised conditions were 80 mM Bz-Ala-OMe, 615 mM Tyr-NH_2, 40 °C, pH 10.0 and 90% v/v DMF; these conditions increased the maximum yield from 0.75% to 26% (% of original ester concentration). A number of cosolvents were also compared; the best peptide yields were observed with acetonitrile and ethyl acetate. Under optimised conditions acetonitrile gave similar yields (20%) as those observed in DMF except the acyl donor and nucleophile concentrations could be reduced to 25 mM and 100 mM respectively. The effect of different nucleophiles was also investigated with respect to peptide yield. Tyr-ßNA and Tyr-pNA were found to be better nucleophiles than Tyr-NH_2. The yields observed were 48%, 61% and 8% respectively (90% v/v DMF, 40 °C, 25 mM Bz-Ala-OMe, 100 mM Tyr-X). No peptide was produced with Tyr-OBu as the nucleophile under the same conditions. With Ala in the P_1 position, and Tyr-pNA as the nucleophile, changing the blocking group or leaving group of the acyl donor had no effect on the peptide yield. Extending the length of the peptide of the acyl donor also had no effect.

Peptide yields with Bz-Ala-OMe and Tyr-ßNA in both DMF and acetonitrile was compared under optimised conditions. Quantitative yields were observed in DMF whereas only 26% yield was found in acetonitrile. Therefore acetonitrile, compared to DMF, increased peptide yields with the Tyr-NH_2 nucleophile, decreased yields with the Tyr-ßNA nucleophile and it was also shown to have no affect on yields with the Tyr-pNA nucleophile.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

ABBREVIATIONS

Ac-	Acetyl-
BSA	Bovine serum albumin
Bz-	Benzyl-
Caps	3-(Cyclohexylamino)-1-propanesulfonic acid
Cbz-	Carbobenzoxy
Ches	4-Cyclohexylaminoethanesulfonic acid
CM-	Carboxymethyl-
CPG	Controlled pore glass
DEAE-	Diethylaminoethyl-
DFP	Diisopropylfluorophosphate
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast performance liquid chromatography
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
IEF	Isoelectric focusing
Mes	4-Morpholinoethanesulfonic acid
PD/GG	Piperazine dihydrochloride/glycylglycine
PITC	Phenylisothiocyanate
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Suc-	Succinyl
SucAAPFpNA	Suc-Ala-Ala-Pro-Phe-pNA
TCA	Trichloroacetic acid
TEA	Triethanolamine

Tes	2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]-amino}-ethanesulfonic acid
TFA	Trifluoroacetic acid
Tos-	4-toluenesulphonyl
$-NH_2$	Amide
-NHPh	Phenyl hydrazide
$-OBu^t$	Butyl ester
-OBz	Benzyl ester
-OEt	Ethyl ester
-OH	Hydroxyl
-OMe	Methyl ester
-ONap	Naphthylamide ester
-ßNA	ß- Naphthylamide
-pNA	p-nitroanilide

CHAPTER 1

INTRODUCTION

1.1 PROTEASES

1.1.1 Historical review

The discovery of proteases dates back to the late 1830's when it was discovered that hydrochloric acid was not the only food digesting agent in gastric juices. This agent was later shown to be pepsin. It was not until 1917, however, that microbial proteases were observed by Weinberg and Seguin, when they detected proteolytic activity in cell free supernatants of *Clostridium histolyticum*. In the 1950's interest in bacterial proteases increased and a number of studies were carried out on proteases from *Aspergillus oryzae, Bacillus subtlis amyloliquifaciens* and *B. subtilis* (Crewther and Lennox, 1950; Fukumoto and Negoro, 1951 and Guntelberg and Ottenson, 1952). Thermostable proteases were not observed until 1962, when Endo isolated thermolysin, a heat stable proteinase from *B. thermoproteolyticus.* Since these initial discoveries many microbial proteases both intra- and extracellular, have been isolated, and characterised (Matsubara and Feder, 1971, Morihara, 1974)

1.1.2 Classification

Protease refers to all enzymes that cleave peptide bonds including endopeptidases and exopeptidases. The name proteinase is given to enzymes that cleave internal peptide bonds (endopeptidases). Exopeptidases cleave either the amino- or carboxy- terminal amino acid from a peptide chain.

Proteases can be classified in a number of ways. Initially they were classified on the basis of molecular size, charge or substrate specificity. More recently classifications have been based on comparisons of active sites, mechanism of action and their three-dimensional structure. This is in contrast to other types of hydrolases which are usually classified according to substrate

specificities. There are four mechanistic classes recognised by the International Union of Biochemistry, within which exists six families: Serine proteases I and II, cysteine (thiol) proteases, aspartic (acid) proteases and metallo-proteases I and II.

Schechter and Berger (1967) introduced a nomenclature to describe the interaction between proteinases and their substrates. This nomenclature is widely used in the literature and has been adopted here. The substrate amino acids are called P (for peptide). The numbering of amino acids on the amino terminal side of the scissile bond is P_1, P_2, P_3 outwards; on the carboxy terminal side of the scissile bond it is P_1', P_2', P_3'. The subsites within the active site of the proteinase are labelled S_3, S_2, S_1, S_1', S_2', S_3' to compliment the substrate residues as shown in Fig 1.1.

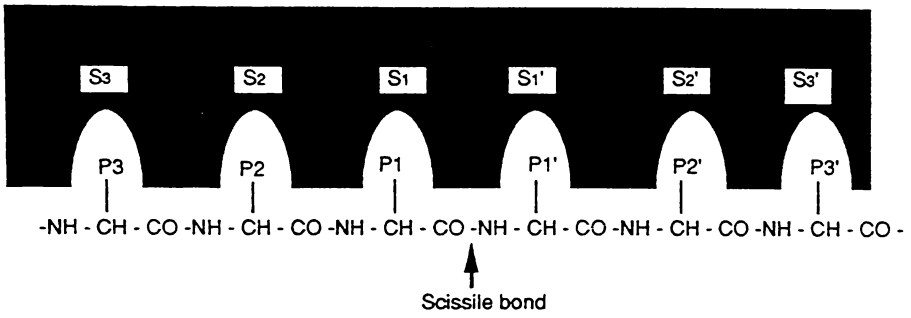


Fig 1.1 Schechter Berger Nomenclature for the Binding of a Peptide Substrate to a Peptidase[a]

The Proteinase is represented by the shaded area. P1...P1' are side chains of amino acids. S1...S1' are corresponding subsites on the enzyme.
[a] Taken from Beynon and Bond (1989).

1.1.2.1 Serine Proteases

There are two distinct families within this group, the first of which is the mammalian serine proteases or the chymotrypsin-like family and the second is the bacterial or subtilisin-like family. Although they are different in amino acid sequence and 3-D structure they have a common active site geometry

and mechanism of reaction. The amino acids involved in the active site are serine, histidine and aspartic acid which form a catalytic triad, catalysis proceeds via a tetrahedral transition state intermediate during acylation and deacylation (Kraut, 1977; Carter and Wells, 1988). All known covalent inhibitors of the serine proteinases also form tetrahedral adducts with similar geometry. All serine proteinases are inhibited by DFP, most are inhibited by PMSF and some by chloromethyl ketones (e.g. N-tosyl-L-lysine chloromethyl ketone), the latter two also inhibit some cysteine proteases (Bond and Butler, 1987).

1.1.2.2 Cysteine (Thiol) Proteases

Cysteine proteases include mammalian lysosomal cathepsins, cytosolic calpains, the plant proteases papain and actinidin and clostripain from *Clostridium histolyticum*. Papain is probably the best studied of these. The major active site amino acid is cysteine which acts in a similar manner to the serine amino acid in serine proteases. Catalysis proceeds via a thiol ester intermediate and is facilitated by adjacent aspartic acid and histidine residues (see Polgar and Asboth, 1986; Pearl, 1987). They are inhibited by low concentrations of p-hydroxymercuribenzoate and alkylating agents such as iodoacetate, iodoacetamide and N-ethylmaleimide. Sulfhydryl inhibiting reagents are not generally specific for cysteine groups at the active site (Bond and Butler, 1987).

1.1.2.3 Aspartic (Acid) Proteases

Aspartic proteases have been widely identified in eukaryotes but seldom in prokaryotes. Penicillopepsin is the model enzyme for this group of proteases. Other examples include mammalian pepsin, rennin, chymosin, and some fungal proteases. Structural data suggests that there is only one family in this group. The basic structure is bilobal which consists mainly of ß-sheets with a deep cleft that contains the active site (Blundell *et al.*, 1980). Two aspartic acid residues, in close proximity, are involved in catalysis. This is thought to be general acid-base catalysis, rather than formation of a covalent enzyme-substrate intermediate (Polgar, 1987). In the pH range 2-3, at which these enzymes are normally optimally active, one of the aspartic acids is ionised

while the other is not. Aspartic proteases are inhibited specifically by pepstatin (which resembles the normal substrate in the transition state) and also by active site-directed affinity labels such as diazoacetyl-norleucine-methyl ester (Kay, 1985). However, the latter type of inhibitor will also react with other proteases.

1.1.2.4 Metallo-Proteases

This group of proteinases can be divided into two families, the first is represented by bovine carboxypeptidase and the second by thermolysin. Metal ions (usually Zn^{2+}) are located in the catalytic centre of the enzyme and may be involved in enhancing the nucleophilicity of water and polarising the peptide bond to be cleaved before attack (Holmes and Matthews, 1981; Bond and Beynon, 1985). Amino acids in the active site also participate in catalysis. For example, carboxypeptidase A has two glutamine residues and one histidine residue interacting in catalysis. Cysteine and serine are also known catalytic residues for this type of protease. Because the metal ions are usually "loosely" bound, metal chelators such as EDTA and 1,10-phenanthroline inhibit metallo-proteases.

1.1.3 Thermostable Proteases

The discussion in this section is limited to those proteases from extreme and hyperthermophiles, i.e., organisms that grow optimally at 65 °C and above.

1.1.3.1 Mechanism of Thermophily

It is well known that enzymes from thermophilic bacteria are generally more thermostable than their mesophilic counterparts. However, other properties such as primary, secondary, tertiary and where applicable quaternary structural parameters and their physiochemical nature are generally similar.

Since the discovery of enzymes with increased thermostability many ideas have been put forward as to the mechanism of thermophily. Two of the early hypotheses were: (1) a high protein turnover of protein molecules which had

similar thermostabilities to mesophilic enzymes and (2) stabilisation by either high molecular weight (e.g. proteins) or low molecular weight (e.g. polyamines) compounds. There has been no experimental evidence to support the former theory. With regards to external stabilising factors there are a few cases where these have been successfully isolated from thermophilic microorganisms (Nakamura, 1960; Prasad and Maheshwari, 1978; Oshima, 1982) but generally this is not the case.

The predominant view that has emerged from extensive research in this area is that thermophilic enzymes are intrinsically more stable than their mesophilic equivalents. It is generally accepted that the stability of proteins relies on the balance of both stabilising (non-covalent interactions within the protein) and destabilising forces (mainly entropy). These forces are in the order of a 4200 kJ/mol and the difference between them is only 21-42 kJ/mol. This means that a small change in either can cause a drastic change in the net free energy. It has been estimated that the 21-42 kJ/mol increase in these stabilising forces (Perutz and Raidt, 1975; Mozhaev and Martinek, 1984) required to increase the thermostability could be provided by a few extra hydrogen bonds or salt bridges (Perutz, 1978; Finney *et al.*, 1980). It is certain that all non-covalent interactions within the protein contribute to the stability of the protein (for review see Mozhaev and Martinek, 1984; Matthews, 1987a,b). Examples of these will be described below.

Zuber (1979) investigated lactic dehydrogenases from mesophilic and thermophilic bacilli which revealed an increase in polar amino acids in the active site with increasing thermostability. The explanation for this was increased ionic and hydrogen-bonding interactions in the interior of the molecule.

Perutz (1978) compared the amino acid sequences of ferredoxin from various clostridial species with varying thermostabilities and concluded that the structures must be similar with the only possible source of increased thermostability arising from extra salt bridges on the protein surface. This was also found to be the case when ferredoxin from lobster and *B. stearothermophilus* were compared by Perutz and Raidt (1975); the salt bridges linked amino acid residues near the N-terminus to residues near the C-terminus. In addition, the results of Matsumura *et al.* (1984) are consistent with these results. The authors compared kanamycin nucleotidyl transferase

from *B. stearothermophilius* and *Staphylococcus. aureus* and found that the only difference in the proteins was the substitution of a lysine for a threonine residue. This substitution enables extra electrostatic bridging within the protein molecule which increases the thermostability.

Amino acid substitutions, for example alanine for glycine and threonine for serine, results in a more compact protein structure (Mozhaev and Martinek, 1984). Increased stability could be achieved in this way by pressing the internal water out of the protein (Walker *et al.*, 1980). Thermostable enzymes have been shown to have higher levels of both alanine and threonine (Argos *et al.*, 1979); therefore their structure is more compact than mesophilic proteins which aids in increasing their thermostability.

Temperature sensitive mutants of T_4 phage lysosome were investigated by Grutter *et al.* (1979) and it was shown that the replacement of a partially exposed arginine with a histidine had no effect on the 3-D structure of the protein. Despite this the thermostability was decreased. The difference was said to be due to subtle changes in the hydrophobic interactions and H-bonding within the molecule.

In addition to the stabilising proteins and polyamines, mentioned above, metal ions have been shown to increase the thermostability of proteins. For example, Ca^{2+} was found to have a role in the stabilisation of thermolysin from *B. stearothermophilus* (Dahlquist *et al.,* 1976; Voordouw *et al.,* 1976) and the proteinase from *Thermus* strain T-351 (Cowan and Daniel, 1982a, Cowan *et al.*, 1982). In addition Co^{2+} was essential in the stabilisation of the aminopeptidase from *Sulfolobus solfataricus*. This could be due to the metal ions increasing the rigidity of thermolabile regions of the protein. The rigidity of thermostable enzymes compared to mesophilic enzymes has been under question for some time. There is now evidence to suggest that thermostable enzymes are in fact more rigid at ambient temperatures, but at physiological temperatures both types of enzymes are similar in flexibility (e.g. Daniel, 1986; Vihinen, 1987). Flexibility of a protein molecule is generally thought to contribute to the catalytic ability of enzymes (Tainer *et al.*, 1984).

As well as a need for thermostable proteins, thermophilic organisms also require thermostable protein-synthesizing systems. Polyamines are suggested to be involved in complexing the 30s ribosomal unit, mRNA and

aminoacyl tRNA at high temperatures (Oshima, 1979). Thiolation of thymidine in tRNA increases its thermostability, possibly via increasing the stacking forces in the molecule. In addition, higher G+C ratios increases the number of H-bonds in thermophilic tRNA and therefore the thermostability (Oshima, 1979). The DNA-dependent RNA polymerases from three extremely thermophilic archaebacteria have been shown to be extremely thermostable (Prangishvilli *et al.*, 1982)

1.1.3.2 Assay Methods

The methods used for screening of extremely thermophilic bacteria for proteolytic activity and subsequent quantification of the proteinases present has been, in most cases, similar to those used for mesophilic proteinases. Typically samples are tested for their ability to resolubilise precipitated para-casein (Sato and Ohmiya, 1966; Lawrence and Creamer, 1969) or to release TCA soluble hydrolysis products from casein (Kunitz, 1947).

Screening of extracellular proteolytic activity can be carried out in several ways. For example, the observation of zones of clearing or precipitation of para casein around bacterial colonies and gelatin solubilisation. Plate diffusion assays have been developed by Cowan and Daniel (1982b) and Montville (1983). The former was based on the procedure of Grimont *et al.* (1977), while the latter uses a combination of gelatin and casein.

As agar melts at temperatures above 65 °C, the temperature required for incubation of extreme thermophiles, Gelrite (Kelco, Merck and Co) with $MgSO_4$ was utilised to solidify media containing casein (see Bragger *et al.*, 1989). This, however is limited to zones of clearing rather than precipitation.

Substrate-PAGE electrophoresis (Heussen and Dowdle, 1980) and substrate overlay gels (Deane *et al.*, 1987) have been used to determine the proteolytic activity of individual protein bands after gel electrophoresis. Both these techniques have been utilised with thermostable proteinases (Connaris *et al.*, 1991; Blumentals *et al.*, 1990) and may be of use in screening experiments.

For quantitative analysis of proteolytic activity casein and haemoglobin (Anson, 1938) are often used, the measurement of the TCA soluble material produced is determined either directly, absorbance at 280 nm, or indirectly via reaction of the protein with dye-binding reagents. Examples of the indirect method include ninhydrin (Moore and Stein, 1954; Reimerdes and Klostermeyer, 1976), Biuret (Hall, 1955), Folin-Ciocalteau reagent (Banga *et al.*, 1959), fluorescamine (Garesse *et al.*, 1979; Udenfriend *et al.*, 1972; Schwabe, 1973), and 2,4,6-trinitrobenzenesulfonic acid (Okuyama and Satake, 1960; Lin *et al.*, 1969). More recently derivatisation of primary amines, without separation from the substrate protein, with o-phthalaldehyde has been used (Church *et al.*, 1985).

Due to the high temperatures utilised in the assay of thermostable proteinases some proteinaceous substrates are denaturated and are therefore, more susceptible to proteolysis than the native protein. Cowan and colleagues (1987a) suggest this is the reason for the higher specific activities found for thermostable proteinases compared to their mesophilic counter parts. In addition, the breakdown pattern of the substrate may differ depending on the temperature of assay; different peptide bonds will susceptible to proteolysis depending on whether the protein is unfolded or in its native state. The fact that the substrate may be denatured at the assay temperature does not limit its use in quantifying the proteolytic activity of thermostable proteinases.

Although activity is found against insoluble substrates, such as collagen (Cowan and Daniel, 1982a; Saravani *et al.*, 1989; Wilson *et al.*, 1991), reproducible results are often difficult to obtain. Similarly, chromogenic substrates are unstable at higher temperatures, especially in the case of ester substrates, p-nitroanilide substrates have proved most stable. Substrate instability has required the use of lower temperatures and in some cases continuous assay methods. Reducing the assay temperature will not affect quantitative determinations but the K_m of the enzyme may be affected. It has also been found, with a number of thermostable proteinases, that for proteolytic cleavage of p-nitroanilide substrates a tripeptide, as a minimum, is required (Cowan *et al.*, 1987b, Saravani *et al.*, 1989).

Dye-linked proteins such as azocasein (Charney and Tomarelli, 1947), azoalbumin (Tomarelli *et al.*, 1949), azocoll (Chavira *et al.*, 1984) and

radioactively labelled proteins (e.g Katchman *et al.*, 1960; Mills and Thomas, 1978) have been used as general substrates for proteases. The binding of a dye to a protein could alter the specificity of the protease for the substrate as was found for caldolase; the activity against collagen was lower than that observed for casein but the activity against azo-coll was greater than azocasein (Saravani *et al.*, 1989). The investigations of thermostable proteases have routinely used casein and azocasein as substrates with direct measurement of the TCA soluble material (Cowan and Daniel, 1982a; Cowan *et al.*, 1987a,b; Saravani *et al.*, 1989; Coolbear *et al.*, 1989; Matsuzawa *et al.*, 1983, 1988; Taguchi *et al.*, 1983). The use of azocasein can present problems, in that, variation between batches is high and non-linearity of the assay is found above certain absorbance values. However, azocasein has been found to be a more sensitive substrate than casein; 3.6 times less Rt41A proteinase will give the same absorbance change with azocasein (420 nm) as is observed with casein (280 nm) (Peek, unpublished results).

1.1.3.3 Examples of Proteases from Extreme Thermophiles

Some of the characteristics of various proteases are illustrated in Table 1.1. Some mesophilic and moderately thermostable proteases have been included for comparative purposes. In general, the thermostability of the proteases correlates well with the growth temperature of the organism. One exception is the proteinase from *B. thuringiensis* which is reasonably stable at 60 °C (22% loss in 7 hr) but the growth temperature of the organism is only 30 °C. The thermostable proteases from extreme thermophiles are either serine or metallo-proteases, with only one example of an acid proteinase. The lack of thiol and acid proteases probably stems from the fact that proteinases of these types, isolated to date, are mostly of eukaryotic origin.

A number of proteinases have been isolated and studied from *Thermus* species, all of which are serine proteases. Caldolysin, isolated from *Thermus* strain T-351 (Cowan and Daniel, 1982a) has been well characterised. Ca^{2+} ions are required for the stability but not activity of this proteinase; six calcium molecules are bound/enzyme molecule. The calcium binding sites show either a high or low affinity for Ca^{2+} ions (Khoo *et al.*, 1984). Caldolysin also

T. aquaticus YT-1 (75 °C) Aqualysin I	Serine	28.5	10.4	>9-10	30 min, 90 °C*	Matsuzawa, *et al*, 1988
T. aquaticus YT-1 (75 °C) Aminopeptidase	Metallo	108.0	8.5	NA	40% loss 4h, 80 °C	Minagawa, *et al*, 1988
T. aquaticus strain T-351 (75 °C) Caldolysin	Serine	20.5	7.7	8.5	1 h, 90 °C*	Cowan *et al*, 1982a
Tc. celer (80 °C) Proteinase	Serine	NA	NA	NA	40 min, 95 °C	Bragger *et al*, 1989
S. solfataricus (80 °C) Proteinase	NA	NA	NA	NA	40 min, 95 °C	Bragger *et al*, 1989
S. solfataricus (85 °C) Aminopeptidase	Metallo	320.0	6.5	4.4	40% loss 15 min , 85 °C*	Hanner *et al*, 1991
Desulfurococcus strain $Tok_{12}S_1$ (88 °C) Archaelysin	Serine	52.0	7.8	8.7	70-90 min,95 °C	Cowan *et al*, 1987d
Pyrococcus furiosus (98 °C) Proteinase	Serine	66.0	7.0	NA	17 min, 105 °C*	Connaris *et al*, 1991

[1] Growth Temperature

* Thermostability carried out in the presence of stabilising metal ions.

B., Bacillus
T., Thermus
Tc., Thermococcus
S., Sulfolobus
NA, Not Available

binds one Zn^{2+} ion/enzyme molecule, the manner of binding and function of which are unknown. Caldolysin is active against a number of natural substrates and also their chromophoric derivatives. Activity against chromogenic peptides is unusual in that a minimum of 3 amino acids must be present in the peptide for hydrolysis with a specificity for small neutral amino acid residues in the P_1 and P_1' position of the bond (Cowan and Daniel, 1982a). No esterase activity was observed for this proteinase. As can be seen in Table 1.1, caldolysin is one of the more thermostable proteinases from *Thermus* species. It is also stable to denaturing agents such as urea and SDS.

Thermus strain Tok3 also produces an extracellular proteinase, the trivial name of which is caldolase (Saravani *et al.*, 1989). No significant amounts of Ca^{2+} or Zn^{2+} are associated with the proteinase and the enzyme is insensitive to metal chelators. Of the natural proteins tested casein and haemoglobin were the most susceptible to hydrolytic attack by caldolase. Activity was also observed against BSA, ovalbumin, collagen and fibrin. High activity was observed against the dye-linked proteins and ester substrates tested. Although tripeptide p-nitroanilide substrates were cleaved, the proteinase was not effective in cleaving the single amino acid p-nitroanilides. Caldolase is resistant to denaturing and reducing agents but was susceptible to SDS. Ionic strength plays a major role in the stabilisation of caldolase; greatest stability was observed with the addition of 0.5 M NaCl + 10 mM $CaCl_2$.

Both of the *Thermus* strains already mentioned were compared with two other *Thermus* strains, Rt6 and Rt41A (Rt6 and Rt41A proteinase), by Cowan *et al.* (1987b). All of the proteinases were active against haemoglobin and casein and all, except Rt6 proteinase, were active against collagen. The cleavage of p-nitroanilide peptide substrates was also investigated. Rt6 proteinase had poor reactivity against these substrates; the other three proteinases exhibited greatest activity against the tetrapeptide substrate and a few of the tripeptide substrates tested. The pH optima observed against casein were similar in all cases (8.8-9.6). However, with azocasein as the substrate a much broader range (7.7-9.3) was observed. Caldolysin was the only proteinase that was calcium stabilised, although subsequent research with Rt41A proteinase has shown that this proteinase does have a calcium requirement for thermostability (Peek, unpublished results; this study). Rt41A proteinase has

also been shown to cleave oxidised insulin B-chain, the first bond hydrolysed being that of Leu^{15}-Tyr^{16}. The cleavage sites have been compared with other microbial proteases in Fig. 1.2. Esterase activity was observed against p-nitrophenol esters, especially that of tyrosine (Peek, unpublished results).

Two proteinases from *Thermus aquaticus* strain YT-1 have been isolated, Matsuzawa *et al.*, 1983), aqualysin I and II. Only aqualysin I has been characterised in detail due to problems with aqualysin II appearing to form complex aggregates of several proteins. Both proteinases are different, as can be seen by the widely different pH optima (Table 1.1). In addition, aqualysin II is chelator sensitive (stabilised by calcium and strontium ions) whereas aqualysin I is not. Aqualysin I exhibits a N-terminal sequence homology with other microbial serine proteinases, especially that of proteinase K. Aqualysin I is stable toward denaturing agents at low temperatures but not as stable as caldolysin at high temperatures. Esterase activity, with a preference for small hydrophobic and aromatic residues, and some elastase activity was observed. Aqualysin I also cleaves oxidised insulin B-chain, the major cleavage sites are similar to those exhibited by Rt41A proteinase, thermitase and proteinase K (see Fig. 1.2).

Proteinase production was also illustrated for *T. caldophilus* strain GK 24 (Taguchi *et al.,* 1983). The proteinase is insensitive to chelating agents and is stable in denaturing agents at low temperatures. Similar reactivities to chymotrypsin were observed against the small molecular weight substrates tested.

Two proteinases from extremely thermophilic bacilli have been isolated and studied, viz, *Bacillus* strain Ok3A.1 and E A.1. These were chosen from a screen of a number of *Bacillus* strains (Coolbear *et al.*, 1991). Both proteinases are neutral metallo-proteinases. although E A.1 proteinase has a narrower pH profile. Ok3A.1 proteinase is not affected by o-phenanthroline which suggests that Zn^{2+} is not required by this proteinase for activity, whereas it is for the activity of E A.1 proteinase. Neither proteinase exhibited elastolytic activity or activity against SucAAApNA or 3-(2-furylacyloyl)-Gly-Leu-NH_2, common synthetic substrates for metallo-proteases. The thermostability of both is greater than that found for the mesophilic proteases from bacilli, and they are more thermostable than thermolysin.

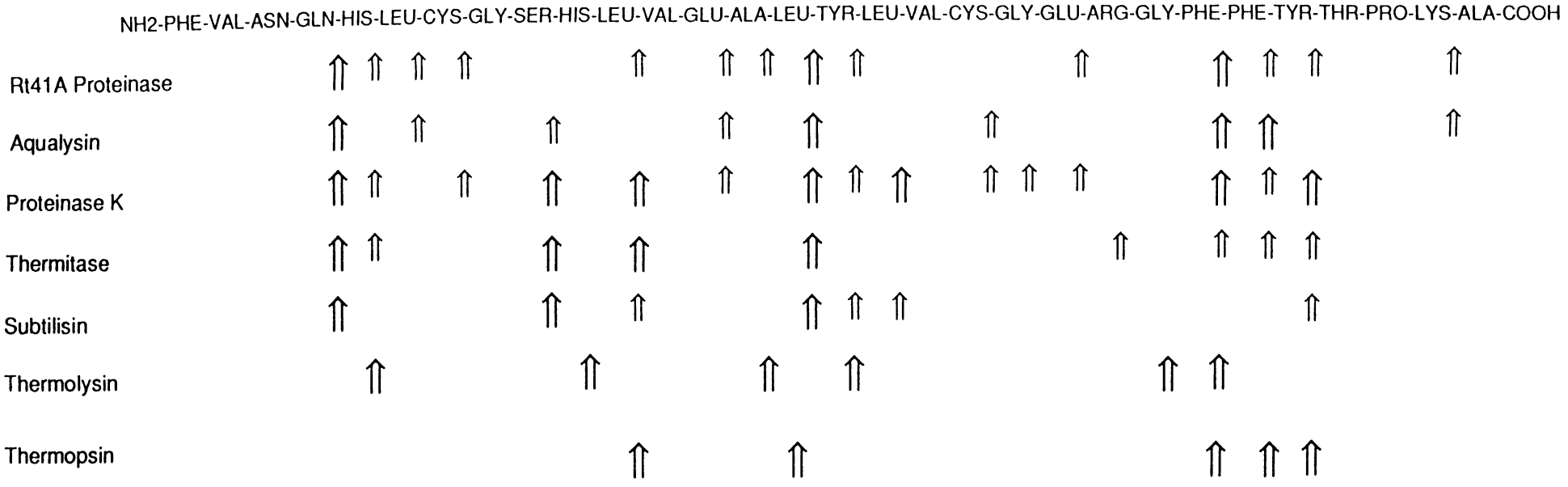


Fig 1.2 Cleavage Sites For Rt41A Proteinase Against The Oxidised Insulin B Chain and Comparison to Other Microbial Proteinases

Rt41A Proteinase from Peek (unpublished results), aqualysin, proteinase K, thermitase, and subtilisin results taken from Matsuzawa (1988), Thermolysin taken from Matsubara (1966), Thermopsin taken from Fusek *et al.*, (1990).
Large arrowheads signify major cleavage sites, small arrowheads signify minor cleavage sites.

A few archaebacteria have been shown to produce proteinases. The classic example is archaelysin, isolated from *Desulfurococcus* strain $Tok_{12}S_1$ (Cowan *et al.,* 1987d). Archaelysin is a serine proteinase with a neutral pH optimum. It hydrolyses a number of native proteins, soluble and insoluble, and has no esterase activity. There is a strong preference for an uncharged aliphatic side chain in the P_1 position. Thermostability was not affected by chelators and exceeded that described for caldolysin.

Multiple active proteinases have been observed for *Pyrococcus furiosus* (DSM 3638); Blumentals and coworkers (1990) demonstrated the presence of five proteolytic bands by SDS-PAGE, whereas Connaris and colleagues (1991) observed 13 proteolytic bands by a similar method. The latter group showed that these bands were due to discrete polypeptides, and suggested that they may exist as active aggregates prior to dissociation by SDS. The data presented in Table 1.1 applies to the most characterised proteinase of those observed. High thermostability was a feature of all the proteinases; this was enhanced by the presence of $CaCl_2$. The proteinase given in Table 1.1 appeared to be the most thermostable. Inhibition data of the proteolytic bands suggest a pre-dominance of serine proteinases. The one band that was insensitive to PMSF was partially inhibited by EDTA (Connaris *et al.*, 1991).

Only one extremely thermostable acid proteinase has been reported to date, thermopsin, which was isolated from *Sulfolobus acidocaldaricus* (Lin and Tang, 1990; Fusek *et al.*, 1990). The presence of a cysteine residue has been demonstrated but appears to be unnecessary for activity. The characteristic active site aspartyl sequence common to aspartic proteases is absent, it is suggested that thermopsin may have a different active site structure to the aspartic protease family. However, a similar transition state may be involved. A broad endopeptidase specificity was observed (Fusek, *et al.,* 1990) with a preference for large hydrophobic residues in both the P_1 and P_1' position of the cleavage site. The specificity of action on oxidised insulin B-chain was similar to that described for Rt41A proteinase and aqualysin above (see Fig. 1.2).

Proteolytic activity has also been observed in screening experiments (Bragger *et al.,* 1989) of various other archaebacteria. Examples from two of these (*Thermococcus celer* and *Sulfolobus solfataricus*) are shown in Table 1.1. Recent studies, carried out by Klineberg and colleagues (1991), have

characterised proteinases from 4 archaebacterial species (*T. celer, Thermococcus* strain AN 1, *T. stetteri, T. litoralis, Staphlothermus marinus*) and the extreme thermophile *Thermobacteroides proteolyticus.* All were found to be serine proteinases, the pH optimum being either neutral (*T. celer, Thermococcus* strain AN 1) or alkaline. High thermostability was observed for all of these, the half lives ranging from 0.25 to 5 hours at 100 °C. It should be noted that the experiments were carried out on impure proteins; the presence of extraneous protein may have some stabilising effect.

The properties of an aminopeptidase from *Thermus aquaticus* YT-1 have been reported by Minagawa *et al.* (1988) and Minagawa (1989). The enzyme was a dimeric metalloenzyme with a pH optimum of 8.5-9.0. The substrate specificity was broad but a preference for hydrophobic amino acids (with the exception of proline) was observed. Also included in the studies of Minagawa (1989) was the data on the carboxypeptidase from the same organism. The enzyme was monomeric with a similar pH optimum as that observed for the aminopeptidase.

A tetrameric aminopeptidase from the archaebacteria *Sulfolobus solfataricus* has also been reported (Hanner *et al.*, 1990). The enzyme is a metalloexopeptidase with a pH optimum of 6.5. Stability was increased by the presence of Co^{2+} whereas Ca^{2+}, Mg^{2+} and Zn^{2+} had little effect.

1.1.3.4 Industrial Potential of Thermostable Proteases

The industrial potential of thermostable organisms and enzymes has been extensively discussed (Doig, 1974; Daniel *et al.*, 1981, 1985/86; Sonnleitner and Fiechter, 1983; Brock, 1985; Godfrey, 1986; Weigel and Ljugdahl, 1986). The industrial potential of thermostable proteinases specifically has also been reported (Cowan *et al.*, 1985; Gusek and Kinsella, 1988).

Economically proteinases have become one of the most important groups of industrial enzymes (Ward, 1983). Examples of these include: the serine proteinase from *B. licheniformis* which is used in detergents, *Mucor* proteinase used in cheese manufacture and the fungal proteinase from

Aspergillus oryzae used in the modification of dough for bread and cracker making.

Thermostable proteases are able to withstand longer periods of exposure to high temperatures than their mesophilic counterparts. In addition there is growing evidence to show that they also have increased stability towards proteolytic attack (Daniel *et al.*, 1982), organic solvents (Owusu and Cowan, 1989), denaturing agents (Oshima, 1979) and variations in pH (Stellwagen, 1984).

When investigating the potential industrial uses of thermostable proteases, the difficulty arises in identifying processes which are currently in place, that may be more profitable if carried out at higher temperatures. Examples of these are industrial protein recovery and hydrolytic reactions (Ward, 1983; Cowan, 1983) in which advantages such as reduced mesophilic microbial contamination and viscosity and increased hydrolysis (Cowan *et al.*, 1985) would outweigh the cost of maintaining the higher reactor temperature.

The potential use of thermostable proteinases in meat tenderisation has been investigated (Wilson *et al.*, 1991). The thermostable proteinases Rt41A and E A.1 were successfully utilised to tenderise meat patties. Their use may prove advantageous in that proteolytic activity is not observed until the meat is cooked, possibly enabling pre-slaughter addition of the proteinase and better control of the tenderisation.

Other advantages of using increased temperatures might include increased solubility of substrates and evaporation of volatile products, either for recovery of products or to reduce inhibition problems. The cost of heating the reactor is likely to be lower than the cost of cooling required by the exothermic microbial growth (Cowan *et al.*, 1985).

The increased stability in the presence of organic solvents may be of use for enzymatic peptide synthesis. Enzymatic peptide synthesis has been extensively studied and often requires the use of organic solvents to reduce the hydrolytic reaction and increase the solubility of substrates.

The immobilisation of a number proteinases has been reported (see Gianfreda and Scarfi, 1991 and Table 1.3) as a means of increasing their

stability. Caldolysin is the only proteinase from an extreme thermophile that has been immobilised (Cowan and Daniel, 1982c). However, thermolysin (Nakanishi *et al.*, 1985; Okahata and Ijiro, 1988; Oyama *et al.*, 1981) and the proteinase from *Thermomonospora fusca* YX (Johnson *et al.*, 1990; Gusek and Kinsella, 1990) have been immobilised, both were isolated from thermophilic organisms. The extra stability conferred by immobilisation may improve the industrial potential of thermostable proteases.

1.2 IMMOBILISATION OF ENZYMES

1.2.1 Introduction

The immobilisation of an enzyme is defined as the confinement of an enzyme in a distinct phase, that is separated from, but is interactive with, the bulk phase (Trevan, 1980). This includes enzymes that are modified in such a way that they are either water-insoluble, restricted by membranes, or attached to macromolecules so that they remain in a soluble form.

The immobilisation of enzymes onto water-insoluble supports has been studied for over 50 years. Early work investigated the adsorption of proteins onto supports such as charcoal, kaolinite, cellulose and glass beads (Nelson and Hitchcock, 1921; Langmuir and Schaefer, 1938;1939). Developments lead to the covalent binding of proteins to supports; two of the earliest reports (Grubhofer and Schleith, 1953 and 1954) involved the immobilisation of carboxypeptidase and amylase to diazotised poly(p-aminostyrene).

The use of enzymes as industrial catalysts has increased in recent years. This is due to a number of advantages they have over chemical catalysts (Whitesides and Wong, 1985). These include high catalytic activity and substrate specificity, mild reaction conditions and minimal by-product formation (Cheetham, 1985). However, enzymes are considered to be relatively unstable and can be inactivated under some operating conditions including extremes of pH, aqueous-organic environments and temperatures at which instability of the enzyme is observed. The stabilisation of enzymes, therefore, has been extensively studied in attempts to overcome these limitations. The methods of enzyme stabilisation that have been investigated to date include the immobilisation of enzymes, chemical modification,

investigation of micro-organisms and their enzymes from extreme environments, and genetic and protein engineering techniques (for review see Gianfreda and Scarfi, 1991).

1.2.2 Methods of Immobilisation

The number of methods that are utilised to immobilise enzymes has increased dramatically. Numerous carriers, linking agents and polymers are now available. The method of choice depends on the enzyme and the operating conditions under which it will be used. An overview of the available methods will be discussed below.

1.2.2.1 Carrier Binding

The binding of enzymes to insoluble supports can be classified into four types, physical adsorption, ionic binding, chelation or metal binding and covalent binding. The selection of the binding method and insoluble support depends on the application. For example, methacrylate or silicone rubber has been shown to be the most appropriate for medical uses (Kennedy and White, 1985a) whereas inorganic supports are normally more suited to industrial purposes.

Physical Adsorption

This was one of the earliest methods used in immobilisation (Nelson and Griffin, 1916) and is also one of the easiest methods. The enzyme molecules are adsorbed onto the support by a static procedure, electrodeposition, reactor loading or by shaking during loading. The latter is most commonly used for laboratory purposes. Reactor loading is popular for commercial uses and involves the mixing of the carrier and the enzyme in the reactor; adsorption takes place as the two are mixed. Electrodeposition involves placing the carrier next to an electrode in the enzyme bath, deposition onto the carrier occurs when the enzyme migrates to the electrode when exposed to the electric current. There is little or no conformational change on adsorption to the support; the effectiveness of adsorption depends on factors

such as the pH, ionic strength, enzyme concentration, amount of carrier, the reaction time and the temperature. Two examples of enzymes immobilised in this way are trypsin onto chitin or chitosan (Goodman and Peanasky, 1982) and urease onto aluminium hydroxide (Grunwald *et al.*, 1979).

Ionic Binding

This method involves the binding of the enzyme to a support that has ion-exchange residues attached, for example, DEAE- and CM- derivatives of sepharose and cellulose. The binding is stronger than in physical adsorption but not as strong as in covalent linkage. High ionic strength and high substrate concentrations can cause the enzyme to leach from the support (Zaborsky, 1973), but conformational changes are reduced, therefore, less enzyme activity is lost due to inactivation. Organic supports are most commonly used for immobilisation by ionic binding (Scouten, 1987). Two enzymes that have been immobilised in this manner are penicillin amidase (Carleysmith *et al.,* 1980) and sucrase (Kaboli and Reilly, 1980); these were immobilised to CM- and DEAE-cellulose respectively.

Chelation or Metal Binding

In this method transition state metals are used to activate the surface of the support, allowing direct coupling of the enzyme through the formation of chelates. The supports utilised include CPG glass (Cabral *et al.*, 1981), cellulose (Woodward and Wiseman, 1978), alumina (Allen *et al.,* 1979) and stainless steel (Oguntimein and Reilly, 1980). Metals that are used include titanium(III), titanium(IV) and zirconium(IV) which form polmeric oxides and hydroxides. Papain has been immobilised to glass fibre paper (Kennedy and Pike, 1980) and titanium oxide (Kennedy *et al.*, 1980) by this method. Some authors have classified both this method and ionic binding as a variation of physical adsorption (Zaborsky, 1973; Scouten, 1987).

Covalent Binding

The binding of the enzyme to the support via covalent linkages is the most widely used immobilisation method. The enzyme is bound tightly to the support and leaching of the enzyme is not observed in most cases. The functional group(s) involved in attachment must not be important for catalytic activity. Care is required, therefore, in choosing the reagent for binding the enzyme. Alleviation of this problem is possible by immobilisation in the presence of a competitive inhibitor or substrate, the use of a reversibly covalently linked enzyme-inhibitor complex, a chemically modified enzyme which is covalently linked through newly incorporated residues or the use of a zymogen precursor (Zaborsky, 1973).

The amino acids that have functional groups reactive enough to be involved in covalent coupling are given in Table 1.2. The amino acid composition of the protein and the hydrophobicity of the amino acid (the more hydrophobic an amino acid the more likely it is to be buried within the molecule) have to be taken into account.

The majority of supports do not have reactive groups with which coupling can take place. However, most have hydroxyl, amino, amide, or carboxyl groups that can be activated. Others are pretreated, as in the silanisation of glass (Weetall, 1976), before activation. Activation reactions used to date include diazotisation, amide bond formation, alkylation and arylation, schiff base formation, Ugi reaction, thiol-disulphide interchange, and mercury-enzyme interactions. The chemistry involved in these reactions is discussed by

Table 1.2 Amino Acids with Reactive Functional Groups[a]

sulfhydryl of cysteine	phenolic of tyrosine
guanidino of arginine	imidazole of hystidine
disulfide of cysteine	indole of tryptophan
thioether of methionine	hydroxyl of serine and threonine

ε–amino group of lysine and the N-terminus group
carboxyl of aspartate and glutamate and C-terminus carboxyl group

[a] Taken from Srere and Uyeda (1976)

Kennedy and White (1985b).

For a discussion of the supports available for immobilisation the reader is directed to Kennedy and White (1985a), Zaborsky (1973) and Methods in Enzymology Vol 44 Chapters 2-10.

1.2.2.2 Entrapment

This method of immobilisation involves the confinement of an enzyme within a polymer or membrane matrix. Substrates and products are able to diffuse in and out. Entrapment includes gel or fibre entrapment and microencapsulation.

Gel Entrapment

Water-insoluble polymers are cross-linked to form gels which entrap the enzyme within interstitial spaces of the gel. Polyacrylamide was first used by Bernfeld and Wan (1964) for the immobilisation of trypsin, papain and other hydrolytic enzymes. Normally the gelling agent is polymerised in the enzyme solution, which can also contain a cross-linking agent. Some leakage of the enzyme from the gel may occur and, in some cases, the formation of free radicals may affect the enzyme activity.

Fibre Entrapment

This involves the entrapment of the enzyme within the microcavities of synthetic fibres and was first developed by Dinelli (1972) and Dinelli *et al.* (1976; 1978). The fibres produce a large surface area for enzyme binding , and are resistant to weak acids and alkalis, high ionic strength and some organic solvents. Low molecular weight substrates must be used. The most commonly used polymer is cellulose acetate.

Microencapsulation

The enzyme is enclosed within a spherical semi-permeable polymer membrane, which substrates and products diffuse across (Chang, 1964;1976). Inactivation is occasionally observed during microencapsulation, but a large surface area for contact of the substrate and enzyme within a small volume is achieved.

1.2.2.3 Cross-linking

The formation of cross-links between enzymes via bi- or multifunctional reagents results in 3-D aggregation of the enzyme. The most common reagent used is glutaraldehyde due to the mild reaction conditions required (Broun, 1976). These covalent linkages can survive extremes of pH and temperature, but activity can be lost by involvement of the active site in bond formation or the active site being in the centre of a cross-linked aggregate.

1.2.2.4 Soluble Enzymes

This method allows the use of the enzyme in its native state, but it is confined by the use of semipermeable membranes, hollow bore fibres or ultrafiltration membranes. The enzyme can also be used in a derivatised form, this can be useful when problems of enzyme containment are observed (Marshall and Rabinowitz, 1976; Vegarud and Christensen, 1975). One of the advantages of this method is that it is well suited to the use of large molecular weight or insoluble substrates. It is also very simple and there is the possibility of simultaneous immobilisation of enzymes.

Combinations of the methods described above are often used for enzyme immobilisation. For example, Kurota and colleagues (1990) have described the binding of trypsin to ion-exchange resins followed by cross-linking with glutaraldehyde. A similar method has also been utilised for the immobilisation of papain, as discussed later.

It is also possible to immobilise more than one enzyme at a time. This is described by Mosbach and Mattiason (1976) and Bouin *et al.* (1976). The product from one reaction is normally the substrate for the other, leading to substrate channelling effects.

1.2.3 Effect of Immobilisation on Enzyme Activity

The immobilisation of enzymes often has a number of effects on their catalytic activity. These effects have been reviewed by the following authors Zaborsky (1973); Goldstein (1976); Engasser and Horvath (1976); Trevan (1980). The following discussion has been based on these reviews. The effects of partitioning, diffusional limitations, conformational changes and steric restrictions are also illustrated in Fig. 1.3.

1.2.3.1 Partitioning

Partitioning of components in the bulk phase can have an effect on the microenvironment that the enzyme is exposed to. This can be caused by diffusional limitations as discussed below or by interactions between the support and the components themselves. One example of this is given by Goldman and coworkers (1968) where papain immobilised in a nitrocellulose membrane was less active than papain in dilute solution, yet was able to digest gelatin more thoroughly. It was suggested that the gelatin was adsorbed and denatured by the membrane and, hence, more susceptible to hydrolysis. Other examples are concerned with attraction or repulsion of substrates or hydrogen/hydroxyl ions due to electrostatic interactions. The resulting microenvironments have different substrate concentrations or pH values to those observed in the bulk phase. Alterations in K_m, V_{max} and pH optima are observed in some cases.

1.2.3.2 Diffusional Limitations

Both external and internal limitations are observed with immobilised enzymes. External limitations are due to the slow diffusion of the substrate across the poorly mixed layer, the "Nernst Planck" layer, that surrounds the

support particle. This results in a substrate concentration gradient between the bulk phase and the support particle. Alleviation of this problem is affected by the use of a more concentrated or less viscous substrate, or by increasing the agitation rate if possible.

Internal diffusional limitations are caused by the small size and tortuosity of the pores, which prevents the forced flow of liquid through the pores. A substrate concentration gradient then forms between the outer and inner regions of the matrix. If the substrate is utilised by enzyme immobilised in the outer regions before it can diffuse to the inner most enzyme particles, the result will be an under estimation of the enzyme activity. The problem of slow internal diffusion can be overcome by increasing the substrate concentration, using small molecular substrates, low enzyme concentrations and increasing the pore size.

The diffusional limitations mentioned above can also apply to the product formed as is illustrated in Fig. 1.3.

1.2.3.3 Conformational Changes

Chemical modifications of the enzyme during immobilisation may alter the shape and the active site of the enzyme, rendering the enzyme completely inactive. The other factor to consider here is that the enzyme must be able to change its conformation to carry out catalysis. If the enzyme is immobilised in such a way that this can not take place, changes in the K_m and V_{max} can be observed. Restriction of the enzyme may also result in a loss of its allosteric properties or could affect the transmission of conformational changes between sub-units. Immobilisation may also affect activator, metal ion (required for stability), or inhibitor binding sites.

1.2.3.4 Steric Restrictions

The position in which the enzyme is immobilised in the matrix can prevent access of the substrate to the enzyme. This can be the result of the orientation of the enzyme with respect to the matrix, which may block access to the active site. Alternatively, if the enzyme is buried within a matrix then small molecular

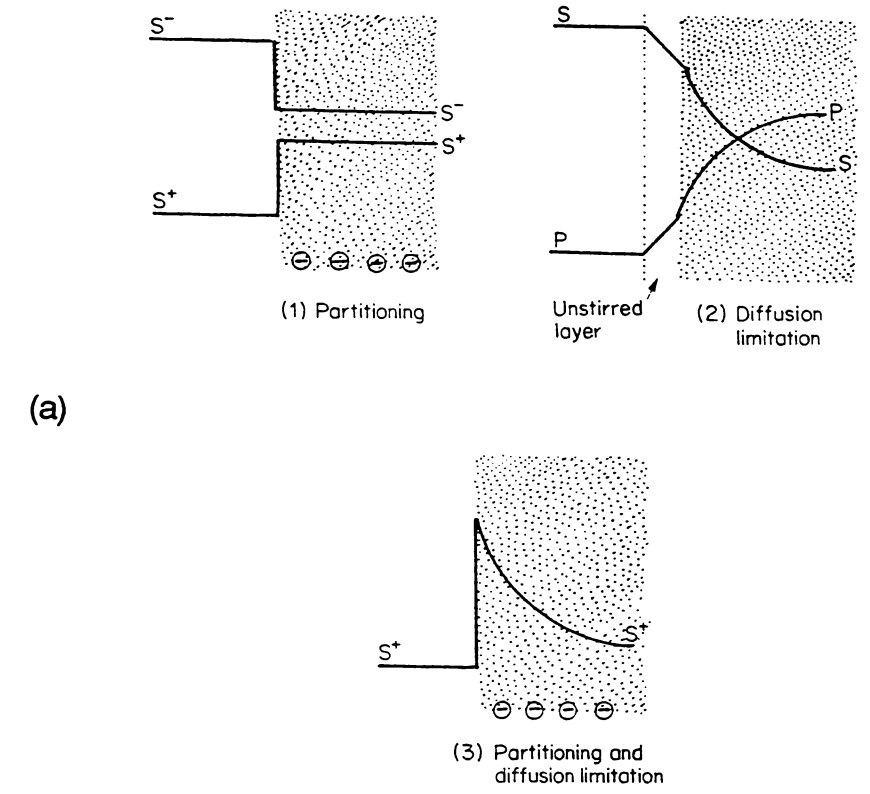


Fig. 1.3 Factors Affecting the Kinetics of Immobilised Enzymes[a]

(a) The effect of partitioning and diffusional limitations on solute concentration profiles in immobilised enzyme particles.
Shaded area represents the immobilised enzyme, S= substrate, P=product.
(b) Effects of steric restrictions of the polymer matrix and conformational changes of the enzyme during immobilisation on the activity of the immobilised enzyme.

[a] Taken from Trevan (1980)

weight substrates or effector molecules will have more chance of reaching the enzyme than large molecular weight molecules.

1.2.3.5 Stability

In many cases the immobilisation of enzymes has produced enhanced stability of the enzyme compared to its soluble counterpart (Klibanov, 1979; Monsan and Combes, 1988). In some cases this may only be an apparent stabilisation. High enzyme loadings can result in preparations that are diffusion limited. That is, the rate of substrate conversion by enzymes immobilised close to the outer regions of the support is higher than the rate of diffusion to enzyme molecules immobilised further inside pores. The enzymes in the internal regions, therefore, "see" little or no substrate in the early stage of stability studies. Once inactivation of the outer enzyme molecules occur, substrate is able to diffuse further into the matrix and is utilised by the enzyme immobilised in this region. An apparent stability of the enzyme is observed until the point at which the active enzyme concentration drops below the point at which substrate diffusion is limiting.

1.2.4 Examples of Immobilised Proteinases

Numerous mesophilic proteinases have been immobilised, examples of which are given in Table 1.3 but will not be discussed further.

Two proteinases from thermophilic organisms have been immobilised, namely, thermolysin and the proteinase from *Thermomonospora fusca* YX. Thermolysin has been immobilised by various methods including adsorption of the proteinase to a support followed by cross-linking with glutaraldehyde (Nakanishi *et al.*, 1985; 1988) and entrapment within polymer matrices (Epton *et al.*, 1976; Kumakura *et al.*, 1984). Oyama and coworkers (1987) compared physical adsorption, ionic binding and covalent binding methods for the immobilisation of thermolysin. Covalent binding produced the most stable enzyme.

The proteinase from *Thermomonospora fusca* YX has been immobilised to cyanogen bromide activated sepharose 4B (Johnson *et al.*, 1990) and CPG

beads glutaraldehyde linkages. The CPG beads were either untreated or pretreated with zirconium oxide (Gusek and Kinsella, 1990). The thermostability of the proteinase was increased on immobilisation in all cases.

Caldolysin is the only reported proteinase from an extreme thermophile that has been immobilised to date (Cowan and Daniel, 1982c); the supports utilised were cyanogen bromide activated sepharose 4B, CM-cellulose and CPG beads. The thermostability of the free proteinase was enhanced when immobilised to the first two of these supports. However, when immobilised to CPG beads a decrease in thermostability was observed. Some activity was lost on immobilisation; this may be a result of steric hindrance due to the large molecular weight substrate utilised in the proteinase assay. Activity recovered was best with the sepharose support. The pH optima of Sepharose-caldolysin and CPG-caldolysin were shifted towards the acid end of the scale, whereas that of CM-cellulose-caldolysin was unchanged. The K_m values for both CM-cellulose-caldolysin and CPG-caldolysin decreased, whereas for sepharose-caldolysin it was unchanged compared to the free enzyme.

1.2.5 Applications of Immobilised Proteinases

A comprehensive list of immobilised enzymes used industrially or at the pilot scale stage is given by Gestrelius and Mosbach (1987). Examples can also be found in Methods of Enzymology Volumes 44 and 136, and in reviews by Chibata and Tosa (1976) and Klibanov (1983). The two main applications of immobilised proteinases, to date, are the resolution of amino acids and the synthesis of peptides. The utilisation of immobilised proteinases for the synthesis of esters has also been investigated (Kise, 1990). Both subtilisin and an aminopeptidase have been utilised to resolve racemic mixtures of amino acids (Gestrelius and Mosbach, 1987).

Research into peptide synthesis utilising both free and immobilised proteinases has grown over recent years. One example of the successful industrial application of enzymatic peptide synthesis is the production of aspartame with thermolysin (Oyama *et al.*, 1981, 1987; Nakanishi *et al.*, 1985). Other immobilised proteinases utilised for peptide synthesis include α–chymotrypsin (Blanco *et al.*, 1989; Serralheiro *et al.*, 1990; Kimura *et al.*, 1990), trypsin and papain (Jakubke and Konnecke, 1987). The utilisation of

proteinases for peptide synthesis in general will be discussed in more detail in the following section.

In addition to industrial applications immobilised enzymes are also used for investigating fundamental biochemical questions. This topic has been reviewed by Martinek and Mozhaev (1985) and examples are also given in Methods in Enzymology Volumes 44 and 135. Immobilised proteinases have been shown to be more useful than their soluble counterparts for the investigation of structural changes in proteins when exposed to denaturing agents (Swaisgood and Catignani, 1987). Immobilisation has also been used to prevent protein-protein interactions such as autolysis and aggregation of proteases (e.g Tanizawa and Bender, 1974)

Table 1.3 Stabilisation of Proteolytic Enzymes

Enzyme	Method of Immob	Support	Stabilisation against
Alkaline protease	(A)	aluminium oxide silica gel	T
Carboxypeptidase-A	(CB)	SMA copolymer;	OS, C, T
	(A)	periodate oxidised cellulose	
Chymotrypsin	(CB)	sepharose CL4B;	P, T
	(E)	polyacrylamide gel;	P,T
	(E)	polymethacrylate, polyacrylamide gel;	T
	(E)	polymeric nanogranes (reverse micelles)	T
Papain	(C)	tetrakis-(P-amino phenyl) ethylene	pH, T
Pronase	(CB)	porous succinamido propyl-glass	U
Trypsin	(CB)	agarose gel;	T
	(E)	polyacrylamide gel;	T
	(E)	polymeric matrix	T, P
	(CB)	A.A.G.M.A.;	T, pH, OS
	(C)	soluble and insoluble co-polymers;	T, pH
	(CB)	aldehyde agarose gels;	T
	(CB)	polyacrylamide gels;	T
	(E)	polymethacrylate, polyacrylamide gel	T

[a] Taken from Gianfreda and Scarfi (1991)
(CB)=covalent binding,(A)=adsorption, (E)=entrapment, (C)=cross-linking
(*)C=generic chemicals, OS= operational stability, P=proteases, T=temperature, U=urea
A.A.G.M.A.= alginic acid-poly (glycidylmethacrylate) copolymer.
S.M.A.= Styrene-maleic anhydride.

1.3 PEPTIDE SYNTHESIS

1.3.1 Introduction

The synthesis of well-defined peptides by proteinases was first described by Bergmann and Fraenkel-Conrat in 1937 and 1938. However, the idea that proteinases should possess this ability dates back to 1898 when it was suggested by van't Hoff that trypsin might have the capacity to catalyse the synthesis of proteins from its own degradation products. Reports of protein synthesis, catalysed by proteases, first appeared in 1901. Sawyalow (1901) identified the precipitate formed by adding rennin to a partial hydrolysate of fibrin as the outcome of a proteo-synthetic process. The precipitate was termed plastein, the formation of which has also been observed on the addition of papain, pepsin, trypsin and chymotrypsin to partial hydrolysates of proteins. The mechanism of this process was not elucidated, however, until the 1960's (Wieland *et al.*, 1960).

From these observations it was generally accepted that protein biosynthesis was a direct reversal of hydrolysis. This was not questioned until the late 1930's when Borsook and Huffman (1938) provided thermodynamic data to suggest that the synthesis of peptide bonds was endergonic under physiological conditions. Borsook (1953) concluded that significant formation of peptide bonds could not result from the mass action reversal of hydrolysis. The theory was not completely dispelled until the existence of the genetic code was proven and the roles of m- and t-RNA in protein biosynthesis were elucidated. Interest in peptide synthesis waned at this time and was not revived until the late 1970's with the prospect of utilising proteases for the synthesis of peptides on a preparative scale (Oka and Morihara, 1977; Morihara and Oka, 1977)

1.3.2 Advantages of Enzymatic Peptide Synthesis

The growing use of proteases in peptide synthesis is part of the trend towards enzymatic organic synthesis in general (Dordick, 1989; Whitesides and Wong, 1985; Kasche, 1986).

To date most peptides are produced chemically rather than enzymatically. The automation of chemical peptide synthesis and newer developments, which reduce the amounts of toxic solvents still favour this process. Nonetheless, the chemical process still has limitations, most of which arise from individual steps which are relatively unspecific. Proteases are advantageous in this respect because of their specificity which is effective on three levels. The first is a structural specificity, which relates to the preference of the protease for a particular amino acid, or structurally related amino acid, in both the S_1 and S_1' binding site. Proteases also display regiospecificity; a protease will only catalyse the formation of bonds between α–carboxy and α–amino groups. Therefore, there is no requirement to block the side chain functions of certain amino acids, as there is in chemical synthesis. Lastly, most proteases demonstrate a strong preference for the L-enantiomer of an amino acid, and can be used to resolve heterochiral mixtures and will produce homochiral products. Recently it has been illustrated that some proteinases will incorporate D-amino acids into peptides (West and Wong, 1986; Margolin and Klibanov, 1987; Kempe *et al.*, 1989). Other advantages conferred by the use of proteases are reduced use of toxic solvents, the availability of a wide variety of proteases and the reuse of the protease if immobilised (Kasche, 1989)

1.3.3 Mechanisms of Enzymatic Peptide Synthesis

Proteases can catalyse peptide synthesis in either a thermodynamically controlled or a kinetically controlled process, as illustrated schematically in Figure 1.4.

In the thermodynamically controlled reaction the protease acts only to increase the rate at which the equilibrium position of the reaction is obtained. It can not influence the position of the equilibrium. The reactions are normally characterised as requiring high substrate concentrations, higher amounts of enzyme and longer reaction times than kinetically controlled synthesis. The maximal yield of a peptide in thermodynamically controlled synthesis is given by:

$$[\text{R-CONH-R'}]_{max} = \frac{K_{app} \cdot [\text{R-COOH}]\,[\text{R'-NH}_2]}{[H_2O]}$$

Where [R-CONH-R'] is the product concentration, [R-COOH] is the acyl component concentration and [R'-NH_2] is the nucleophile concentration. The yield, therefore can be increased either by the law of mass action or by increasing K_{app}. The law of mass action can be affected in three ways. Firstly, by precipitation of the product, due to its insolubility in the reaction media, which drives the reaction towards synthesis. High concentrations of reactants may be required for this depending on the solubility of the product (Morihara and Oka, 1981). Secondly, by partitioning of the product into a nonpolar phase that is not miscible with water. The peptide product is removed from the equilibrium, again driving the reaction towards synthesis. Lastly, increasing the concentration of one of the reactants, normally the nucleophile, may also cause a shift in the equilibrium (Shin and Takahashi, 1989). The factors affecting K_{app} will be discussed later.

Kinetically controlled synthesis requires the formation of an acyl intermediate, which is then deacylated either by water or another nucleophile. Proteases that do not form this covalent intermediate (e.g metallo- and carboxyproteases) can be utilised in this type of synthesis providing they have non-overlapping binding sites for the acyl donor and the nucleophile. For deacylation the nucleophile must first bind to the acyl-enzyme intermediate (Reichmann and Kasche, 1985). At this stage hydrolysis of the resulting complex can still occur. Evidence for this hydrolysis is indicated by a non-linear increase in the aminolysis/hydrolysis ratio with increasing nucleophile concentration. That is, if hydrolysis of the acyl-enzyme-nucleophile complex occurs, increasing the nucleophile concentration will increase both the aminolysis and hydrolysis reactions and hence, a decline in the increase in the ratio of the two is observed. Due to the requirement of an acyl-enzyme intermediate this process is restricted to serine and thiol proteinases, although porcine pancreatic lipase (Margolin and Klibanov, 1987) and esterases can also catalyse this reaction. For significant yields in kinetically controlled reactions it is necessary for the protease to have a higher transferase activity compared to hydrolase activity, otherwise once formed the peptide will undergo hydrolysis. There are two types of kinetically controlled peptide synthesis (see Fig. 1.5), case (i) is observed when there is significant hydrolysis of the peptide product and case (ii) when secondary hydrolysis is negligible. This factor has to be taken into account when determining maximum yields.

$$\text{R-COOH} + \text{R'-NH}_2 \overset{K_{app}}{\rightleftharpoons} \text{R-CONH-R'} + \text{H}_2\text{O} \qquad (1)$$

$$\text{R-COOX} + \text{R'-NH}_2 + \text{H}_2\text{O} \overset{\text{transferase}}{\rightleftharpoons} \text{R-CONH-R'} + \text{XOH} + \text{H}_2\text{O}$$

hydrolase ↗↙ hydrolase (2)

$$\text{R-COOH} + \text{XOH} + \text{R'-NH}_2$$

Fig. 1.4 Scheme for Enzymatic Peptide Synthesis

(1) Thermodynamically controlled synthesis

(2) Kinetically controlled synthesis

Where R-COOH = carboxy component, R-COOX = activated carboxy component, R'-NH_2 = nucleophile, R-CONH-R'= peptide product.

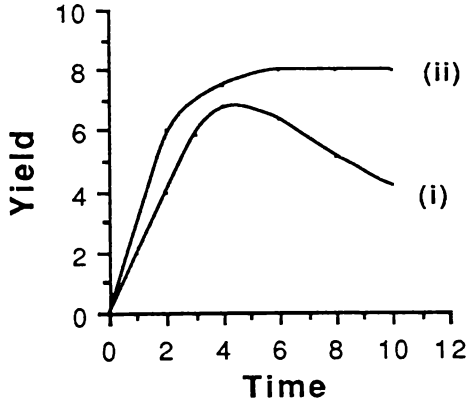


Fig. 1.5 Effect of Time on Kinetically Controlled Peptide Synthesis

(i) rate of synthesis is equal to hydrolysis
(ii) rate of synthesis >> rate of hydrolysis

1.3.4 Yield Determining Factors

1.3.4.1 pH-optimum

Thermodynamically controlled synthesis

The ionic forms of the acyl component and nucleophile are unreactive; the degree of protonation depends on the pH of the systems and the pK values of the substrates. The coupling of two unprotected amino acids is a highly endergonic process. This is due to the strong acidity and basicity of the α–carboxy and α-amino groups of the reactants. Blocking the non-reactive end of both components shifts the pK values, such that the pK of the acyl component increases and that of the nucleophile decreases, making peptide synthesis more favourable (Jakubke *et al.*, 1985). The pH optimum for the reaction is usually between 6-7, the mid point of the pK values of the reactants. The blocking groups also ensure that only one defined peptide is produced. The particular blocking group utilised, especially in the case of the nucleophile, can also influence the reaction yield (e.g see Morihara and Oka, 1981; Kullman, 1980; Aso, 1989).

Kinetically controlled synthesis

Deacylation of the acyl-enzyme complex is the rate determining step in kinetically controlled synthesis (Jakubke and Konnecke, 1987), hence the yield of the reaction will depend on the pK_b of the nucleophile (Kasche, 1986). The pH optimum for this process is, therefore, more alkaline than for thermodynamically controlled reactions as the pK_b of the nucleophile is usually ≥ 8.0. It should be noted that Tris buffer also has the ability to act as a nucleophile and therefore its use as a buffer should be avoided. The requirement of blocking the non-reactive groups also holds for kinetically controlled reactions.

1.3.4.2 Temperature

Thermodynamically controlled synthesis

As the formation of a peptide bond from uncharged precursors is endothermic, (Jakubke, 1987) the K_{app} should increase with temperature. Machini de Miranda and co-workers (1986) illustrated that this was the case after a 1 h reaction but if the reaction was continued for 3 h the temperature did not affect the yield. Stability of the proteinase (thermolysin) at the higher temperatures would have been a problem in the longer incubation. Generally the effect of temperature should be studied empirically in each case, although very few studies investigate this parameter.

Kinetically controlled synthesis

From the effect of temperature on the kinetic constants in the maximal yield equation and free energy diagrams, Kasche (1986) predicted that maximum yields should generally decrease with increasing temperature. This prediction was supported with experimental data. Other studies to date (Nilsson and

Table 1.4 Potential Advantages of Employing Enzymes in Organic as Opposed to Aqueous Media[a]

1) Increased solubility of nonpolar substrates.
2) Shifting of thermodynamic equilibria to favour synthesis not hydrolysis
3) Reduction in water-dependent side reactions.
4) Immobilisation is often unnecessary; most enzymes are insoluble in organic solvents. Recovery is possible by filtration.
5) If immobilisation is desired, adsorption is satisfactory and enzyme s are unable to desorb from these surfaces in most non-aqueous media.
6) Ease of product recovery from low boiling point solvents.
7) Enhanced thermal stability of some enzymes; water is required to inactivate at high temperatures.
8) Elimination of microbial contamination.
9) Potential of enzymes to be used directly within a chemical process.

[a] Taken from Dordick (1989)

Mosbach, 1984; Reichmann and Kasche, 1985; West and Wong, 1986; this study) also support this. One case, however has reported an increase in the transferase to hydrolase ratio with increasing temperature (Jonczyk and Gattner, 1981).

1.3.4.3 Solvent Composition

Enzyme catalysis in organic media has become increasingly popular (Dordick, 1989, Whitesides and Wong, 1985). This can be ascribed to the numerous advantages to be gained by catalysis in this medium compared to aqueous solutions as is illustrated in Table 1.4 (some of these advantages may not apply for a given solvent system). There are many different systems that have been utilised, these range from near anhydrous organic solvents (Zaks and Klibanov, 1984;1985; Yamane *et al.*, 1988) to biphasic systems including water-immiscible solvents (Halling, 1984;1987; Brink and Tramper, 1985) and the formation of reverse micelles (Martinek *et al.*, 1981; Castro and Cabral, 1989), to water-miscible solvent systems (Kise and Shirato, 1988). Both water- miscible and -immiscible solvents have the advantage of increasing the substrate solubility. The use of water-immiscible solvents can prevent the solvent from directly affecting the stability and reactivity of the enzyme. However, denaturation of enzymes can occur at the solvent/aqueous interface. Another problem can arise if monophasic systems of water-immiscible solvents are utilised in combination with more polar substrates. Partitioning of the substrates and/or product into the micro-aqueous phase can result in high concentrations of both surrounding the enzyme, and may cause inhibition of the enzyme. It should be noted that a certain amount of water is required for activity of the enzyme (Zaks and Klibanov, 1988).

The effect of an organic solvent on synthesis is finely balanced with the decrease in enzyme stability and activity that is often observed as a consequence of its addition. This is because water-miscible solvents tend to disrupt the hydration shell surrounding the enzyme causing its denaturation (Khmelnitsky *et al.*, 1991; Laane *et al.*, 1987). However, with water-miscible cosolvent systems no separate aqueous phase is present and therefore the concentration of substrates and products around the enzyme can be easily controlled (Cassells and Halling, 1989). Another advantage of monophasic

systems is that diffusional resistance across the water/organic interface is eliminated. This reduces the complexity of kinetic analysis and the determination of substrate specificities and in some cases may increase the overall reaction rate. Due to the expansive nature of the topic, further discussion of organic solvent systems will be limited to the effects on peptide synthesis.

Thermodynamically controlled synthesis

The presence of a water-miscible solvent increases the pK_a of the acyl component, while the amino component is virtually unaffected, and peptide synthesis is enhanced. As mentioned earlier, the effect of the solvent on enzyme activity must be taken into account as well. The use of a biphasic system affects the pK of both substrates in such a way as to increase synthesis (Homandberg *et al.*, 1978; Martinek and Semenov, 1981). However, the extent of the effect depends on the volume ratio of the organic to aqueous phase and the partition coefficient of the substrates.

Kinetically controlled synthesis

To date no systematic studies have been presented on the effects of different organic solvents on the kinetic constants and hence maximal yields of these reactions. The use of organic solvents in these cases are generally aimed at lowering the water activity of the system in order to reduce hydrolytic reactions. It has been illustrated in numerous cases that although the activity of the protease is decreased, the reduced water activity increases yields (e.g see Reslow *et al.*, 1988; Kise and Shirato, 1988; Kuhl *et al.*, 1980). This has been observed in both water-miscible and immiscible solvents.

1.3.4.4. Ionic Strength

Thermodynamically controlled synthesis

Increasing the ionic strength of a system stabilises isolated ions (Kasche, 1989). The pK value of the carboxyl groups decrease while the amino groups

are hardly affected by increasing ionic strength. The use of buffers in condensation reactions therefore should be minimised.

Kinetically controlled synthesis

If the acyl-enzyme complex is charged, two ion-ion interactions are involved. Reactions involving like charges should show an increase in yield with increasing ionic strength. When opposite charges are involved a decrease in yield is expected, as was observed by Kasche (1986) in the synthesis of ß-lactam antibiotics. The use of ions other than those used for peptide synthesis in the latter situation should therefore be avoided.

1.3.4.5 Substrate Specificity

This has been shown to have a major influence on the yields in peptide synthesis. The blocking groups of both substrates are important. This is due to the specificity of S_2 and S_2' subsites of the protease. In the case of the acyl component this can also be due to the solubility of the substrate (Florsheimer *et al.*, 1990) and hence depends on the reaction media. Machini de Miranda and coworkers (1986) showed that the Cbz- blocking group gave better yields than the Boc- group; this was ascribed to the thermostability of the substrates. Another example is in papain-catalysed synthesis where replacement in the acyl component, of the Bz- or Cbz- group with an Ac- group renders the amide bond formed resistant to hydrolytic cleavage (Fruton, 1982).

The nucleophile blocking group generally has more influence on the reaction than the blocking group of the acyl component. One example of the effect of the nucleophile blocking group is in the synthesis of Cbz-Gly-Phe-Leu-X by pepsin where the yield was found to depend on X, the order of reactivity was given as -NHPh> -OBu> $-NH_2$, -OEt (Morihara and Oka, 1981). In addition, the synthesis of Ac-Phe-Lys-X by chymotrypsin was increased with Lys-OBu as the nucleophile compared to Lys-OEt and Lys-OBz (Aso, 1989).

In some cases the activating group of the acyl component is able to influence peptide synthesis. Kuhl *et al.* (1986) showed that by using carboxyamidomethyl (CAM) esters instead of -OMe and -OEt, shorter reaction

times were observed. In addition, the CAM esters were more soluble in aqueous media and therefore, less solvent was required. Schellenberger *et al.* (1991d) also showed that by using an inverse substrate (when a cationic centre is included in the leaving group rather than the acyl moiety), trypsin was able to synthesise bonds that it could not hydrolyse.

When choosing substrates for peptide synthesis, a knowledge of the specificity of the protease is essential. The same structural features that determine the rate of hydrolysis of a peptide bond also influences the synthesis of the same bond (Fruton, 1982). Both primary (P_1, P_1' positions) and secondary (P_2, P_2' positions) specificity can be involved depending on the protease. The secondary specificity of some proteinases has already been discussed above in relation to the blocking groups. A large amount of data has been amassed on the P_1 and P_1' specificity of a number of proteinases, for more information the reader is directed to reviews by Fruton (1982), Kullman (1987) and Kasche, (1989). A few selected examples, with respect to synthesis will be discussed below.

Chymotrypsin, one of the more studied proteinases in the area of peptide synthesis, has a specificity for bulky hydrophobic amino acids (Trp, Tyr, Phe and Leu) in the P_1' position (Fruton, 1982; Fersht *et al.*, 1973). Activity was observed against Ala-NH_2, Tyr-NH_2, Trp-NH_2, Gly-NH_2 and Lys-OMe as nucleophiles (Fersht *et al.*, 1973). Oka and Morihara (1978) have shown in the chymotrypsin-catalysed synthesis of Cbz-Phe-X-NH_2, that Val-NH_2, Ileu-NH_2, Gly-Leu-NH_2 and Leu-NH_2 had similar reactivities but poorer yields were observed for Ala-NH_2 and Gly-NH_2.

Trypsin has a high specificity for Arg and Lys in the P_1 position (Fruton, 1982) with a preference for hydrophobic or bulky amino acids in the P_1' position. In particular, Oka and Morihara (1977) showed that Phe- and Leu-NH_2 were more effective nucleophiles than Val- and Ala-NH_2 and poor yields were observed with Gly- and Glu-NH_2.

As a last example thermolysin exhibits a broad specificity in the catalysis of peptide bonds. Isowa *et al.* (1977) and Isowa and Ichikawa (1979) showed that Cbz- derivatives of all hydrophobic amino acids (or others whose hydrophilic groups were blocked) are suitable for synthesis, except those of

Val, Ileu, Pro, Tyr and Trp. The best amino acid esters or amides in the condensation reaction were Ileu, Phe, Val, Leu and Met.

The length of the peptide chain in the acyl component is often important as well; this was observed for subtilisin by Morihara and Oka (1981). No synthesis was observed for the condensation of Cbz-Phe-OH and Leu-NH_2, whereas with Leu-NHPh an 89% yield was attained. When Cbz-Gly-Pro-Leu was substituted as the acyl donor the yields were 84% and 100% respectively with the same nucleophiles.

A number of factors, therefore, must be taken into account when investigating the potential of a protease for synthesising peptides. If one is interested in synthesising peptides enzymatically, careful consideration of all of these factors is imperative. Even if in the first instance yields are low, there are a number of options for improving productivity.

1.3.5 Enzymatic Synthesis of Biologically Active Peptides.

Although much of the work on peptide synthesis has been concerned with the synthesis of model dipeptides, there are a number of examples where biologically active peptides have been produced. Examples of these are given below.

1.3.5.1 Semisynthesis of Human Insulin

Insulin consists of two peptide chains (A-and B-chain). The primary structures of human and porcine insulin vary only in the C-terminal amino acid of the B-chain, an alanine in porcine insulin is replaced with a threonine in human insulin. The semisynthesis of human insulin is therefore a realistic approach and as first described by Inouye *et al.* (1979). The conversion of porcine insulin into human insulin was achieved via trypsin-catalysed fragment condensation. A desoctapeptide-(B23-B30)-insulin (acyl component), derived by tryptic hydrolysis of porcine insulin, was condensed with a synthetic octapeptide (nucleophile component), which corresponds to the sequence B23-B30 of human insulin. The equilibrium of the reaction is controlled by a 10-fold excess of the nucleophile. Enzymatic synthesis is advantaged over chemical synthesis in this case as the blocking and deblocking of side chain

functions is avoided; the reaction is highly specific and better yields are obtained.

Morihara *et al.* (1979) have also carried out the semisynthesis of insulin by selective removal of the Ala_{B30} residue from porcine insulin. A 50-fold excess of the nucleophile (Thr-OBu) was added in the presence of trypsin and human insulin was obtained with a 73% yield.

1.3.5.2 Aspartame

The precursor (Cbz-Asp-Phe-OMe) of Aspartame (Asp-Phe-OMe) has been synthesised by utilising thermolysin to couple Cbz-Asp with Phe-OMe (Isowa *et al.*, 1979). Both the D- and L- enantiomers of the nucleophile are present in the reaction. Only the L-enantiomer is incorporated into the peptide product. The D-enantiomer forms an insoluble salt with the synthesised peptide (Cbz-Asp-Phe-OMe.Phe-OMe) which drives the reaction in the synthetic direction. The elimination of the blocking of the side chain -COOH of the Asp residue favours enzymatic synthesis as opposed to chemical synthesis and being able to use a racemic mixture of the nucleophile reduces the expense of the substrate.

1.3.5.3 Synthesis of [Leu] and [Met] Enkephalin

Papain- and chymotrypsin-catalysed synthesis of both [Leu] and [Met] enkephalin, Tyr-Gly-Gly-Phe-Leu(Met), has been demonstrated by Kullman (1979; 1980; 1984). Amino acid phenyl hydrazides were used as nucleophiles and Boc- amino acids and peptides, or ethyl esters of these, were utilised as acyl components. The peptide-bond-forming steps are controlled by the insolubility of the products enhancing the yields and simplifying purification procedures. The use of a monophasic aqueous-organic systems for all but one of the steps also helps to shift the equilibrium toward synthesis and enhances the solubility of the reactants. Carboxypeptidase Y has also been shown to catalyse the synthesis of [Leu] enkephalin (Widmer *et al.*, 1981). The exopeptidase is utilised in both condensation reactions and desamidation reactions, which are carried out in aqueous solutions. Before elongation of the peptide carboxypeptidase Y

removes the C-terminal -NH_2 group and it is replaced with an -OEt group, condensation with next nucleophile takes place. Advantage is taken of the fact that carboxypeptidase Y will only act at the C-terminal of the peptide and therefore will not cleave internal bonds of the growing peptide. However, transpeptidation reactions are a risk at each elongation step.

1.4 Objectives

The first aim of this project was to investigate the immobilisation of the proteinase from *Thermus* strain Rt41A (henceforth called Rt41A proteinase), a thermophilic bacteria isolated from the Rotorua thermal region, in New Zealand (Lim, 1983). The immobilisation of enzymes is widely used in attempts to increase their thermostability and stability towards other denaturing agents such as organic solvents (as described in section 1.2). Because of their inherent stability thermostable enzymes have potential application in industry (Daniel *et al.*, 1986). Increasing the stability further by immobilisation may increase this potential.

The second aim was to investigate the potential use of Rt41A proteinase in enzymatic peptide synthesis. The research in this area is expanding rapidly, but the use of proteinases from extreme thermophiles has not been reported to date. The resistance of thermostable enzymes to denaturation by organic solvents (Owusu and Cowan, 1989), which are often used in peptide synthesis, suggests that they may have an advantage over the mesophilic proteinases currently used. The immobilisation of the proteinase may also prove useful in peptide synthesis as it may add to the inherent stability of the enzyme.

CHAPTER 2

PURIFICATION AND IMMOBILISATION OF *THERMUS* STRAIN Rt41A PROTEINASE.

2.1 METHODS

2.1.1 Production and Purification of Rt41A Proteinase

Rt41A proteinase was produced in two batches. The first was utilised for preliminary experiments (sections 2.1.4 and 2.1.5) and to investigate the effect of pore size of controlled pore glass beads on the percent recovery of the proteinase after immobilisation (section 3.2.1). The second batch of proteinase was used for all other experiments described in Chapters 3 and 4.

The pH adjustment of all buffers was carried out at the temperature at which they were to be used.

2.1.1.1 Batch 1

Thermus strain Rt41A was grown in a 600 l fermenter, in a medium described by Janssen *et al.* (1991). The cells were removed from the media by continuous centrifugation using a Sharples centrifuge (model 6, 12 600 g) The proteinase was precipitated from the cell-free supernatant with ammonium sulphate (60% saturation, room temperature). The precipitate was redissolved with 20 l of 10 mM Hepes, pH 7.5 (buffer A), containing 1 mM $CaCl_2$ followed by diafiltration and concentration using a Ym10 spiral wound membrane (Amicon). The concentrated proteinase was bound to a Fast Flow S Sepharose column (Pharmacia, 300 ml bed volume, 5 cm x 15 cm, 20 ml/min) equilibrated with buffer A and the protein eluted stepwise first with buffer A + 100 mM NaCl followed by buffer A + 500 mM NaCl. The proteinase containing fraction (500 mM NaCl) was then desalted with buffer A + 5 mM $CaCl_2$ and applied to a Mono S 10/10 FPLC column (Pharmacia) which had been equilibrated with 10 mM Tes, pH 7.5 (buffer B), containing 5 mM $CaCl_2$. The bound enzyme was eluted with a 90 ml linear gradient (0-500 mM NaCl in buffer B), at a flow rate of 3 ml/min. The active fractions were pooled and loaded directly onto a Preparative TSK-Gel filtration column (GW3000SW, Toyo Soda Co., Japan), which had been equilibrated in buffer B. The fractions

containing the proteinase were pooled and dispensed into aliquots which were fast frozen in liquid N_2 prior to storage at -70 °C.

2.1.1.2 Batch 2

Production of the Rt41A proteinase was carried out as described above, except the culture was grown in 3 x 10 l amounts in a CF2000 fermentation plant with a type 1 fermenter vessel (Chemap, Mannedorf, Switzerland) as described by Jannsen *et al* (1991). The cells were removed from the whole culture by continuous centrifugation as above. The remaining cell debris was then removed with a Hollow fibre PM30 ultrafiltration cartridge (Amicon, H10P30-20). The resulting cell free supernatant was then concentrated and desalted with a Ym10 spiral wound membrane. The proteinase was then loaded onto the Fast Flow S Sepharose column, followed by the Mono S FPLC column as described above. The active fractions from this last step were pooled and desalted (Ym10 membrane, Amicon) and reapplied to the Mono S column with the salt gradient extended from 90 to 135 ml. The active fractions were pooled, desalted in buffer B + 0.01% Triton X-100 and stored at -70 °C, as described for Batch 1.

2.1.2 Protein Determination

The protein content of the preparation at each stage of both the purification procedures was determined by the method of Lowry *et al.* (1951). Samples containing Hepes buffer were dialysed against deionised water as this buffer interferes with the protein assay.

2.1.3 Electrophoresis

SDS-PAGE (Pharmacia Phast System) was utilised to determine the molecular weight of Rt41A proteinase. The samples and standard marker proteins (low molecular weight marker kit, Pharmacia) were run on 10-15% T Phast gels. The samples were prepared for electrophoresis as described by Pharmacia (separation technique file No.110), except the proteinase was inhibited with PMSF prior to this treatment. After electrophoresis the gels were stained with silver stain (Pharmacia development file No. 210)

2.1.4 Isoelectric Point Determination

The isoelectric point was determined by focusing on a 1% agarose gel (Pharmacia) containing 6.3% Pharmalyte 8.0-10.5 (Pharmacia) and 12% w/v sorbitol. The gel was focused at 600 Volts for 3 h at 10 °C on a Pharmacia flat bead electrophoresis unit. After focusing the gel was fixed with 10% w/v TCA/5% w/v sulphosalicyclic acid and stained with Coomasie Blue as described in *Isoelectric focusing: principles and methods* (Pharmacia).

2.1.5 Quantitative Determination of Proteolytic Activity

Proteolytic activity was determined by the method of Charney and Tomarelli (1947) using azocasein as a substrate. The substrate solution was prepared using 50 mM Hepes, pH 8, + 5 mM $CaCl_2$ and 0.2% w/v azocasein. Assays were started by the addition of 10 µl of sample to 1 ml of substrate at 75 °C and stopped after 10 min (unless other wise stated) by the addition of 0.5 ml of 15% w/v TCA. The tubes were then cooled either at room temperature for 30 min or at 4 °C for 10 min, to promote flocculation. This was followed by centrifugation at 13 000 g (Runne-Heidelberg model 85-1) for 5 min. The absorbance of the supernatant was determined at 420 nm. One unit (Au) of activity is defined as equivalent to a change in absorbance of 1.0/h at 420 nm.

2.1.6 Quantitative Determination of Endopeptidase Activity

Endopeptidase activity was determined against other synthetic p-nitroanilide substrates (Sigma and Bachem) and assayed according to the procedure of Del Mar *et al.* (1979). Stock solutions of the peptides were made in methoxyethanol, the final concentration in the assay was 10% v/v; this did not affect the activity of the enzyme. Assays were initiated by the addition of 10 µl of enzyme to 500 µl of preincubated 0.25 mM substrate in 50 mM Hepes, pH 8.0, + 5 mM $CaCl_2$. The reaction was carried out at 75 °C. The reaction was stopped after 5 or 2 min. by the addition of 100 µl of glacial acetic acid. The absorbance was determined at 410 nm. One unit (U) of activity is equivalent to 1 µmole of pNA released/min. The extinction coefficient of pNA is 8880 $M^{-1}cm^{-1}$ at 20 °C.

Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SucAAPFpNA) was chosen for use in routine assays. The assay procedure was as above except the stock solution were prepared in 10% acetonitrile and the final substrate concentration was 2 mM. The reaction temperature was also reduced to 40 °C.

The proteolytic and endopeptidase activity of the immobilised proteinase was determined in an identical manner to the free proteinase regardless of substrate, except that tubes were shaken in a Griffin flask shaker (5 cycles/sec) during the assay.

2.1.7 Immobilisation of Rt41A Proteinase to Polymer Carrier VA-Epoxy Biosynth

The immobilisation of Rt41A proteinase to VA-Epoxy Biosynth (Riedel-de Haen AG, Germany) was based on the method of Burg *et al.* (1988). 800-2400 Au of enzyme, in 1.2 ml of 1 M KH_2PO_4 buffer, pH 7.5, was combined with 200 mg of carrier. The mixture is then shaken reciprocally (60 cycles/min) for 3 days at room temperature. The reaction buffer was then removed by filtering through sintered glass and the resin washed with 3 x 5 ml volumes of 0.1 M KH_2PO_4 buffer, pH 7.0. After resuspending in 50 mM KH_2PO_4 buffer, pH 7.0, containing 0.02% w/v sodium azide, the immobilised enzyme was stored at 4 °C.

2.1.8 Immobilisation of Rt41A Proteinase to Controlled Pore Glass Beads

Rt41A proteinase was immobilised to controlled pore glass (CPG) beads via covalent coupling. The method employed was based on that used by Weetall (1976). At each stage of the immobilisation process the beads were degased by sonication for 5 min. 1 g of acid washed CPG beads were silanized in 30 ml of 2% v/v q-aminopropyl triethoxysilane (adjusted to pH 3-4 with 6N HCl). After 2 hours at 75 °C, the beads were washed with 100 ml of de-ionised water and dried overnight at 85 °C. 32 ml of glutaraldehyde, in 2.5% v/v in 50 mM Hepes, pH 7.5, was then linked to 1 g of the beads at room temperature for 60 min, the residual glutaraldehyde was removed by exhaustive washing with de-ionised water. 2 ml of the enzyme solution (54 Au/m^2 of beads, unless otherwise stated) was then added to 1 g of the beads

and left to react for 4 hours at 20°C in a reciprocal shaker (60 cycles/min). Finally the preparation was washed successively with 50 ml/g of beads with de-ionised water, 1 M NaCl and finally de-ionised water. After resuspending the beads in 50% v/v glycerol/100 mM Hepes, pH 7.5, + 10 mM $CaCl_2$ the immobilised proteinase was stored at 4°C. After immobilisation, leaching of the enzyme from the glass beads and Polymer VA-Epoxy Biosynth resin was investigated, under assay conditions. A sample of azocasein was removed at the end of the assay, before TCA addition, and was reassayed for azocasein activity. As no activity was found in the assay supernatant it was concluded that the enzyme did not leach from the matrix under these conditions.

2.2 RESULTS and DISCUSSION

2.2.1 Purification of Rt41A Proteinase

2.2.1.1 Batch 1

Table 2.1 illustrates the change in specific activity, and hence the increase in purity of the proteinase during the purification procedure detailed in section 2.1.1.1. The $(NH_4)_2SO_4$ precipitation step was unsuccessful in that very little increase in purity was achieved and the losses in activity were high. Similar results have been observed with this step in previous purifications of this proteinase and also with the production of caldolase (Saravani *et al.*, 1989). This is due to the low grade $(NH_4)_2SO_4$ that is used in large scale production. Collingwood *et al.* (1989) have shown that Rt41A proteinase can be flocculated with Fe(III) and it is likely that the proteinase adsorps to iron colloids in the $(NH_4)_2SO_4$ which hinders its extraction from the pellet (Don Cowan, unpublished results). The Fast Flow S sepharose column proved very successful in that the purification was increased approximately 40 fold with approximately 70% recovery of the enzyme. The Mono S column also gave a reasonable increase in the specific activity, with good enzyme recovery. The gel-filtration step was another unsuccessful step in that 50% of the proteinase activity was lost with little gain in purity. The resolution on the column was poor; it was later discovered that the column had deteriorated quite significantly which is likely to have caused this poor resolution (see Fig. 2.3). The fractions collected were chosen so as to avoid possible contamination from the neighbouring protein peak. The elution profiles for the

columns utilised in this procedure are illustrated in Figs. 2.1, 2.2 and 2.3. The final specific activity was 9150 Au/mg of protein with a 60 fold purification.

Table 2.1 Purification of *Thermus* strain Rt41A Proteinase from 600 l Fermenter

Purifcation Step	Total Protein (mg)	Total Activity (Au x10^4)	Specific Activity (Au/mg)	Purification Factor	% Recovery
Cell Free Supernatant	9050	144	151		100
$(NH_4)_2SO_4$ Pellet	4647	99	202	1.4	68
Extraction from $(NH_4)_2SO_4$ pellet	2657	67	247	1.6	47
Diafiltrate	1356	34	252	1.7	24
Fast Flo S Sepharose	35.5	23	6558	43.4	16
Mono S FPLC	19	17	8750	57.8	12
Preparative TSK-Gel	9.7	8.8	9150	60	6

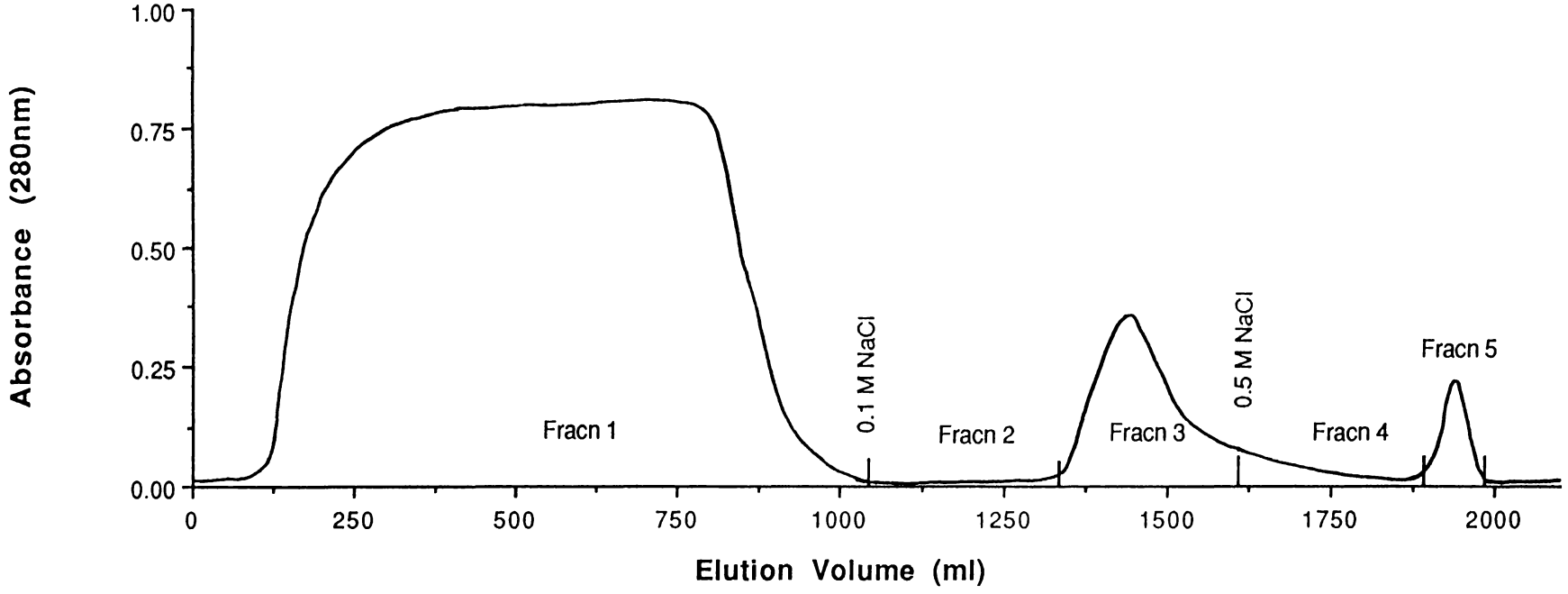


Fig 2.1 Stepwise Elution of *Thermus* strain Rt41A Proteinase from a Fast Flow S Sepharose Column

Fraction 1 contains 9456 Au; Fraction 2, no Au; Fraction 3 6972 Au; Fraction 4 3075 Au; Fraction 5 84231 Au.

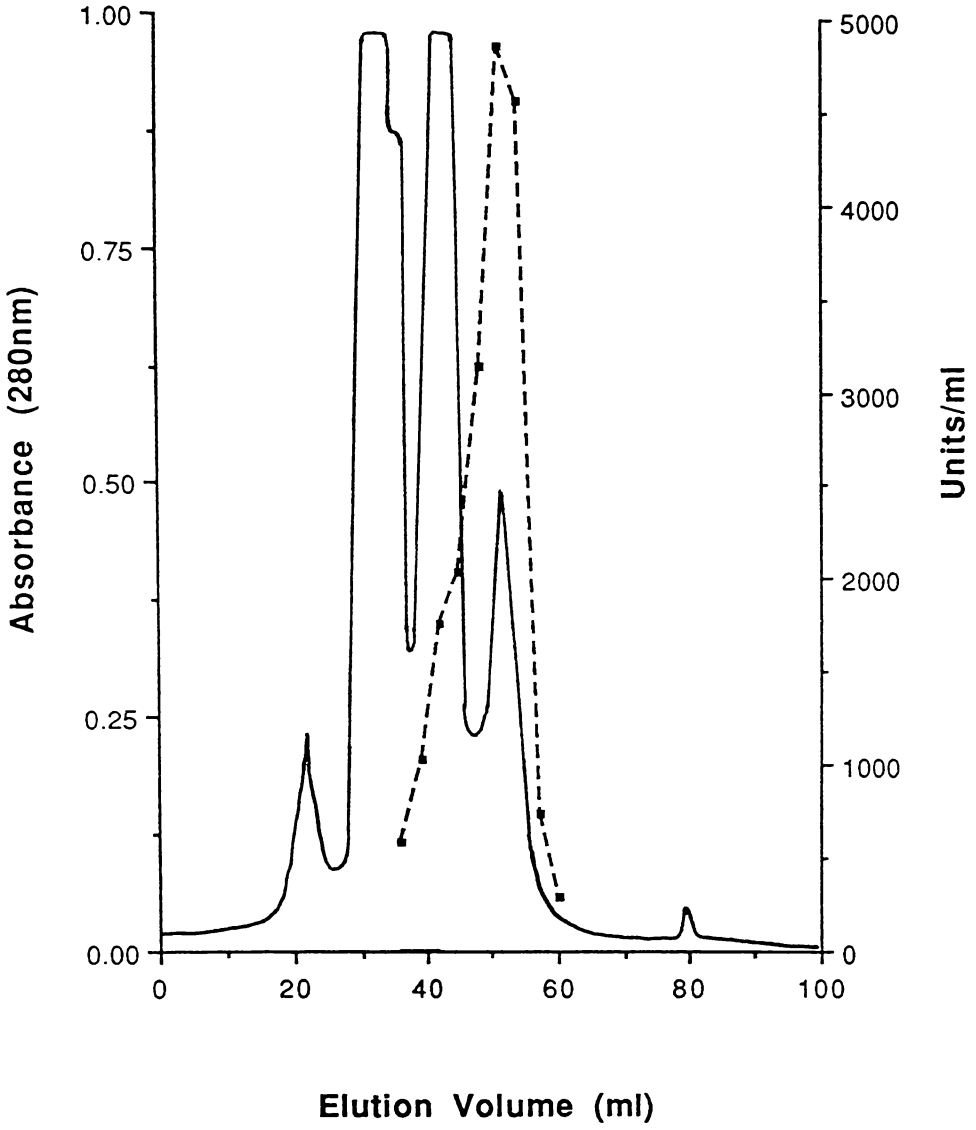


Fig 2.2 Elution Profile of *Thermus* Strain Rt41A Proteinase from a Mono S 10/10 FPLC Column (1).

Elution gradient was 0-0.5 mM NaCl over 90 ml (3 ml/min)
The solid line represents the change in absorbance and the dashed line represents the proteolytic activity detected.

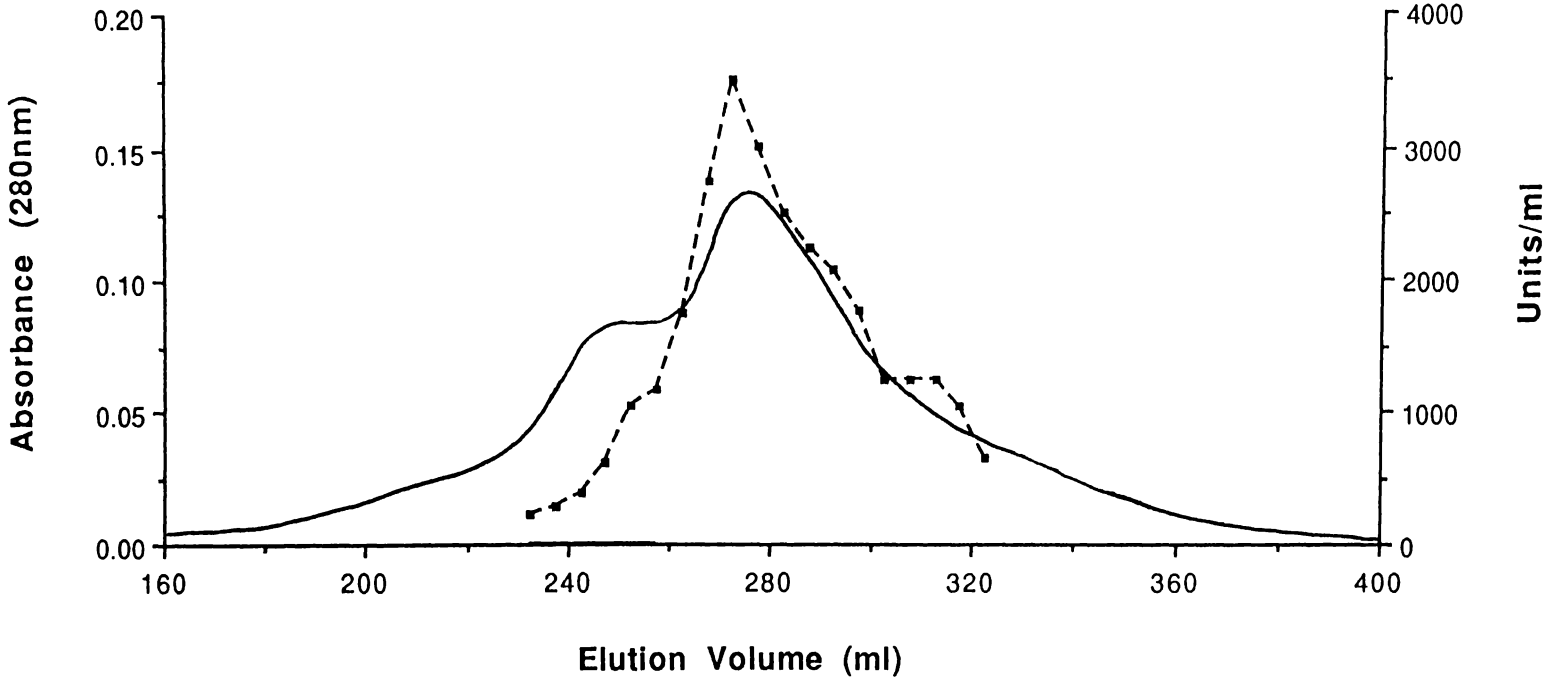


Fig 2.3 Elution Profile of *Thermus* Strain Rt41A Proteinase from a TSK-Gel Filtration Column.

The solid line represents the change in absorbance and the broken line represents the proteolytic activity detected.

2.2.1.2 Batch 2

The second purification scheme for Rt41A proteinase (section 2.1.1.2) yielded an enzyme preparation of similar purity to that above (see Table 2.2). Due to the lower total protein content of the cell free supernatant and the problems experienced previously, the $(NH_4)_2SO_4$ precipitation step was avoided. Large activity losses were observed while desalting the enzyme preparation prior to application to the Fast Flow S Sepharose column. The diafiltration buffer, in this case, did not contain $CaCl_2$, as precipitation of calcium salts was a potential problem due to the presence of phosphate in the culture medium. The proteinase, therefore, may have lost activity due to autolysis, although the concentrate was maintained at 4 °C to reduce this possibility. More likely, the proteinase may have been denatured due to foaming caused by the presence of Triton X-100, the forces acting under these conditions being intensified by the absence of $CaCl_2$. The Fast Flow S Sepharose step was not as successful as in the preceding purification scheme. As more activity was found in the unbound protein fraction and there was some activity associated with the protein eluted in the first wash, this is likely to be caused by overloading of the column. The recovery of activity and separation of proteins achieved by the Mono S column in the first instance was much the same as that found above. When the proteinase was applied to this column for a second time the separation of peaks was not as expected. It has been found by Peek (unpublished results) that the peaks were better separated with the extended gradient, however, this was not the case here. It is possible that the protein concentration applied in this case was too high to allow good separation of the peaks. However, the separation was better than that observed in the first application of the proteinase to the Mono S column. The proteinase fractions kept for further use were chosen to eliminate the contamination of other proteins. The elution profiles of the columns utilised in this purification scheme are illustrated in Figs. 2.1- 2.4. The final specific activity of the preparation was 8935 Au/mg of protein, the purity of this preparation is very similar to that found for Batch 1 in terms of specific activity.

Table 2.2 Purification of *Thermus* strain Rt41A Proteinase from Chemap

Purification Step	Total Protein (mg)	Total Activity (Au x10^4)	Specific Activity (Au/mg)	Purification Factor	% Recovery
Cell Free Supernatant	2635	83	315		100
PM30	2190	78	356	1.13	94
Diafiltrate	1805	31	390	1.24	38
Fast Flow S Sepharose	42	17	4035	12.8	21
Mono S FPLC[a]	5.3	4.7	8935	28.4	6

[a] Values quoted are for the enzyme preparation after it had been applied to the mono S column for the second time

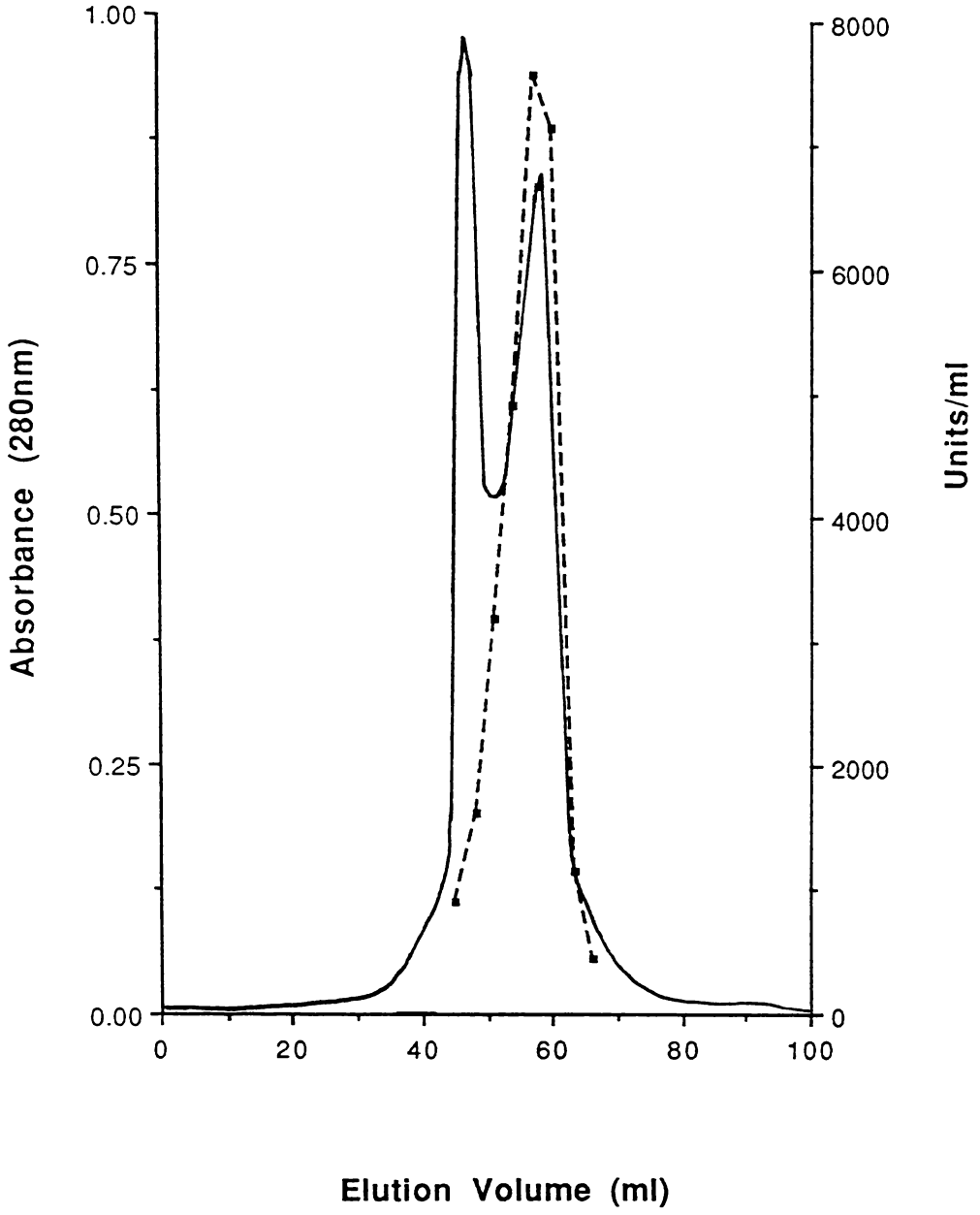


Fig 2.4 Elution Profile of *Thermus* Strain Rt41A Proteinase from Mono S 10/10 FPLC Column (2).

Elution gradient was 0-0.5 mM NaCl over 135 ml (3 ml/min)
The solid line represents the change in absorbance and the broken line represents the proteolytic activity detected.

2.2.2 Electrophoresis

The SDS-PAGE gel of the proteinase prepared as described in section 2.1.3 is illustrated in Fig. 2.5. This was the proteinase preparation used for the bulk of the work on the comparison of the free and immobilised proteinase and all of the peptide synthesis work. The molecular weight of the proteinase band was determined from plotting the log of the molecular weight standards against the relative distance they travelled through the gel (Fig. 2.6) and was found to be 32.2 kDa. This value is the same as that found by Peek (unpublished results) by the same method. The other faint band that is seen on the gel is likely to be due to autolytic action of the proteinase as PMSF does not completely inhibit the activity of Rt41A proteinase during the sample preparation. The molecular weight of this band is 25.0 kDa.

2.2.3 Isoelectric Point Determination

After isoelectrofocusing only one protein band was observed (see Fig. 2.7). This suggests that the preparation was and the extra band observed is most likely due to autolysis. Alternatively two proteins are present with the same pI. Even if this was the case the contaminating protein observed after separation on the Mono S column has no proteolytic activity, as is seen in Fig. 2.4 and would be less than 10% of the final preparation. As the proteinase band focused beyond the last pI marker (pI 10.25), but before the end of the pH gradient (pH 10.5) the isoelectric point is estimated to be between these two values.

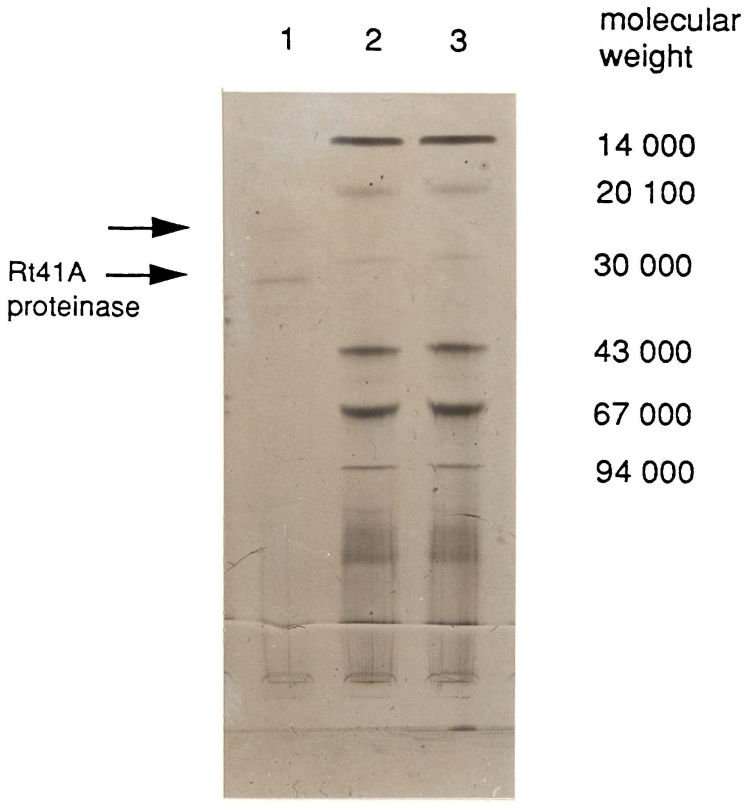


Fig 2.5 SDS-PAGE of Purified *Thermus* strain Rt41A Proteinase

Lane 1 Purified Rt41A proteinase
Lane 2 & 3 Molecular weight markers (Pharmacia):
phosphorylase B, 94 000
BSA, 67 000
ovalbumin, 43 000
carbonicanhydrase, 30 000
soybean trypsin inhibitor, 20 100
α-lactoglobulin A 14 400

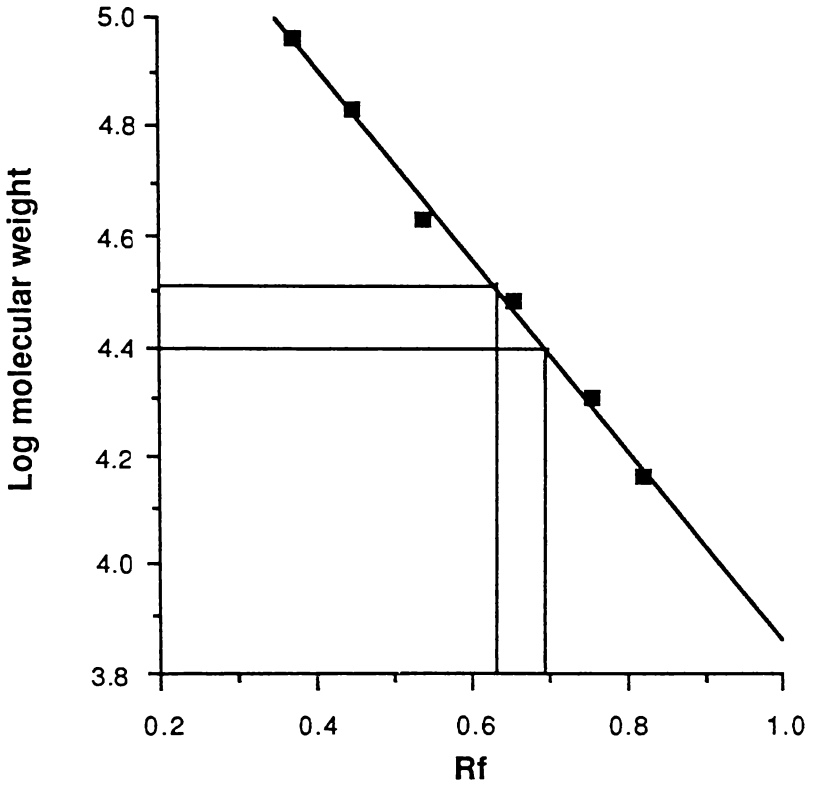


Fig. 2.6 Standard Curve for Molecular Weight Determination by SDS-PAGE.

Molecular weight standards utilised are given in Fig. 2.5
Rf for Rt41A proteinase was 0.63 and 0.693 for the other band.

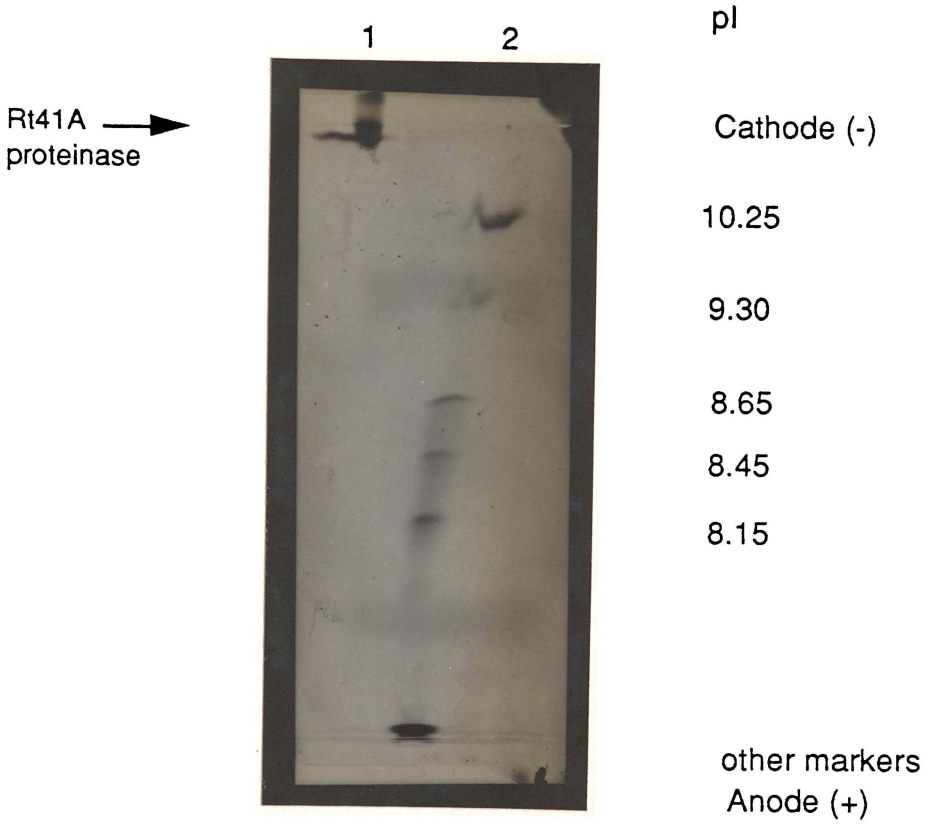


Fig 2.7 IEF Gel of Purified *Thermus* strain Rt41A Proteinase

Lane 1 Purified Rt41A proteinase
Lane 2 pI markers (Pharmacia):
cytochrome C, 10.25
trypsinogen, 9.30
lentil lectin-basic band, 8.65
lentil lectin-middle band, 8.45
lentil lectin-acidic band, 8.15
horse myoglobin-acidic band, 7.35
human carbonic anhydrase B, 6.55
bovine carbonic anhydrase B, 5.85
ß-lactoglobulin A, 5.20

2.2.4 Substrate Specificity of Rt41A Proteinase

The activity of Rt41A proteinase against a number of p-nitroanilide substrates was investigated. The results of this study are illustrated in Table 2.3. To enable a more complete description of the specificity of Rt41A proteinase the results of Cowan *et al.* (1987b), Table 2.4, and Peek (unpublished results), Table 2.5 and Fig. 2.8, have also been presented here.

2.2.4.1 Specificity of the S_1 Subsite

The activity of Rt41A proteinase against p-nitroanilide and p-nitrophenyl ester substrates is illustrated in Tables 2.3, 2.4 and Table 2.5 respectively. From these results Rt41A proteinase demonstrates a preference for aromatic amino acids in the P_1 site of the scissile bond. Leucine, arginine, alanine and lysine can also be accommodated in the S_1 subsite in some cases.

Activity against p-nitroanilide substrates is dependent on the amino acid in the P_1 position of the peptide bond but also on the chain length of the peptide. This is clearly shown by the fact that Ac-Phe-pNA and Cbz-Phe-pNA are not cleaved by the proteinase (Table 2.4) yet the highest activity was found when Phe was in the P_1 position of a tetra- and tri-peptide p-nitroanilides (Table 2.3). Similarly Bz-Arg-pNA is not cleaved yet Arg is in the P_1 position of several tri-peptides that are cleaved. It appears that three amino acids is the minimum requirement for cleavage of p-nitroanilide substrates. This effect of chain length on activity was also found for the three other proteinases from *Thermus* strains T351, Rt6 and Tok3. that were compared with Rt41A proteinase by Cowan *et al.* (1987b). Similar results were also found by Saravani *et al.* (1989) with caldolase.

Table 2.3 *Thermus* strain Rt41A Proteinase Activity Against p-Nitroanilide Substrates[a]

Substrate	K_m (mM)	V_{max} (μmoles/min)
Suc-Ala-Ala-Pro-Phe-pNA	0.6	0.067
Suc-Ala-Ala-Pro-Leu-pNA	4.00	0.047
Bz-Val-Ser-Arg-pNA	2.86	0.039
Suc-Phe-Leu-Phe-pNA	0.29	0.098

Substrates tested which showed no activity: Bz-Val-Gly-Arg-pNA, Cbz-Gly-Gly-Leu-pNA, D-Val-Leu-Lys-pNA, Suc-Ala-Ala-Ala-pNA, Suc-Tyr-Leu-Val-pNA, Bz-Pro-Phe-Ala-pNA, Tos-Gly-Pro-Ala-pNA, Cbz-Lys-Arg-pNA, Bz-Arg-pNA, and Boc-Lys-pNA.

Table 2.4 Comparative Hydrolysis of Chromogenic Peptide Substrates with *Thermus* strain Rt41A Proteinase[a]

Values quoted have been expressed as a ratio of peptidase to caseinase activity.

Substrate	Normalised hydrolysis Rate
Bz-Ileu-Gly-Glu-Arg-pNA	12.40
Bz-Phe-Val-Arg-pNA	4.80
Suc-Ala-Ala-Ala-pNA	0.30
L-Val-Leu-Lys-pNA	0.25

The following substrates were not detectably hydrolysed by Rt41A proteinase: Suc-Val-Val-Arg-pNA, Tos-Gly-Pro-Lys-pNA, Suc-Ala-Ala-Val-pNA, Suc-Arg-Gly-Pro-pNA, Bz-Arg-pNA, Cbz-Phe-pNA and Ac-Phe-pNA

[a] Table taken from Cowan et al. (1987b)

Table 2.5 Esterase Activity of *Thermus* strain Rt41A Proteinase[a]

Cbz- amino acid p-nitrophenyl ester	% Tyrosine rate
Tyrosine	100
Alanine	40
Leucine	15
Tryptophan	15
Phenylalanine	11
Benzyl Cysteine	5
Valine	2
Proline	0.1

Cbz- amino acid p-nitrophenyl esters not cleaved were glycine, arginine, isoleucine, lysine and benzyl aspartate.

[a] Taken from Peek (unpublished results)

2.2.4.2 Specificity of the S_1' Subsite

The cleavage of oxidised insulin B chain by Rt41A proteinase, with time, is shown in Fig. 2.8 (taken from Peek unpublished results) The first cleavage site is Leu^{15}-Tyr^{16} with the second and third cleavage site being Gln^{4}-His^{5} and Phe^{24}-Phe^{25} respectively. Minor cleavage sites after 3 h were His^{5}-Leu^{6}, Leu^{6}-cysteic $acid^{7}$, cysteic $acid^{7}$-Gly^{8}, Glu^{13}-Ala^{14} and Phe^{25}-Tyr^{26}. After 24 h other minor cleavage sites included Leu^{11}-Val^{12}, Tyr^{16}-Leu^{17}, Glu^{21}-Arg^{22}, Tyr^{26}-Thr^{27} and Lys^{29}-Ala^{30}. From these results it is clear that Rt41A proteinase has a preference for aromatic amino acids in the P_1' position of the scissile bond, with a preference for aliphatic amino acids in the minor cleavage sites. The proteinase is also able to accept positively and

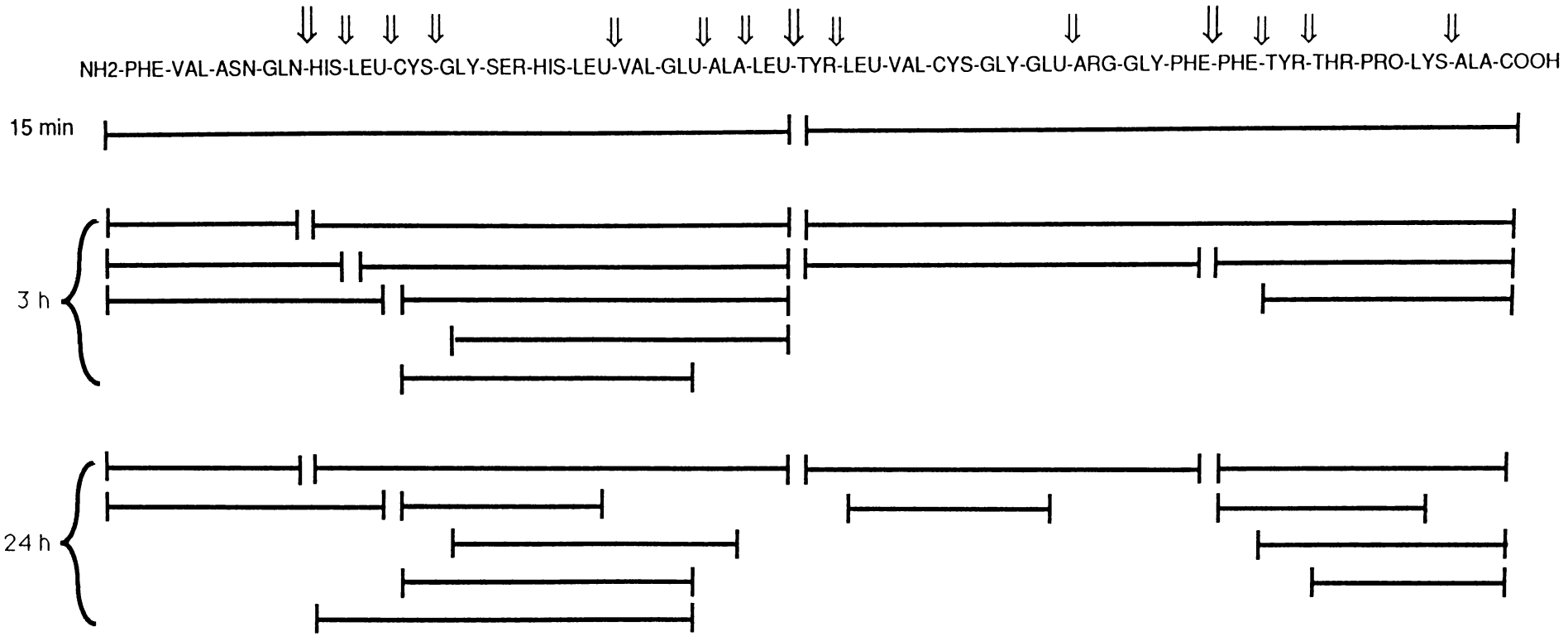


Fig 2.8 Cleavage of Oxidised Bovine Insulin B Chain by Thermus strain Rt41A Proteinase.

The diagram shows the peptides recovered by HPLC after 15 min, 3 h, and 24 h incubation with Rt41A proteinase. Major cleavage sites (large arrowheads) were determined from the large peak areas of HPLC traces and by the high recovery of amino acids from these peptides. Minor cleavage sites (small arrowheads) had relatively smaller peak areas and a lower yield of amino acids. Taken from Peek (unpublished results)

negatively charged amino acids in the S_1' subsite (Leu^6-cysteic $acid^7$ and Glu^{21}-Arg^{22}.), however, these are very minor cleavage sites and may not be significant.

As mentioned in the introduction Rt41A proteinase displays a similar cleavage pattern of oxidised insulin B chain to that found for other microbial serine proteinases (see Fig. 1.2).

2.2.5 Validation of Assay Methods

From the results presented in Table 2.3 Suc-Ala-Ala-Pro-Phe-pNA (SucAAPFpNA) was the substrate of choice for further characterisation of the free and immobilised proteinase on the basis of the affinity of the enzyme for the substrate. Although the K_m for Suc-Phe-Leu-Phe-pNA is lower, solubility problems were associated with this substrate. Subsequent kinetic studies carried out in this laboratory (Peek, unpublished results) have shown that the SucAAPFpNA substrate also has a higher specificity constant (K_{cat}/K_m) than Suc-Phe-Leu-Phe-pNA.

The hydrolysis of SucAAPFpNA by Rt41A proteinase (detailed in section 2.1.6) was found to be linear over a period of 5 min providing the absorbance change in that time did not exceed 0.45 at 410 nm for both the free and immobilised proteinase.

The azocasein assay was linear for a period of 10 min, for both the free and immobilised proteinase, providing the absorbance change was less than 0.2 at 420 nm.

When assaying the immobilised proteinase with either SucAAPFpNA or azocasein it was essential that the tubes were shaken; without it the activity observed was significantly lower. A speed of 5 cycles/min was found to give optimal activity, speeds greater than this actually resulted in a decrease in activity.

2.2.6 Comparison of Polymer Carrier VA-Epoxy Biosynth and CPG Beads as Immobilisation Supports

At all enzyme loadings tested (800-2400 Au/ 0.4 ml of CPG beads and /400mg of VA-Epoxy Biosynth) there was no significant difference in the % recovery of active Rt41A proteinase with either support (Table 2.6). The maximum recovery (41-43%) was found with the lowest enzyme loading. All further studies were carried out with the CPG beads as they are thermostable at temperatures >70 °C and were also advantageous in that they are resistant to microbial attack.

Table 2.6 Comparison of Binding Efficiency of *Thermus* strain Rt41A Proteinase to Controlled Pore Glass Beads and Polymer Carrier VA-Epoxy Biosynth.

	% Enzyme Recovered	
Enzyme added (Au)	VA-Epoxy Biosynth[a]	CPG Beads[b]
800	41	43
1600	24	35
2400	28	28

[a] VA-Epoxy Biosynth particle size=50-200 µm with a pore size of 80 nm.
[b] Rt41A proteinase immobilised to 23 nm pore size beads, 125-177 µm diameter

2.2.7 Storage Stability of Immobilised Rt41A Proteinase.

A detailed study was not carried out on the storage stability of the immobilised proteinase. The most successful procedure for storing the immobilised proteinase was in 50% v/v glycerol in 50 mM Hepes buffer pH 8 + 5 mM $CaCl_2$ at 4 °C. In this form the immobilised proteinase completely retained its activity for 18 months. The activity of the immobilised proteinase was lost when dried either by lyophilisation, or air drying at 75°C. No activity was lost when the storage buffer was removed by filtration with the aid of a venturi pump. The latter procedure was utilised when resuspension of the immobilised proteinase was required for experimental purposes, e.g. pH stability and thermostability experiments.

CHAPTER 3

COMPARISON OF FREE AND IMMOBILISED *THERMUS* STRAIN Rt41A PROTEINASE

3.1 INTRODUCTION

There has been increased interest in the use of immobilised enzymes in industry because of the advantages they confer over their soluble counterparts. This includes increased stability to temperature, pH and organic solvents, recovery and reuse of the enzyme. When proteases are immobilised an additional benefit is the prevention of autodigestion (Klibanov, 1979, Martinek and Mozhaev, 1985). Examples of immobilised enzymes that are used on an industrial scale are immobilised glucose isomerase for the production of fructose-enriched syrups from corn starch hydrolysates (Jenssen and Rugh, 1987), and immobilised aminoacylase in the preparation of L-amino acids from racemic mixtures (Chibata and Tosa, 1976). Immobilised enzymes are also utilised in biochemical studies, for example to isolate subunits from oligomeric enzymes, investigation of the tertiary structures of proteins and studying the mechanisms of enzyme denaturation (Martinek and Mozhaev, 1985).

Covalent attachment to a solid support was utilised in this study due to the high temperatures (>70 °C) preferred for determining enzyme activity. *Thermus* strain Rt41A proteinase (Rt41A proteinase) was immobilised to controlled pore glass, as described in Chapter 2, and studies were carried out on the effect of immobilisation to CPG beads with regards to the activity against two substrates, pH optima, temperature-activity relationships, and stability towards pH, organic solvents and temperature. The effect of pore size on the immobilisation onto CPG beads was also investigated.

3.2 MATERIALS AND METHODS

Rt41A proteinase was purified as described in section 2.1.1.1 (Batch 1) and 2.1.1.2 (Batch 2). Batch 1 was utilised in section 3.2.1, all other experiments were carried out with Batch 2. All buffers were adjusted to the required pH at the temperature of use.

3.2.1 Immobilisation of Rt41A Proteinase to CPG Beads with Various Pore Sizes.

The effect of CPG beads with different pore sizes on the percent recovery of Rt41A proteinase bound was investigated. The proteinase was immobilised as described in section 2.1.8 and was applied either at a fixed level of activity/m^2 of beads or a fixed level of activity/g of beads. The specifications of the different CPG beads are given in Appendix 1. The amount of protein bound to the glass beads (for calculation of specific activity) was determined by the difference between the protein added to the system and that left in the supernatant after immobilisation.

3.2.2 Comparison of Free and Immobilised *Thermus* strain Rt41A Proteinase

3.2.2.1 pH Activity and Stability

The activity of the free and immobilised proteinase, in the range pH 5 to 12.4, was determined as in section 2.1.5 and 2.1.6, except that 10 mM Piperazine dihydrochloride/Glycylglycine (PD/GG) + 5 mM $CaCl_2$ was used as a buffer. The effect of different buffers on the pH activity profile was also investigated with 50 mM Ches, 50 mM Hepes and 10 mM Glycylglycine; all of which contained 5 mM $CaCl_2$.

The pH stability of both the free and immobilised enzyme was determined, at 80 °C. The free enzyme stock was diluted 1:10 with 10 mM PD/GG buffer + 5 mM $CaCl_2$ at the appropriate pH. 60 µl aliquots were dispensed into preincubated tubes, and tubes removed at set time intervals frozen in liquid N_2 and stored at -70 °C until assayed. The immobilised enzyme was first washed in de-ionised water and then reconstituted in the same volume of 50% v/v glycerol

in de-ionised water. 10 µl aliquots were then dispensed into assay tubes and mixed with 500 µl of preincubated PD/GG buffer of the appropriate pH. After incubation three tubes were removed and stored at -70 °C until assayed. To assay the immobilised enzyme the incubation buffer was removed prior to assay and replaced with preincubated (75 °C) azocasein, the assay completed as described in section 2.1.6.

3.2.2.2 Thermostability

The thermostability of the free and immobilised proteinase was investigated at 50, 65, 70 and 90 °C in the absence and presence of 10 µM and 5 mM $CaCl_2$. Enzyme solutions containing various $CaCl_2$ concentrations were prepared as follows. The free proteinase containing 5 mM $CaCl_2$ was used as a stock solution. To obtain enzyme in 10 µM $CaCl_2$, the stock proteinase was dialysed against 100 µM $CaCl_2$ and then diluted 1:10 with 50 mM Hepes, pH 8. Removal of all $CaCl_2$ was achieved by diluting the stock enzyme with 100 mM Hepes + 20 mM EDTA.

At 50 and 70 °C 1 ml of the proteinase was placed in a plastic reaction tube (1.5 ml) and 60 µl aliquots were removed at selected time intervals and frozen at -70 °C until assayed. For the 90 °C samples, 60 µl of enzyme was added to preincubated plastic tubes (0.5 ml) and tubes removed after appropriate time intervals.

The immobilised proteinase was washed and reconstituted in 50% v/v glycerol and water as in section 3.2.2.1. From this stock, 10 µl was pipetted into assay tubes and 500 µl of 50 mM Hepes pH 8, containing 5 mM $CaCl_2$, 10 µM $CaCl_2$ or 10 mM EDTA. The buffer was preincubated before the addition of the enzyme. Three tubes were removed at each time interval and frozen at -70 °C. The immobilised proteinase was then assayed as described in section 3.2.2.1.

All thermostability experiments were carried out in the presence of 0.01% v/v Triton X-100. The final concentration of the proteinase, before incubation, was the same for all $CaCl_2$ concentrations.

3.2.2.3 Solvent Stability

The stability of the free and immobilised proteinase was investigated in the presence of varying concentrations of DMF and heptanol, at 40, 60 and 80 °C. Heptanol is water-immiscible and does not affect the buffer concentration. Therefore, 48 Au , in 50 µl, of the free proteinase was added to 450 µl of heptanol/buffer (50 mM Hepes + 5 mM $CaCl_2$, pH 8) to give final solvent concentrations of 25, 50 and 90% v/v. However, DMF is water-miscible and will affect the buffer concentration. To compensate for this 10 µl of the free proteinase containing 240 Au was added to 40 µl of buffer (625 mM Hepes + 62.5 mM $CaCl_2$) and 450 µl of DMF/deionised water to give final solvent concentrations as above. In both cases 0.01% Triton X-100 was included in the incubation mix. For both solvent systems, 500 µl of the solvent/buffer mix was was added to a 15 µl aliquot of the immobilised proteinase (pre-treated as in section 3.2.2.1). The free and immobilised proteinase samples in heptanol were shaken as described in section 2.1.6.

Samples were removed at 0, 3 and 6 h and frozen at -70 °C until assayed. Samples were assayed as described previously (section 3.2.2.1) except that the free proteinase samples in heptanol had appropriate amounts of buffer added to them to adjust the aqueous phase volume to 500 µl, from which samples were extracted.

3.3 RESULTS AND DISCUSSION

3.3.1 Immobilisation of Rt41A Proteinase to CPG Beads with Various Pore Sizes

The effect of pore size on the proteinase recovered at a fixed level of activity/m^2 of support or at a fixed level of activity/g of support is illustrated in Table 3.1. The percent recoveries are higher when SucAAPFpNA is the substrate. A comparison of the specific activity of Rt41A proteinase immobilised to the 50 nm pore size beads with the free enzyme, using SucAAPFpNA, illustrated that all of the enzyme bound was active. No activity remained in the supernatant at the end of the immobilisation process. Because azocasein has a high molecular weight the lower activity observed

with this substrate could be due to a number of reasons. These include conformational changes of the enzyme on immobilisation, steric hindrance of the substrate due to the orientation of the enzyme with respect to the matrix or internal diffusional limitations (Trevan, 1980). It is likely that a combination of these effects are occurring.

When a fixed amount of Rt41A proteinase was added /m^2 of support and assayed with both substrates, the percent recovery of the immobilised proteinase increases with increasing pore size up to 105 nm, and then decreases at the largest pore size (187 nm). The increase in percent recovery is most likely due to an increase in the accessibility of the proteinase into the pores with increasing pore size. The available surface area for immobilisation is therefore larger. It is not certain why there is a decrease in percent recovery with the 187 nm pore size beads. Borrego *et al.* (1989)

Table 3.1 Percent Recovery of *Thermus* strain Rt41A Proteinase Immobilised on Controlled Pore Glass Beads with Varying Pore Sizes.

Rt41A proteinase was immobilised either at a fixed level of enzyme/g of beads or a fixed level of enzyme/m^2 of beads[a].

	% Recovery from 54 Au/m^2 of beads		% Recovery from 3750 Au/g of beads.	
Pore Size	Suc[b]	Azo[c]	Suc	Azo
15 nm	27.3	10.7	50.6	15.4
23 nm	47.0	16.2	68.7	24.7
50 nm	42.0	21.3	44.7	20.0
105 nm	64.0	26.5	59.3	22.0
187 nm	39.0	18.5	64.0	22.0

[a] When using the 50nm pore size beads binding 54 Au/m^2 of beads is equivalent to 3750 Au/g of beads and 5µg of protein. Results given are the average of three replicates.
[b] Activity assayed with SucAAPFpNA.
[c] Activity assayed with Azocasein

found a similar effect with immobilised pectinesterase. It was suggested that with a bigger pore size the enzyme is immobilised further inside the bead. This causes an increase in internal diffusional limitations because of the high molecular weight pectin substrate. It is not clear why the decrease in activity recovered is observed with the SucAAPFpNA substrate. It is possible that although the substrate diffuses within the pores more readily than azocasein, that the utilisation of the substrate by the enzyme at the outer regions of the pores is faster than the rate of diffusion into the very internal area of the beads. This would result in an under-estimation of the total enzyme activity enzyme as described in section 1.2.3.2.

When Rt41A proteinase is immobilised at a fixed activity/g of CPG beads, pore size has very little effect on the percent proteinase recovered with the SucAAPFpNA assay. The percent recovery when assaying with azocasein is also similar for all pore sizes, except that a slightly lower recovery is obtained with the 15 nm pore size beads. This suggests that similar amounts of proteinase have been immobilised in all cases.

As the pore size of the beads decreases the surface area for immobilisation/g of bead increases (see appendix 1). Therefore, even though the smaller pores restrict entry of the proteinase, as described above, the surface area to which the proteinase can bind is similar in each case. This surface area may be at the very mouth of the pores or on the outside of the beads. The apparent lower recovery, against azocasein, with the 15 nm pore size beads is most likely due to the proteinase blocking the pores. If some of the proteinase blocking the mouth of the pore is immobilised in the wrong orientation, with respect to the outside of the pore, an underestimation of the total activity would result (see Fig 3.1).

3.3.2 The Effect of the Proteinase Concentration on Enzyme Recovery After Immobilisation

Fig. 3.2a,b illustrates the amount of proteinase activity recovered as a function of enzyme units applied; this is expressed both in absolute terms and as a percentage of the enzyme units added. Saturation of the 105 nm beads is reached after the addition of 21 SucAAPFpNA units and 540 Au/m^2 of beads. At the highest enzyme concentration the number of units bound actually

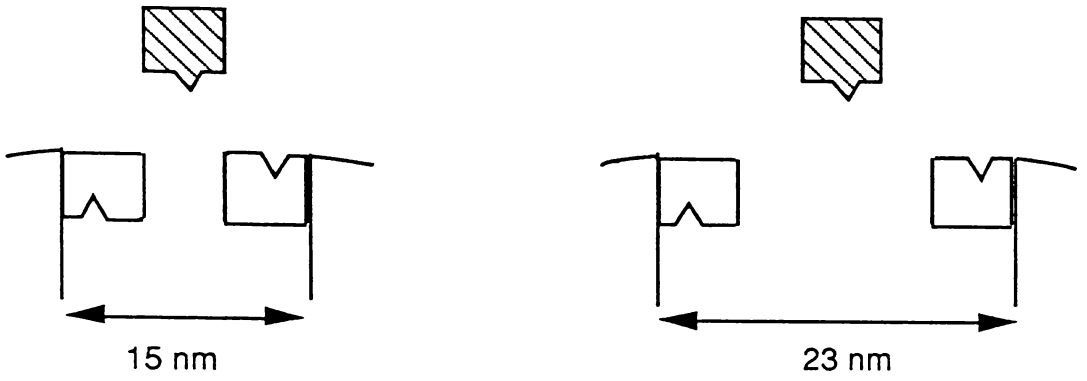


Fig. 3.1 Effect of Pore Size on the Accessibility of Azocasein to the Active Site of Immobilised *Thermus* strain Rt41A Proteinase[a].

Azocasein

Immobilised Rt41A Proteinase

[a] Diagram has been drawn to scale assuming both the proteinase and substrate are globular proteins with approximate diameters of 5 nm.

decreases. The results show that the level of proteinase added to the CPG beads in the pore size experiment does not cause saturation of the beads. The decrease in proteinase recovered at the highest concentration applied could be due to autolysis during immobilisation. There was a significant amount of active proteinase in the supernatant after immobilisation, whereas very little if any activity was found in other cases.

At the lower enzyme loadings the percent recovery of proteinase activity does not vary much, although the recovery with SucAAPFpNA as a substrate is higher. At higher enzyme loadings the percent recovery decreases with increasing proteinase concentration; when azocasein is the substrate the decrease occurs at a lower enzyme loading (40 Au) than that observed when using SucAAPFpNA as substrate (7 U). This is not surprising when one considers the internal diffusional limitations that are observed when using azocasein as a substrate as described in section 3.3.1. Low enzyme loadings are, therefore, more economical in terms of maximal recovery of the proteinase activity applied.

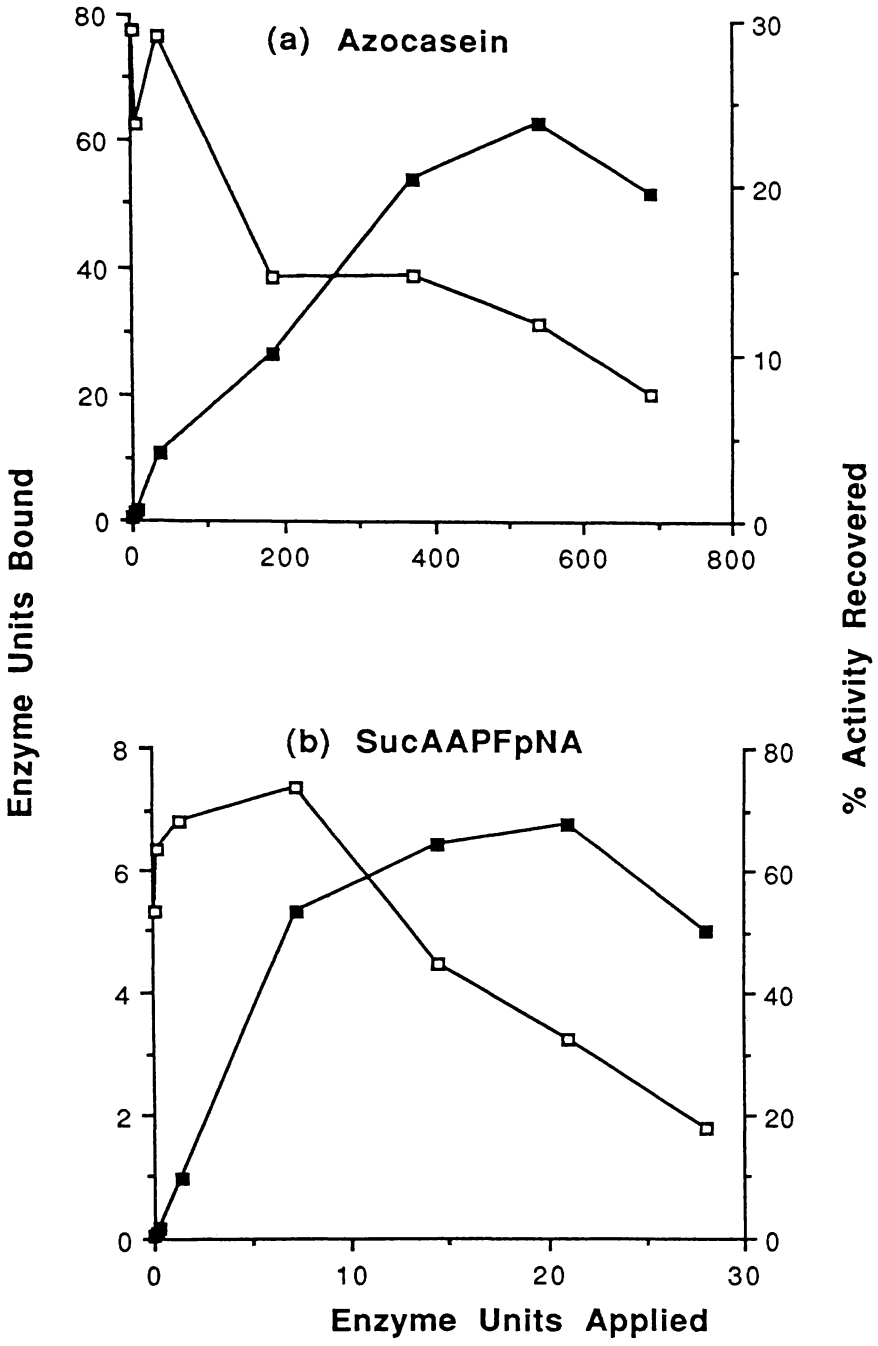


Fig 3.2 Immobilisation of *Thermus* strain Rt41A Proteinase onto Controlled Pore Glass Beads (105 nm) as a Function of Enzyme Concentration.

Enzyme activity was applied / m^2 of beads. The proteinase activity after immobilisation was determined with either (a) azocasein or (b) SucAAPFpNA and is expressed as either units of enzyme bound [■] or % of enzyme activity recovered [□].

3.3.3 Kinetic Effects of Immobilisation

3.3.3.1 K_m Effects

The apparent K_m (azocasein as substrate) for Rt41A proteinase immobilised onto 15 and 105 nm pore size beads (54 Au/m^2) was 0.07% w/v in both cases. The K_m of the free proteinase is 0.02% w/v. The difference in K_m between the free and immobilised enzymes could be due any one of a number of factors. These include the external and internal diffusional limitations as discussed in section 1.2.3.2, which causes saturation to occur at a higher substrate concentration than for the soluble enzyme (Zarborsky, 1973; Engasser and Horvath, 1976). Alternatively, electrostatic interactions between the matrix and the substrate can cause partitioning of the substrate affecting its concentration in the microenvironment of the enzyme. In addition, conformational changes of the enzyme on immobilisation may alter the active site of the enzyme and hence the affinity for the substrate (Trevan, 1980). There is no evidence to suggest which of these effects is occurring, however, it is likely that a combination of these effects is influencing the K_m.

The K_m for the free proteinase when assayed with the SucAAPFpNA substrate is 0.6 mM whereas for the immobilised proteinase (50 nm pore size beads) it is 1.5 mM. The difference in K_m between the free and immobilised proteinase will be caused by the same factors that were mentioned above for the azocasein substrate. However, external and internal diffusional limitations will be less with the SucAAPFpNA substrate, hence the difference between the K_m values for the free and immobilised proteinase is less than that observed for the azocasein substrate. Although the SucAAPFpNA substrate concentration when assaying the immobilised enzyme was not much higher than the K_m, the absorbance change over the assay period was linear. Therefore, the true initial rate of the reaction was observed.

3.3.3.2 Substrate Inhibition of Free and Immobilised Rt41A Proteinase

The free proteinase is inhibited by concentrations of azocasein above 0.1% w/v (K_i= 0.57% w/v) as shown in Fig. 3.3a,c. The immobilised proteinase was inhibited at concentrations above 1.25% w/v azocasein (K_i= 5.1% w/v)

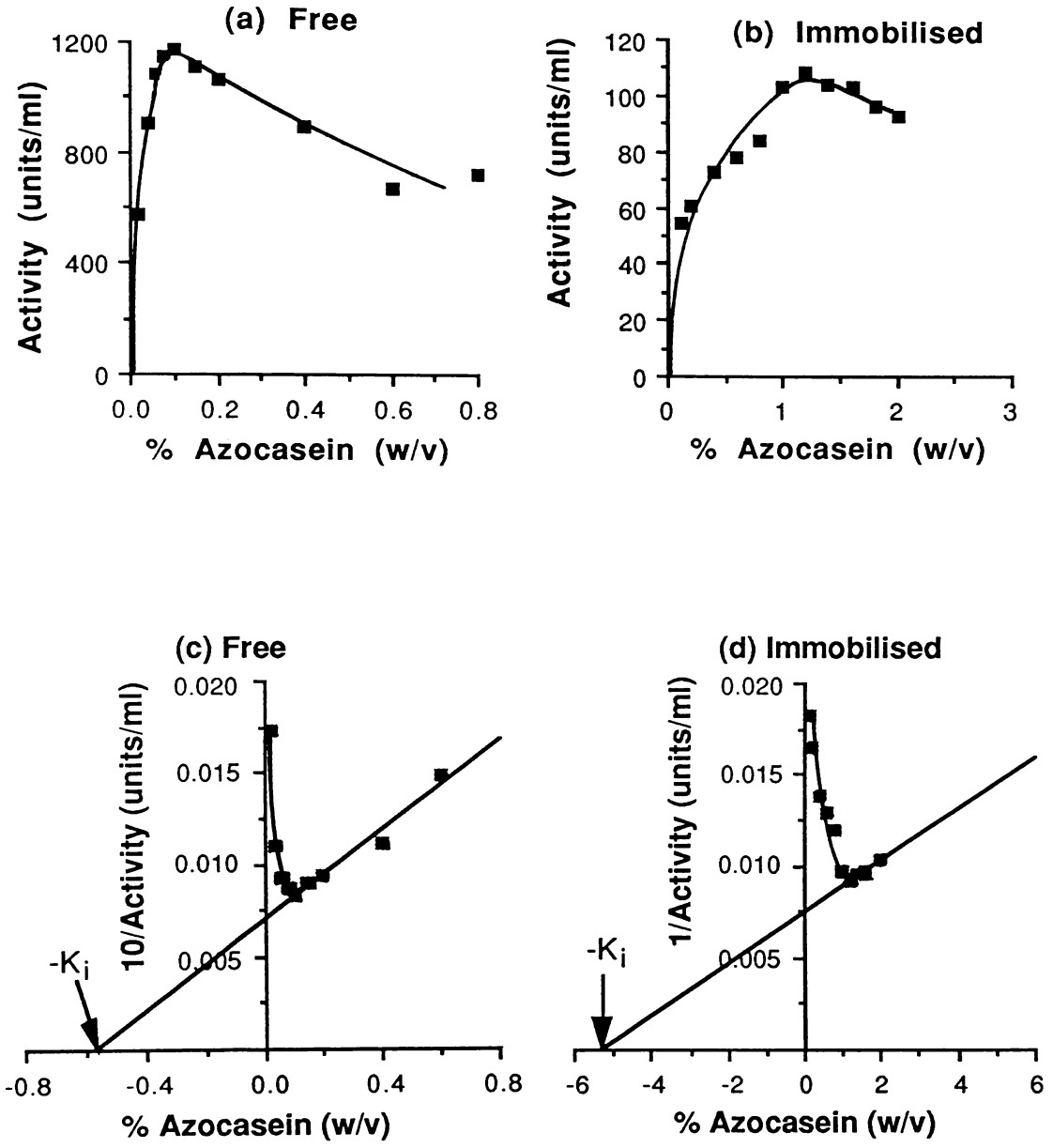


Fig 3.3 Effect of the Substrate Concentration on the Activity of Free and Immobilised *Thermus* strain Rt41A Proteinase.

(a) and (b) Activity vs substrate concentration for free and immobilised Rt41A proteinase respectively.
(c) and (d) 1/V vs S plot for K_i determination (Dixon and Webb, 1979) for free and immobilised Rt41A proteinase respectively.

as is illustrated in Fig. 3.3b,d. The K_i values were determined from linear regression analysis of 1/V vs S plots as shown in Fig. 3.3c,d. Enzymes that are substrate inhibited in free solution, are generally inhibited when immobilised (Trevan, 1980), although Cowan (1980) found substrate inhibition was eliminated on immobilisation. The level of substrate required to cause this inhibition compared to the free depends on both partitioning and diffusional effects as mentioned above. In this case the substrate concentration in the microenvironment surrounding the immobilised enzyme is less than that in the bulk solution, this is also confirmed by the higher K_m that is observed. A higher concentration of substrate, therefore, is required to produce inhibition.

3.3.4 Effect of Temperature and Pore Size on Activity of Immobilised Rt41A Proteinase

The effect of temperature and pore size on the ratio of activity against SucAAPFpNA and azocasein is shown in Table 3.2. Activity against azocasein increases in comparison to the SucAAPFpNA substrate as temperature increases for both the free and immobilised proteinase. This is because the Q_{10} for azocasein (1.54) is much higher than that for SucAAPFpNA (1.01), as calculated from the Arrhenius plots (Fig. 3.6 and 3.7); this will cause a decrease in the SucAAPFpNA/Azocasein ratio with an increase in temperature, regardless of whether the enzyme is free or immobilised. Therefore it is difficult to infer whether temperature is having an affect on the diffusional limitations of the immobilised proteinase. However these results do show that at both temperatures, by increasing the pore size of the CPG bead there is an increase in the activity of the azocasein relative to SucAAPFpNA which seems to be approaching that of the free enzyme. This suggests that as the pore size is increased the internal diffusional limitations are decreased.

3.3.5 Comparison of Free and Immobilised *Thermus* strain Rt41A Proteinase

At this stage one particular pore size bead (50 nm) was chosen to complete further studies. This pore size was chosen on the basis of good recoveries and ease of pipetting. Although the 105 nm pore size beads gave better

Table 3.2. Effect of Pore Size and Temperature on the Ratio of SucAAPFpNA/Azocasein Activity of Immobilised *Thermus* strain Rt41A Proteinase.

	Free	Immobilised (23 nm)[a]	Immobilised (187 nm)[b]
50 °C			
SucAAPFpNA	47.2	10.9	16.5
Azocasein	118	7.2	31.2
Suc/Azo	0.40	1.52	0.53
80 °C			
SucAAPFpNA	97.2	10.2	20.2
Azocasein	972	16.2	87.8
Suc/Azo	0.10	0.63	0.23

[a] Pore size of CPG beads.

recoveries they settle out of solution quickly, because of a higher density, resulting in large sample variation during pipetting. An enzyme loading of 50 Au/m^2 of beads was chosen (below saturation levels) to reduce pipetting errors due to handling very small quantities of beads. The resulting enzyme preparation had specific activities of 5284 Au/mg and 144 U/mg of protein for azocasein and SucAAPFpNA respectively. Comparing these to the free enzyme the azocasein specific activity is 40% lower, whereas with the SucAAPFpNA substrate it is unchanged. This suggests that 100% of the enzyme bound is active against the SucAAPFpNA substrate. This disparity between high and low molecular weight substrates has been found by others (Cheetham, 1985; Beeby, 1979 and Johnson *et al.*, 1990) and suggests that the azocasein is not accessible to all of the proteinase immobilised or it is diffusion limited as has been mentioned previously (section 3.3.1).

3.3.5.1 pH Optimum

pH activity profiles of free and immobilised Rt41A proteinase, for both SucAAPFpNA and azocasein substrates, are illustrated in Fig. 3.4a,b. The SucAAPFpNA substrate could not be used above pH 11 as significant non-enzymatic hydrolysis of the substrate occurs.

The pH optimum of the free enzyme was 10.5 for both substrates. The immobilised proteinase has an optimum pH of 9.5 and 8.0 for azocasein and SucAAPFpNA respectively. Interestingly, the immobilised proteinase retains >60% of its activity at pH 12 when using azocasein as the substrate, compared to the free proteinase which has only 10% of the optimal activity at this pH. Shifts in the pH optimum of enzymes, either to a more acid or alkaline pH, are often observed when the enzyme is immobilised. This is caused by unequal partitioning of H^+ and OH^- ions between the micro- and macroenvironment of the enzyme; this is due to electrostatic interactions with the matrix (Engasser and Horvath, 1976). Therefore the enzyme is exposed to a different pH than is measured in the bulk phase.

Caldolysin immobilised to CPG beads also produced a shift in the pH optimum towards a more acidic pH with casein as the substrate (Cowan and Daniel, 1982b). Gusek and Kinsella (1990), however, found the proteinase from *Thermomonospora fusca* YX, immobilised in a similar manner, showed an increase in the pH optimum as compared to the soluble enzyme. They suggested that this was because negative charges from unreacted silanizing reagent, and on the glass itself, attracted H^+ ions creating a more positive microenvironment than the bulk phase. Other enzymes immobilised to CPG beads have shown no alteration in the pH optimum (Kidima and Pickard, 1990; Borrego *et al.*, 1989). It is clear, therefore, that the difference in the micro- and macroenvironmental pH is not the only factor affecting the pH optimum of the immobilised enzyme in this case.

The free proteinase has a higher pH optimum than has been seen for this enzyme previously (Peek, unpublished results). The authors used 4 buffers (Mes, Hepes, Ches and Caps) over the pH range, possibly one the buffers is stabilising or inhibiting the proteinase in some way. To investigate this possibility three buffers were then utilised over the pH range 7-12. namely,

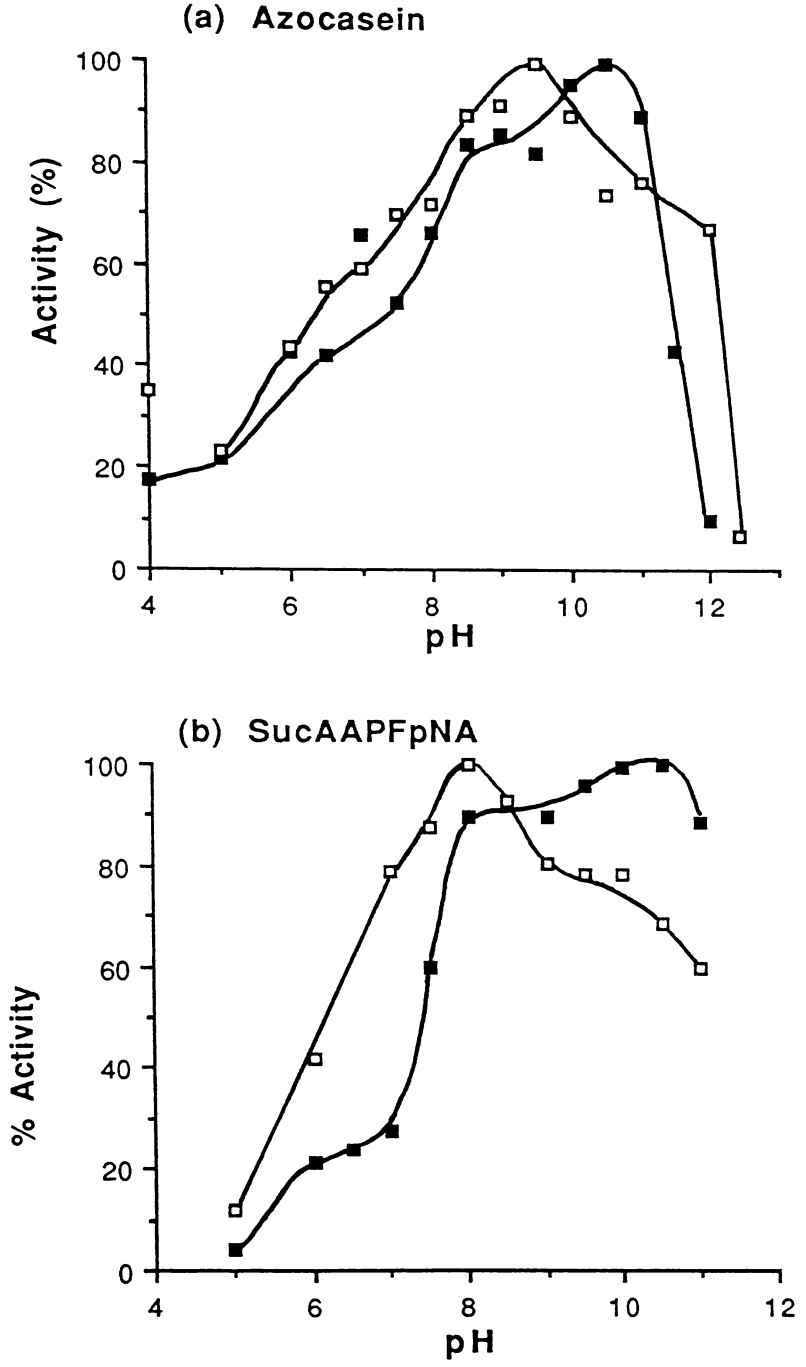


Fig 3.4 pH Profile for Free and Immobilised *Thermus* strain Rt41A Proteinase.

The activity of the free [■] and immobilised [□] proteinase was assayed with either (a) Azocasein or (b) SucAAPFpNA. Details as described in section 2.1.5 and 2.1.6 except Piperazine dihydrochloride/Glycylglycine buffer was utilised to vary the pH.

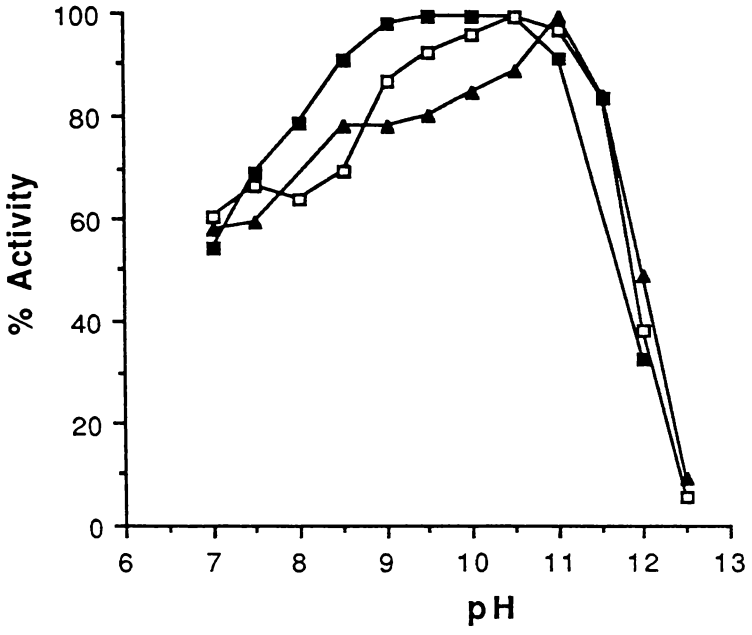


Fig 3.5 Effect of the Buffer on the pH Optimum of Free *Thermus* strain Rt41A proteinase.

Activity determined with azocasein.

- ■ 50 mM Ches
- □ 50 mM Hepes
- ▲ 10 mM Glycylglycine

Hepes and Ches (50 mM + 5 mM $CaCl_2$) and glycylglycine (10 mM + 5 mM $CaCl_2$). The pH optimum for Hepes and Ches was 10.5 and for glycylglycine it was 11.0 as is shown in Fig. 3.5. Although these buffers were utilised at some pH values outside of their buffering range the pH before and after the reaction was determined; as no pH change was observed the results were considered to be valid. No further study was undertaken in this area.

3.3.5.2 pH Stability

The pH stability of the free and immobilised proteinase was compared at 80° C over the pH range 5-11, the approximate half-lives are displayed in Table 3.3. In all cases the immobilised proteinase had greater stability with respect to pH than the free proteinase. The free proteinase was most stable at pH 5 and 6, the half-lives being 9 and 9.5 h respectively, at pH 7-11 the half-lives were 4 h or less. The immobilised proteinase was most stable at pH 6 and 7 with a half-life of greater than 50 h in both cases. At pH 5 and in the pH range 8-10 the half-lives varied from 10.5-18 h. The immobilised proteinase was least stable at pH 11 with a half-life of 3.5 h. The increased stability of the free proteinase at lower pH values is most likely due to lower autolytic activity, as the proteinase is less active. The stability of the both the free and immobilised proteinase could not be investigated at pH 12 due to the precipitation of the $CaCl_2$ during the pre-incubation of the buffer. However, the activity remaining after 10 min was determined; no activity remained for either the free or immobilised proteinase after this time. As shown below (section 3.3.5.4) the immobilised proteinase is much more stable at low calcium concentrations These combined results suggest that the lower activity observed with the free proteinase at pH 12 (section 3.3.5.1) is likely to be due to its lower stability under the reaction conditions, compared to the immobilised. The fact that any activity is observed at all is surprising, it is likely that the proteinases are more stable in the presence of the substrate. Alternatively, the proteinases are more active at this pH and the activity observed is within the first few minutes of the reaction.

Table 3.3 pH Stability of Free and Immobilised *Thermus* strain Rt41A Proteinase at 80 °C.

pH	Half-Life (h)[a]	
	Free	Immobilised[b]
5	9.0	18.0
6	9.5	>50.0
7	4.0	>50.0
8	1.8	10.5
9	1.8	12.5
10	2.0	14.5
11	1.8	3.5

[a] Half-life determined as described in section 3.2.2.1
[b] Rt41A proteinase immobilised to CPG beads with a 50nm pore size.

3.3.5.3 Temperature-Activity Relationship

The effect of temperature on the activity of the free and immobilised proteinase was investigated in the presence and absence of 5 mM $CaCl_2$. With azocasein as the substrate and in the presence of $CaCl_2$ the Arrhenius plots (Fig. 3.6) show a downturn in the slope at 90 °C for the free proteinase and at 85 °C for the immobilised enzyme. In the absence of $CaCl_2$ this break occurs between 60-65 °C in both cases. These breaks are due to thermal denaturation of the proteinase. It is unusual that the downturn for the immobilised proteinase occurs at a slightly lower temperature than the free proteinase. The former is known to be more thermostable (section 3.3.5.4), therefore the break point should occur at a higher temperature than the free proteinase. It is likely, however, that this may be related to the substrate; the arrhenius plots for the free and immobilised proteinase with the SucAAPFpNA are identical. It is possible that the azocasein substrate is unfolded at the higher temperatures, due to denaturation, and hence, the internal diffusional limitations would be increased and the activity decreased significantly.

The apparent activation energy for free Rt41A proteinase in the presence and absence of $CaCl_2$ is approximately 77 kJ/mol, whereas for immobilised Rt41A proteinase it is approximately 57 kJ/mol. These plots show that even when immobilised the proteinase requires Ca^{2+} for thermal stability as denaturation occurs at a lower temperature without it. The change in activity with increasing temperature (up to the point where denaturation dominates), however, is unaffected by the presence of $CaCl_2$ as shown by the similar activation energies. The apparent activation energy of immobilised Rt41A proteinase is lower than the free enzyme. It is generally accepted that diffusion limitation in immobilised enzymes can lead to a reduction in the activation energy, sometimes to half the original value (Engasser and Horvath, 1976; Trevan, 1980; Gemeiner *et al.*, 1986). The reason being that as the temperature increases the substrate diffusion within the matrix becomes rate-limiting. At high temperatures the reaction is controlled by external diffusional limitations which causes a further decrease in the apparent activation energy. This may also help to explain why the downturn for the immobilised proteinase is observed at a lower temperature compared to the free proteinase.

The Arrhenius plots (Fig. 3.7) with SucAAPFpNA as a substrate are more difficult to interpret. Although the plots for the free and immobilised enzyme in the presence of $CaCl_2$ have been drawn as 3 linear sections, they could be represented as curves, the middle section in both cases being very flat. There are many reasons for curvilinear plots, such as changes in pH optima, and K_m changes with increasing temperature. However, in this case the most likely one is that the substrate concentration is not sufficiently larger than the K_m. This can give non-linear Arrhenius plots depending on the heat of substrate binding (Han, 1972). These results also show that $CaCl_2$ is required for stability, but not for activity.

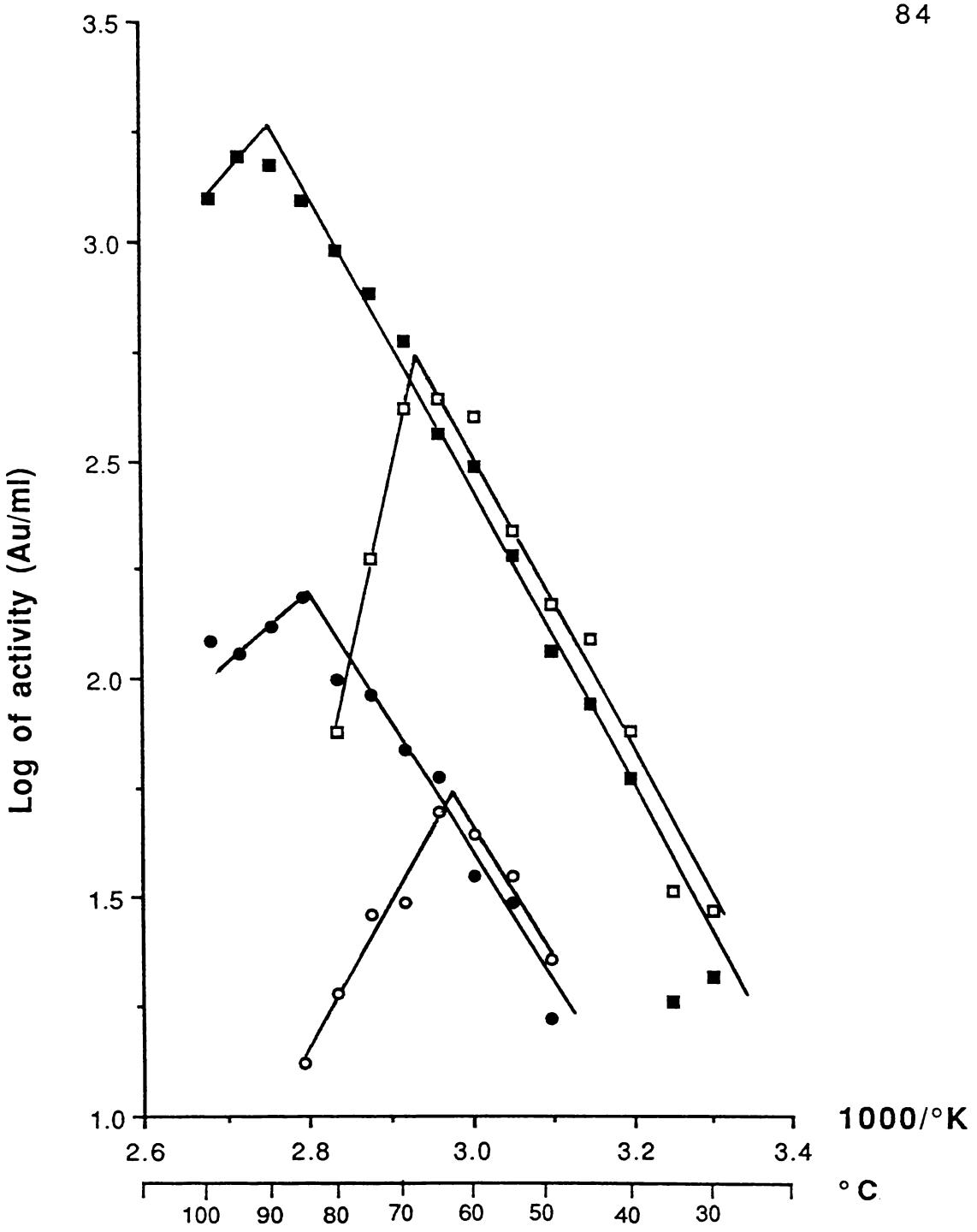


Fig 3.6 Arrhenius Plot for Free and Immobilised *Thermus* strain Rt41A Proteinase, Azocasein as Substrate.

Activity was determined for the free [■,□] and immobilised [●,O] proteinase in the presence [■,●] and absence [□,O] of $CaCl_2$.

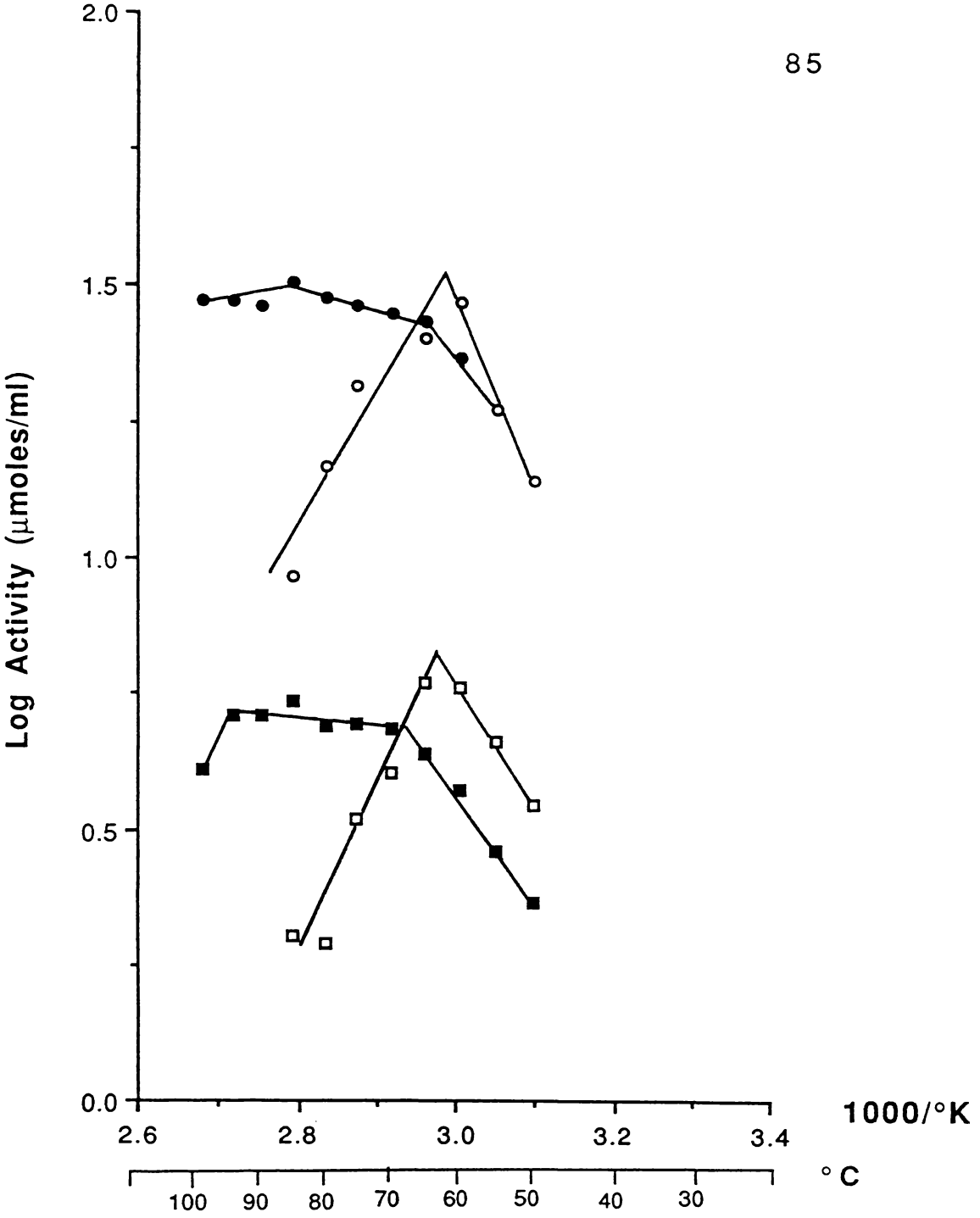


Fig 3.7 Arrhenius Plot for Free and Immobilised *Thermus* strain Rt41A Proteinase, SucAAPFpNA as Substrate.

Activity was determined for the free [■,□] and immobilised [●,O] proteinase in the presence [■,●] and absence [□,O] of $CaCl_2$.

3.3.5.4 Effect of Temperature and $CaCl_2$ Concentration on the Thermostability

Table 3.4 illustrates the results of thermostability experiments with free and immobilised Rt41A proteinase. The denaturation curves from which the half-lifes were calculated from are illustrated in Appendix 2. At 50 °C in the presence of both 5 mM and 10 μM $CaCl_2$ the free and the immobilised enzyme is stable for >4 days. However, in the absence of $CaCl_2$ the free enzyme had a half-life of 4 h compared to the immobilised enzyme which had only lost approximately 25% of its activity after 72 h.

The immobilised proteinase proved more thermostable at 65, 70 and 90 °C than the free enzyme at both $CaCl_2$ concentrations, which is shown by the increase in half-life in each case (see Table 3.4). This increase in stability was most significant in the presence of 10 μM $CaCl_2$.

Kinetic analysis of the thermostability data was also undertaken; the first and second order plots of the denaturation curves are shown in Figs. 3.8-3.15.

Table 3.4 Effect of $CaCl_2$ on the Thermostability of Free and Immobilised *Thermus* strain Rt41A Proteinase.

Values are quoted as half-lifes (h)

	5 mM $CaCl_2$		10 μM $CaCl_2$		0 $CaCl_2$	
Temp	Free	Immob[b]	Free	Immob	Free	Immob
50 °C	>96	>96	>72	>72	4(T)	>72
65 °C	85	115	17(A)	115	0.11(T)	0.10
70 °C	67(T)	110	5(A)	110	0.04(T)	0.08
90 °C	0.5	1.5	0.2(T)	1.33(T)	ND	ND

[a] half-life determined as described in section 3.2.2.2
[b] Rt41A proteinase immobilised to CPG beads, 50 nm pore size.
(T) Thermal denaturation is major cause of loss of activity
(A) Autolysis is major cause of loss of activity
ND= Not determined.

Figs 3.8-3.15 Kinetic Analysis of the Effect of $CaCl_2$ Concentration and Temperature on the Thermostability of Free and Immobilised *Thermus* strain Rt41A Proteinase.

Panel (a) First order plot (straight line indicates thermal denaturation).
Panel (b) Second order (straight line indicates autolysis).
Data is presented only where a clear distinction between autolysis and thermal denaturation was observed. The temperature and $CaCl_2$ concentration are stipulated in the individual figure legends.

Fig 3.8 50° C, Free Proteinase, No $CaCl_2$

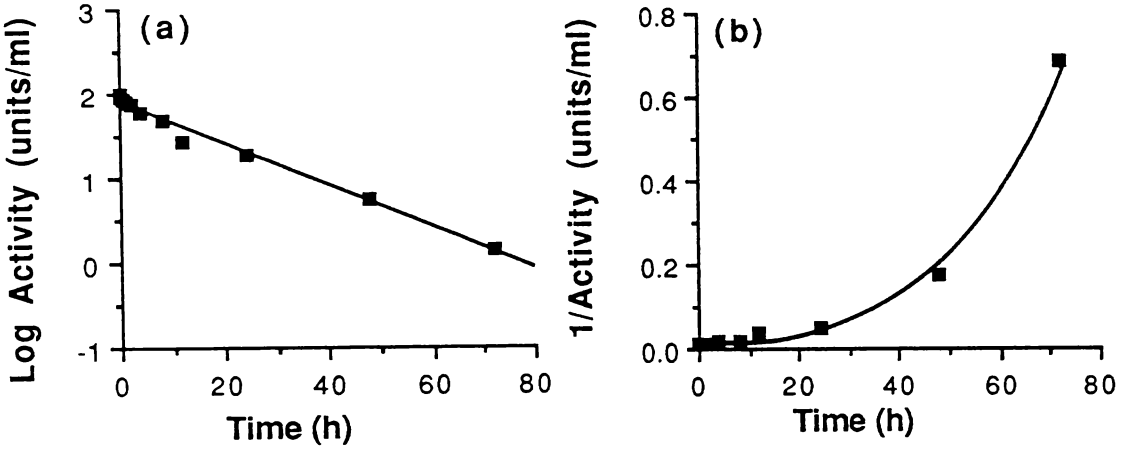


Fig 3.9 65 °C, Free Proteinase, No $CaCl_2$

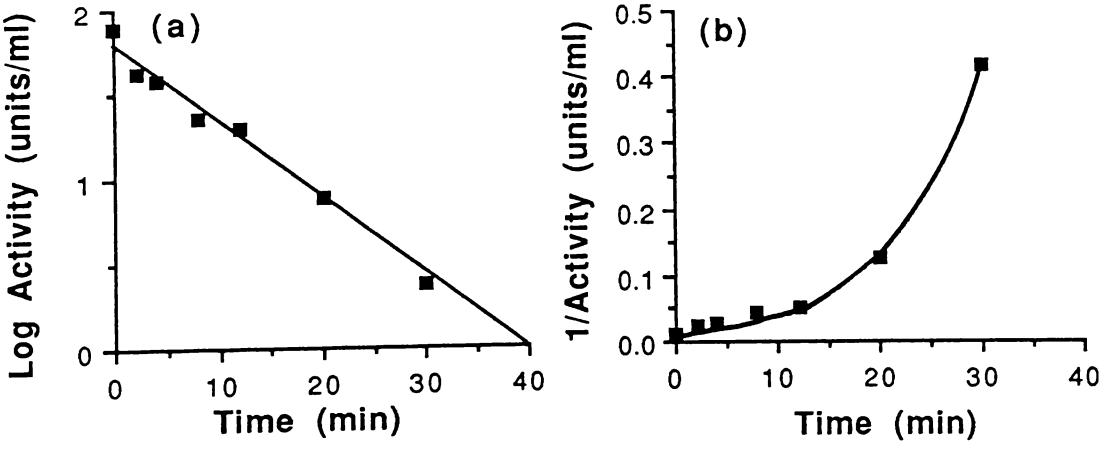


Fig 3.10 65 °C, Free Proteinase, 10 μM $CaCl_2$

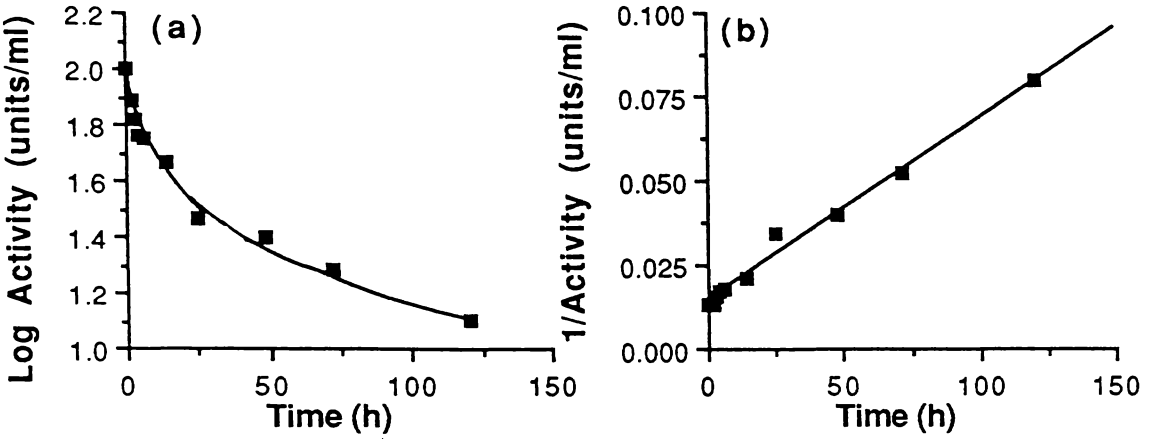


Fig 3.11 70 °C, Free Proteinase, No $CaCl_2$

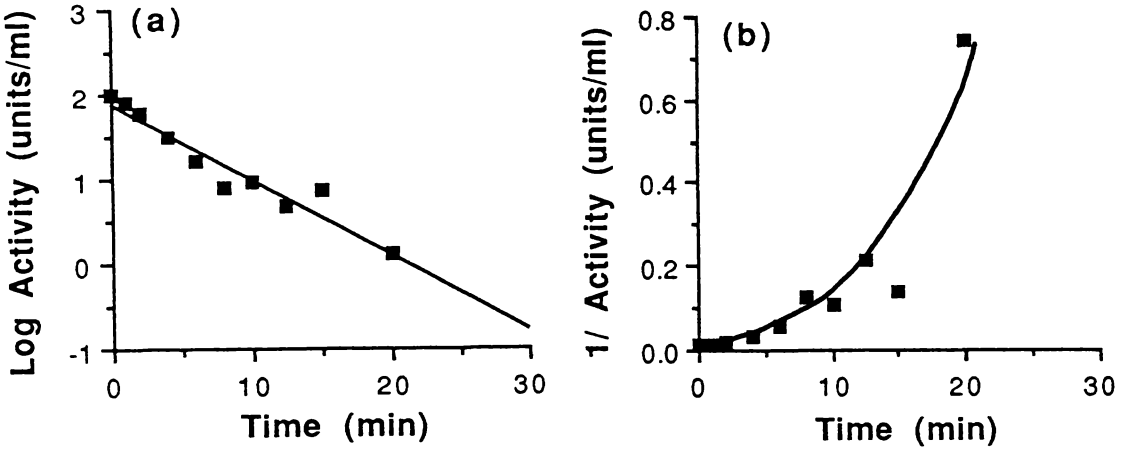


Fig 3.12 70 °C, Free Proteinase, 10 μM $CaCl_2$

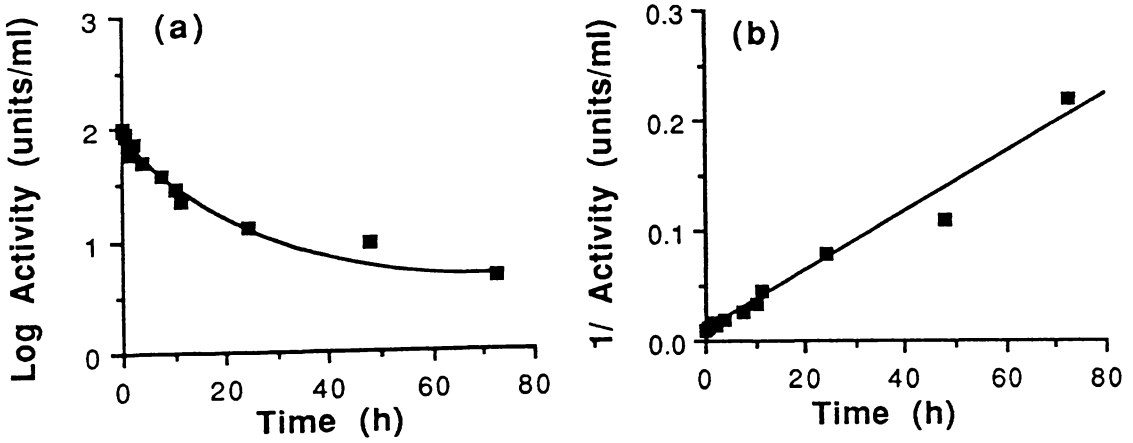


Fig 3.13 70 °C, Free Proteinase, 5 mM $CaCl_2$

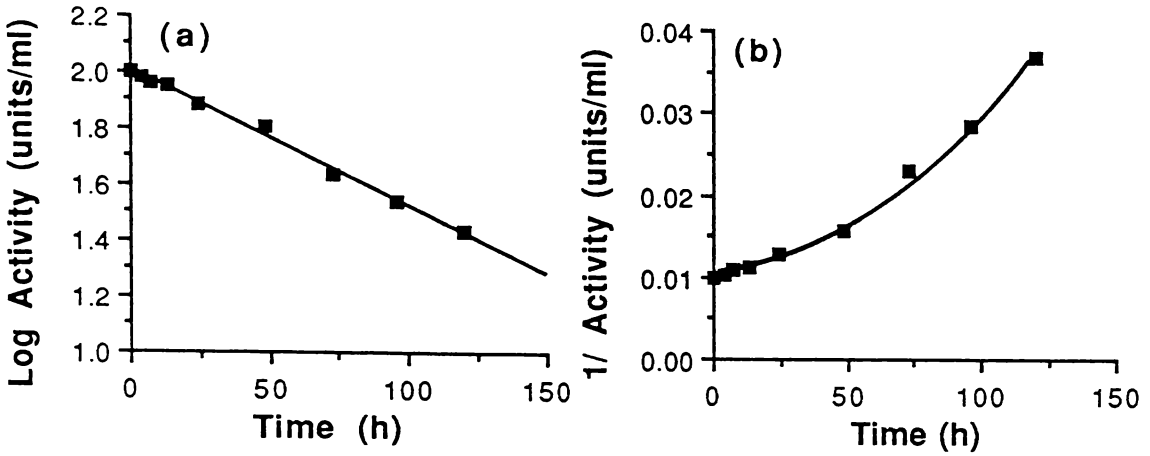


Fig 3.14 90 °C, Free Proteinase, 10 μM $CaCl_2$

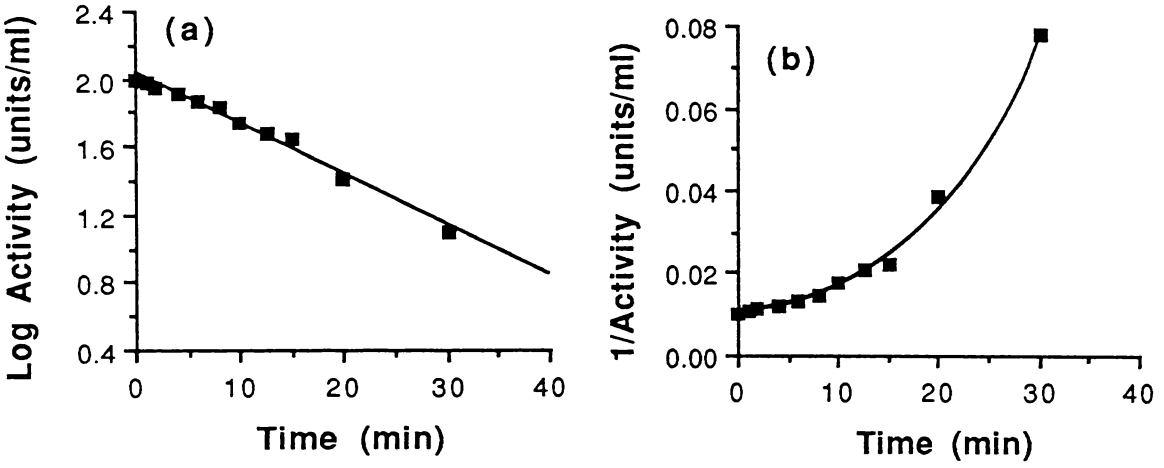
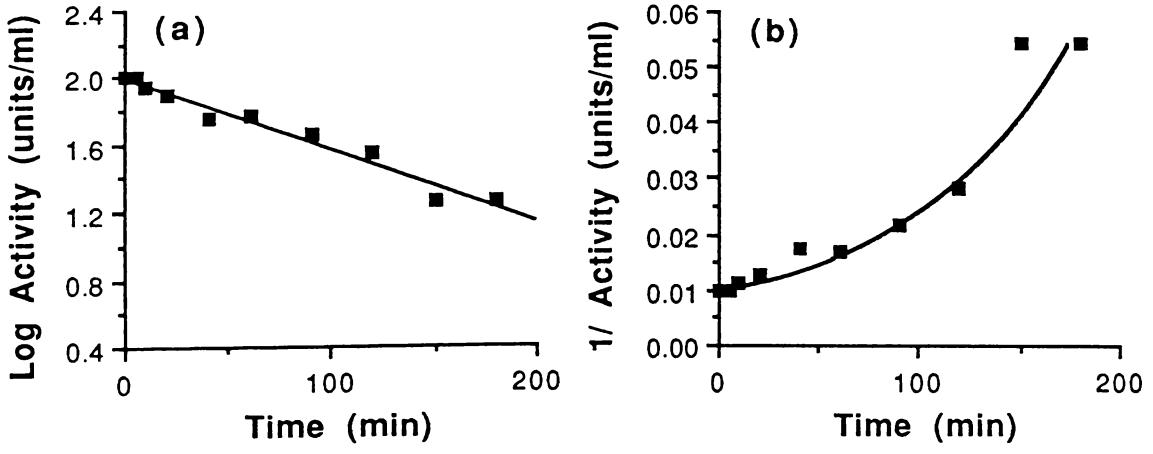


Fig 3.15 90° C, Immobilised Proteinase, 10 μM $CaCl_2$



Only the plots that showed a clear distinction between thermal denaturation and autolysis are given. The findings of this kinetic analysis are indicated in Table 3.4.

These results suggest that at all temperatures tested, except 90 °C , low levels of $CaCl_2$ stabilise the proteinase against thermal denaturation and higher levels stabilise against autolysis. If this is so, then for the immobilised enzyme, where autolysis of the proteinase is eliminated (as mentioned in section 3.1) then the stabilisation conferred by immobilisation should be most significant in the presence of 10 μM $CaCl_2$. This is what is found and also explains why the thermostability of the immobilised proteinase in the presence of 5 mM and 10 μM $CaCl_2$, and the free proteinase in the presence of 5 mM $CaCl_2$ are so similar. That is, both 5 mM $CaCl_2$ and immobilisation protect against autolysis. No additional stability is gained when using 5 mM $CaCl_2$ with the immobilised proteinase.

At 90 °C in the presence of 10 μM $CaCl_2$ thermal denaturation is the dominating cause of loss of activity, as opposed to the autolysis found at other temperatures. At this temperature 10 μM $CaCl_2$ appears to be insufficient to protect against thermal denaturation.

The effect of Ca^{2+} ions on the thermostability of both thermolysin and caldolysin has been investigated. In both cases increased thermostability is observed with calcium. Four calcium ions are bound per molecule of thermolysin. The binding affinity of the first calcium binding site is high and this site is thought to be responsible for thermal stabilisation of the proteinase (Voordouw *et al.,* 1976); the fourth binding site has a lower affinity for calcium and is thought to stabilise against autolysis (Fassina *et al.,* 1986). This suggests that low levels of calcium would stabilise against thermal denaturation and saturating levels of calcium would stabilise against autolysis. This is consistent with the results described here. Caldolysin has binding sites for 6 calcium ions per enzyme molecule (Khoo *et al.*, 1984), and the authors suggest that the extra 2 ions are responsible for the higher thermostability observed by this proteinase compared to thermolysin.

The thermostability of enzymes after immobilisation can either be enhanced, decreased or unchanged, numerous examples exist for each case (see Zaborsky, 1973). The immobilisation of caldolysin to CPG beads (Cowan and Daniel, 1982c) reduced its thermostability, whereas immobilisation to Sepharose and CM-cellulose enhanced its thermostability. Apparent stabilisation of enzymes is also observed when high enzyme loadings are used (Trevan,1980). This is because high concentrations of the enzyme can contribute to internal diffusional restrictions such that all of the potential immobilised activity is not realised. This means that enzyme molecules located in the outer regions of the support react at a rate greater than the diffusion rate of the substrate, consuming most or all of the substrate molecules. Enzymes deeper within the support, therefore, are exposed to little or no substrate and the total activity of the immobilised enzyme is underestimated. This results in no apparent change in activity initially, or an effective stabilisation of the enzyme. In this case however, enzyme loadings were used below saturating levels (section 3.3.2), therefore stabilisation of the proteinase as described here is real.

It has also been observed by a number of authors (e.g. Ulbrich and Schellenberger, 1986; Epton *et al.*, 1973; Martinek *et al.*, 1977) that the inactivation of immobilised enzymes is biphasic in nature. Although at 50 °C and 65 °C in the absence of $CaCl_2$ (Fig. A2.1a and A2.2a) the loss of activity does level off, generally the denaturation curves of both the free and immobilised proteinase are very similar. The increased stabilisation, as described above, is mostly due to the removal of autolysis, with some stabilisation against thermal denaturation in the presence of $CaCl_2$.

3.3.5.5 Solvent Stability

Immobilisation of the free proteinase has increased its stability towards the denaturing effects of varying concentrations of DMF and heptanol, as is shown in Table 3.5.

At 40° C the free proteinase looses very little activity after 6 h in 25 and 50% v/v DMF but, losses 38% of its activity in 90% v/v DMF over the same incubation period. Similar results are seen at 60 °C, however in 90% v/v DMF no activity remains after 3 h. The immobilised enzyme was stable in the

presence of DMF for 6 h at both 40 and 60 °C, except at 90% v/v DMF at 60 °C where only 16% of its activity remained.

At 80 °C the stability of the free proteinase decreases with increasing solvent. The activity of the immobilised proteinase is also reduced as the solvent concentration increases, however it is more stable than the free. For example, 47% activity is retained for the immobilised proteinase after 6 h in 50% v/v DMF compared to 11% for the free. No activity remains for either proteinase at 90% v/v DMF at this temperature after 3 h.

The stability in the presence of heptanol at 40 °C is interesting because the stability of the free proteinase increases with increasing solvent, there being no activity loss at 90% v/v heptanol after 6 h. This is because enzymes can be denatured at the solvent/buffer interface (Cassells and Halling, 1989), the surface area of which is larger at lower heptanol concentrations. More enzyme, therefore, is exposed to denaturing conditions at 25% v/v than at 90% v/v heptanol. The immobilised proteinase is stable at all heptanol concentrations at this temperature.

At 60 and 80 °C the stability of both the free and immobilised proteinase decreases with increasing solvent concentration. Very little activity is retained by the free proteinase at 60 °C at all solvent concentrations after 3 h, and only 4% activity remains in 25% v/v heptanol after 6 h. At 80 °C the free proteinase retains no activity after incubation in the lowest concentration of heptanol. The immobilised enzyme is again more stable, activity being retained for 6 h at 40 °C at all concentrations. At 60 °C increased stability is also observed after 6 h at 25% v/v heptanol and 3 h at 50 and 90% v/v heptanol.

The stability that immobilisation confers on the proteinase in heptanol is likely to be due to shielding of the proteinase via attachment to the CPG beads, the solvent being repelled by water associated with the glass beads. In the case of the DMF, the bonds between the enzyme and the support due to immobilisation may help stop the denaturing effects by increasing the rigidity of the proteinase (Kennedy and White, 1985a).

Table 3.5. Solvent Stability of Free and Immobilised *Thermus* strain Rt41A Proteinase[a].

Values are quoted as the % activity remaining after the given time.

	Solvent Concentration (%v/v)					
	25%		50%		90%	
	Free	Immob[b]	Free	Immob	Free	Immob
DMF[b]						
40 °C 3 h	100	100	100	100	77	100
6 h	91	100	93	100	62	100
60 °C 3 h	100	100	100	100	0	20
6 h	100	100	100	94	0	16
80 °C 3 h	70	100	34	65	0	0
6 h	53	70	11	47	0	0
Heptanol						
40 °C 3 h	85	100	95	100	100	100
6 h	62	100	77	100	100	100
60 °C 3 h	43	100	28	100	13	100
6 h	4	100	0	89	0	49
80 °C 3 h	0	62	0	46	0	0
6 h	0	57	0	20	0	0

[a] Stability determined as described in section 3.2.2.3
[b] Immobilised Rt41A proteinase

Other proteinases have been immobilised with a view to increase the thermostability towards organic solvents. α-chymotrypsin was immobilised in a polyacrylamide gel, the resulting preparation showed no loss in activity in 100% v/v ethanol at either 20 °C or 70 °C. The free proteinase, however, had a half-life of <1 h under the same conditions at 20 °C (Mozhaev *et al.*, 1990). Trypsin and α-chymotrypsin immobilised to alumina-phosphocolamine complex also showed increased thermostability to organic solvents (Pugniere *et al.*, 1986). In a number of cases this improved stability towards organic solvents has been taken advantage of, for use of these proteinases in peptide synthesis (Pugniere *et al.*, 1986; Kise *et al.*, 1990; Nilsson and Mosbach, 1984).

CHAPTER 4

PEPTIDE SYNTHESIS WITH *THERMUS* STRAIN Rt41A PROTEINASE

4.1 INTRODUCTION

There is a growing interest in the use of proteases to catalyse the formation of peptide bonds, and numerous studies have been reported to this end (for review see Fruton (1982) and Kullman (1987)). Proteases can catalyse the synthesis of peptide bonds in either a thermodynamically controlled or a kinetically controlled manner.

Thermodynamically controlled synthesis is a direct reversal of the hydrolytic reaction. The protease is used only to increase the rate at which the reaction equilibrium is established (Kasche, 1989). All proteases are able to synthesise peptides in this way. Thermodynamically controlled synthesis is disadvantaged in that longer reaction times and larger amounts of enzyme are required. The equilibrium of the reaction can be shifted towards synthesis allowing quantitative yields (e.g. Cassells and Halling, 1990; Nakanishi *et al.*, 1986) by the use of organic solvents, altering the pH, ionic strength or temperature of the reaction and by the formation of an insoluble product (see section 1.3.3).

Kinetically controlled synthesis requires the formation of an acyl or thiol ester-enzyme intermediate, as is illustrated in Fig. 4.1. Alternatively, proteases with non-overlapping binding sites for the acyl component and nucleophile can be used. Only serine and cysteine proteases are able to form the covalent intermediates. Kinetically controlled synthesis is characterised by shorter reaction times, lower enzyme concentrations, and the use of an activated acyl donor (Jakubke *et al.*, 1985). Proteases with a high transferase to hydrolase activity are necessary for attaining high yields. The yields is also influenced by the properties of the enzyme, acyl donor, and nucleophile, as well as the factors mentioned for thermodynamically controlled synthesis.

Immobilised proteinases are becoming popular for use in peptide synthesis (e.g. Nilsson and Mosbach, 1984; Pugniere *et al.*, 1986; Aldercreutz *et al.*,

Immobilised proteinases are becoming popular for use in peptide synthesis (e.g. Nilsson and Mosbach, 1984; Pugniere *et al.*, 1986; Aldercreutz *et al.*, 1990) due to their increased stability in organic solvents. The ease of separating the enzyme from the products and the potential to reuse the proteinase also makes immobilisation an attractive option.

The free and immobilised proteinase from *Thermus* strain Rt41A has been investigated for their potential use in kinetically controlled peptide synthesis. A number of factors are investigated for the optimisation of Bz-Ala-Tyr-NH_2 synthesis, attention being focused on the catalytic activity of the proteinase; different solvents and the water content of the system was also investigated.

To our knowledge this is the first proteinase from an extreme thermophile (optimum growth temperature >65 °C) to be utilised in this process. The use of a thermostable esterase has been reported (Owusu and Cowan, 1990), however, this was used in transesterification reactions.

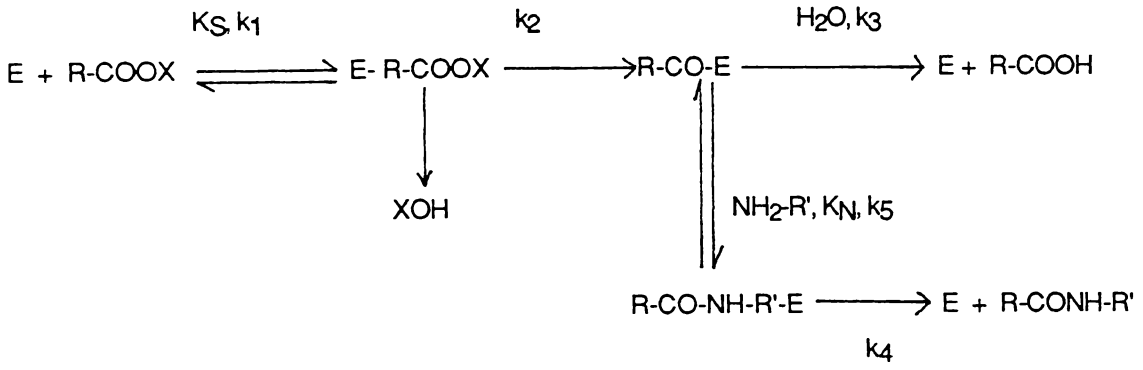


Fig. 4.1. Scheme for Kinetically Controlled Synthesis of Peptides by Serine and Cysteine Proteinases.

E = enzyme, R-COOX = acyl donor, R-CO-E= acyl enzyme intermediate, NH_2-R' = nucleophile and R-CO-NH-R'-E = acyl- enzyme-nucleophile complex, R-CONH-R' = peptide product.

4.2 MATERIALS AND METHODS

All buffers were adjusted to the desired pH at the temperature of use.

4.2.1 Peptide Synthesis with Free and Immobilised *Thermus* strain Rt41A Proteinase

The general procedure for producing peptides with the free and immobilised proteinase is described below. Any deviations from these conditions are detailed in the text or figure legends. The free and immobilised proteinase were prepared as described in section 2.1.1.2 and 2.1.8 respectively. For immobilisation approximately 77 Au/m^2 of beads was applied to 50 nm pore size CPG beads.

N-terminally blocked amino acid esters were incubated with amino acid nucleophiles (generally amides). Approximately 400 Au/ml of the free proteinase was added to the reaction volume. The immobilised proteinase (100 µl/500 µl reaction volume) was washed in de-ionised water on a sintered glass filter. Small amounts of water may still have been present within the pores of the beads, however, this would have resulted in less than a 1.5% change in the final concentration. The beads were then added to the reaction vial. The solvent used was 50% v/v DMF. Stock solutions of 1 M $CaCl_2$ and 625 mM Caps (pH 10.5) containing 0.25% Triton X-100 were added to give final concentrations of 5 mM $CaCl_2$, 50 mM Caps (pH 10) and 0.01% v/v Triton X-100. It should be noted when using this buffer at such a high concentration, the final pH of the solvent/buffer mix dropped significantly when diluted in the presence of the solvent. The 625 mM Caps buffer was therefore, adjusted to pH 10.5 at the temperature of use, which gave the desired final pH of 10 when diluted. The concentrations of the acyl component and nucleophile were 25 and 100 mM respectively.

4.2.2 HPLC Analysis

The amino acid substrates and dipeptide products were separated on a reverse phase C_{18} column (5 µm, Brownlee Labs, U.S.A.) which was connected to a Waters HPLC system. An 18 ml gradient of 10-100% v/v

acetonitrile (Hipersolv) containing 0.1% v/v TFA was utilised to elute the substrates and products from the column, at a flow rate of 1 ml/min. The column temperature was maintained at 30 °C. The products were detected at 240 nm or 280 nm with a Shimadzu UV spectrophotometer (model SPD-6A).

4.2.3 Amino Acid Analysis: PICO-TAG Method.

Product peaks from HPLC analysis were collected in borosilicate glass vials which had been washed in 6N HCl (Analar, 40 °C for 4 h). After lyophilisation the samples were prepared for acid hydrolysis by the addition of 100 µl of 6N HCl (Aristar) containing 1% v/v phenol. The vials were then purged with oxygen free nitrogen, followed by evacuation. This procedure was repeated three times, the vials were then sealed under vacuum. After hydrolysis for 1 h at 150 °C or 4 h at 110 °C the samples were dried under reduced pressure. Redrying agent (ethanol:water:TEA, 2:2:1) was added and removed under vacuum.

Following the procedure of Bidlingmeyer *et al.* (1984) the derivatization of the amino acids was carried out by the addition of a mixture of ethanol, TEA, water and PITC in the ratio of 7:1:1:1 respectively. After mixing and reacting for 20 min at room temperature the samples were dried under vacuum as before.

The derivatised sample was reconstituted in 5 mM disodium orthophosphate buffer (adjusted to pH 7.4 with 10% v/v phosphoric acid), containing 5% v/v acetonitrile. The sample was then injected onto a Novapak C_{18} reverse phase column which was maintained at 40 °C. The two eluting solvents used were: Eluent A- 85 mM anhydrous sodium acetate pH 6.4 (with acetic acid), containing 0.05% v/v TEA, 0.02% w/v EDTA. This was filtered (0.2 µm) and acetonitrile added to a final concentration of 6% v/v. Eluent B - 60% acetonitrile solution containing 0.02% w/v EDTA. This was filtered using a solvent-resistant 0.45 µm filter (Durapore). Both eluents were continuously sparged before and during use with helium. The gradient utilised to elute the amino acids is shown in Table 4.1.

Amino acid standards (BDH Sepramar 'A') were treated in an identical manner as the samples, except without acid hydrolysis. Comparisons of these

Table 4.1 Amino Acid Analysis Gradient Profile

Time (min)	Flow Rate (ml/min)	%A	%B	Grad. Curve[a]
0	1.0	100	0	*
10	1.0	54	46	5
10.2	1.0	0	100	6
11.7	1.0	0	100	6
12.0	1.5	0	100	6
12.2	1.5	0	100	6
12.5	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	6

a Gradient curve profile number as specified by Waters (see Appendix 3)

standards with samples enabled identification of peaks and the calculation of yields.

4.2.4 Optimisation of Bz-Ala-Tyr-NH_2 Synthesis

Bz-Ala-OMe and Tyr-NH_2 were incubated with free and immobilised Rt41A proteinase, under varying conditions to optimise the yield. The amount of Bz-Ala-Tyr-NH_2 produced was determined by quantitatively relating amino acid analysis to HPLC peak areas, as described in appendix 4. The amount of Bz-Ala-OMe remaining and Bz-Ala-OH produced were calculated from standard curves (see appendix 4.2, Figs. A4.3 and A4.4). The initial rates of Bz-Ala-Tyr-NH_2 production and Bz-Ala-OMe hydrolysis with the free enzyme were calculated from duplicate samples (50 µl) taken at 1, 2, 4, 6, 8 and 10 min, the reaction was stopped by the addition of 50 µl of 1% v/v TFA in DMF. Maximum yields for both the free and immobilised proteinase were determined once the ester substrate had been completely utilised or no enzyme activity remained.

4.2.5 Calculation of the Partition Constant

From the initial rates of peptide bond formation it was possible to calculate the partition constant, p, of the reaction, which is defined as an efficiency parameter for the nucleophile in acyl transfer reactions (Schellenberger *et al.*, 1991c). A decrease in the partition constant indicates an increase in aminolysis compared to hydrolysis. The ratio of the rate of hydrolysis (V_H) and aminolysis (V_A) is dependent on the nucleophile concentration [N] and is given by equation. (1):

$$\frac{V_H}{V_A} = \frac{k_3 K_N + [N] k_5}{[N] k_4} = \frac{p}{[N]} \qquad (1)$$

Where k_3, k_4 and k_5 represent the rate constant for the production of the hydrolysis and aminolysis products and hydrolysis of the acyl-enzyme-nucleophile complex respectively (see Fig. 4.1). As there is a 4 fold excess of the nucleophile in relation to the acyl donor and the yields observed in the initial stages of the reaction are less than 1%, the concentration of the nucleophile was considered to be constant. The partition constant was calculated from equation (2).

$$p = \frac{V_H [N]}{V_A} = \frac{[P_2] [N]}{[P_3]} \qquad (2)$$

Where $[P_2]$ and $[P_3]$ are the concentrations of the hydrolysis (R-COOH) and aminolysis products (R-CONH-R') respectively. The relationship between p and the concentration of the nucleophile is described by equation (3):

$$p = \frac{k_3 K_N}{k_4} + \frac{[N] k_5}{k_4} \qquad (3)$$

Both the maximum yields and the partition constants were calculated from duplicate samples; the standard error of the values given is ≤ 5%.

4.2.6 Immobilised *Thermus* strain Rt41A Proteinase Column

An HPLC column (Merck, 4 x 250 mm, 3.14 ml) was packed with Rt41A proteinase immobilised onto 2 ml of CPG beads (see sections 2.18 and 4.2.1) by gravity . The remainder of the column was filled with CPG beads of the same dimensions, but without immobilised proteinase.

4.2.6.1 Hydrolysis of SucAAPFpNA

The immobilised enzyme column was connected to HPLC pumps (Waters, USA, model 45). The flow rate was maintained at either 0.7 or 1.5 ml/min. The column was equilibrated in 50 mM Hepes (pH 8) containing 5 mM $CaCl_2$. 1 µmole of SucAAPFpNA was then applied. The release of pNA, in the collected fractions, was measured spectrophotometrically at 410 nm. Yields were calculated from the extinction coefficient (8880 $M^{-1}cm^{-1}$).

4.2.6.2 Synthesis of Bz-Ala-Tyr-NH_2

For peptide synthesis an Eldex meter pump (USA, model A-30-5-2) was utilised which enabled flow rates of less than 0.1 ml/min to be achieved. The column was equilibrated with 50% v/v DMF containing 50 mM Caps (pH 10) + 1 mM $CaCl_2$ (buffer C) at 40 °C. 5 ml of the substrates (25 mM Bz-Ala-OMe + 100 mM Tyr-NH_2 in buffer C) was then applied. The column was then reequilibrated with buffer C. Fractions (20 x 0.5 ml) were collected and yields determined by HPLC analysis as described in section 4.2.4.

4.3 RESULTS and DISCUSSION

4.3.1 Substrate Specificity, Initial Screen.

Potential substrates for peptide synthesis were chosen on the basis of Rt41A proteinase cleavage sites in oxidised insulin B chain and esterase activity (Peek, unpublished results, see Fig 2.8 and Table 2.5). Combinations of Ac-

Phe-OEt, Ac-Trp-OEt, Ac-Tyr-OEt and Bz-Ala-OMe (acyl donors) were tested with Phe-, Tyr-, Trp-, Ala-, Glu-and His- amides (as nucleophiles) using the free proteinase. Substrates were added in equimolar concentrations (25 mM) to 50 mM Hepes (pH 8.0) containing 5 mM $CaCl_2$ and 10% v/v DMF. 300 Au of the free proteinase was added prior to incubation at 70 °C.

The production of Ac-Phe-Tyr-NH_2, Ac-Tyr-Trp-NH_2, Bz-Ala-Trp-NH_2, Bz-Ala-Tyr-NH_2, Bz-Ala-Phe-NH_2 was confirmed by amino acid analysis. The presence of tryptophan in the product peak was proven spectrophotometrically at 280 nm due to its instability in the acid hydrolysis step of the amino acid analysis. It was also suspected, from the appearance of 2 new peaks (possibly Ac-Tyr-OH and Ac-Tyr-Tyr-NH_2), that Ac-Tyr-Tyr-NH_2 was produced. However yields were very low and conclusive evidence could not be obtained. From this screen the synthesis of Bz-Ala-Tyr-NH_2 was arbitrarily chosen to investigate conditions for yield optimisation. The yield observed for Bz-Ala-Tyr-NH_2 was subsequently determined to be 0.75%.

4.3.2 Optimisation of Bz-Ala-Tyr-NH_2 Synthesis with Free and Immobilised *Thermus* strain Rt41A Proteinase

Rt41A proteinase, section 2.1.1.2, was immobilised to compare the ability of the free and immobilised proteinase to synthesise peptides. A higher enzyme loading was utilised in this case (compared to section 3.3.4) to try and concentrate the proteinase. It has generally been found that larger amounts of enzyme are required for synthesis as opposed to hydrolysis reactions, as observed by Oka and Morihara (1977). The percent recovery, of the enzyme applied, was determined with azocasein and SucAAPFpNA as substrates and was 18% and 50% respectively. The lower percent recovery in the case of the azocasein assay compared to previous results may be due to the higher enzyme loading. Less substrate may have access to the proteinase due to steric hindrance or increased internal diffusional limitations as discussed in section 3.3.1. The percent recovery, in the case of the SucAAPFpNA substrate, was also lower than found previously (section 3.3.5). This could be due to the rate of hydrolysis of the substrate being faster than the rate of diffusion, therefore the total activity is under-estimated as discussed in section 1.2.3.2. The actual protein bound in this case was not determined.

The scheme for the synthesis of Bz-Ala-Tyr-NH_2 is illustrated in Fig. 4.2. The optimisation of the production of this peptide is outlined in the following sections. Initial rates for both Bz-Ala-Tyr-NH_2 and Bz-Ala-OH production were calculated for the free proteinase only. This is due to rapid settling of the immobilised proteinase during sampling, making it difficult to maintain a constant concentration of the proteinase, in relation to the reaction volume, throughout the experiment.

Representative traces showing the disappearance of the Bz-Ala-OMe substrate and the appearance of product peaks are given in Fig. 4.3a,b.

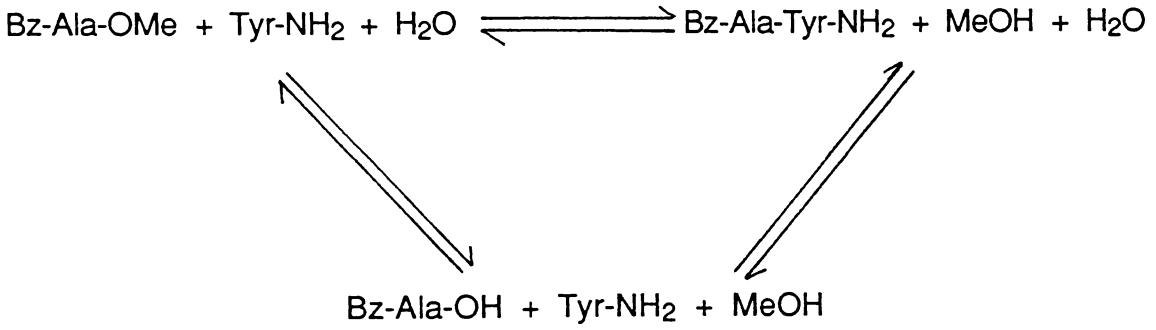


Fig. 4.2 Scheme for Synthesis of Bz-Ala-Tyr-NH_2 with *Thermus* strain Rt41A Proteinase.

4.3.2.1 Effect of the Reaction pH

The effect of pH on the partition constant and the maximal yield for Bz-Ala-Tyr-NH_2 production with the free and immobilised proteinase is illustrated in Fig. 4.4a,b. The partition constant decreases with increasing pH until pH 9, above this value there is little change in the partition constant. The maximal yields for both the free and the immobilised proteinase are best at the alkaline end of the pH scale. Although the variation in yields within the pH range 9-11 are small the optimal pH is 10 for both proteinases.

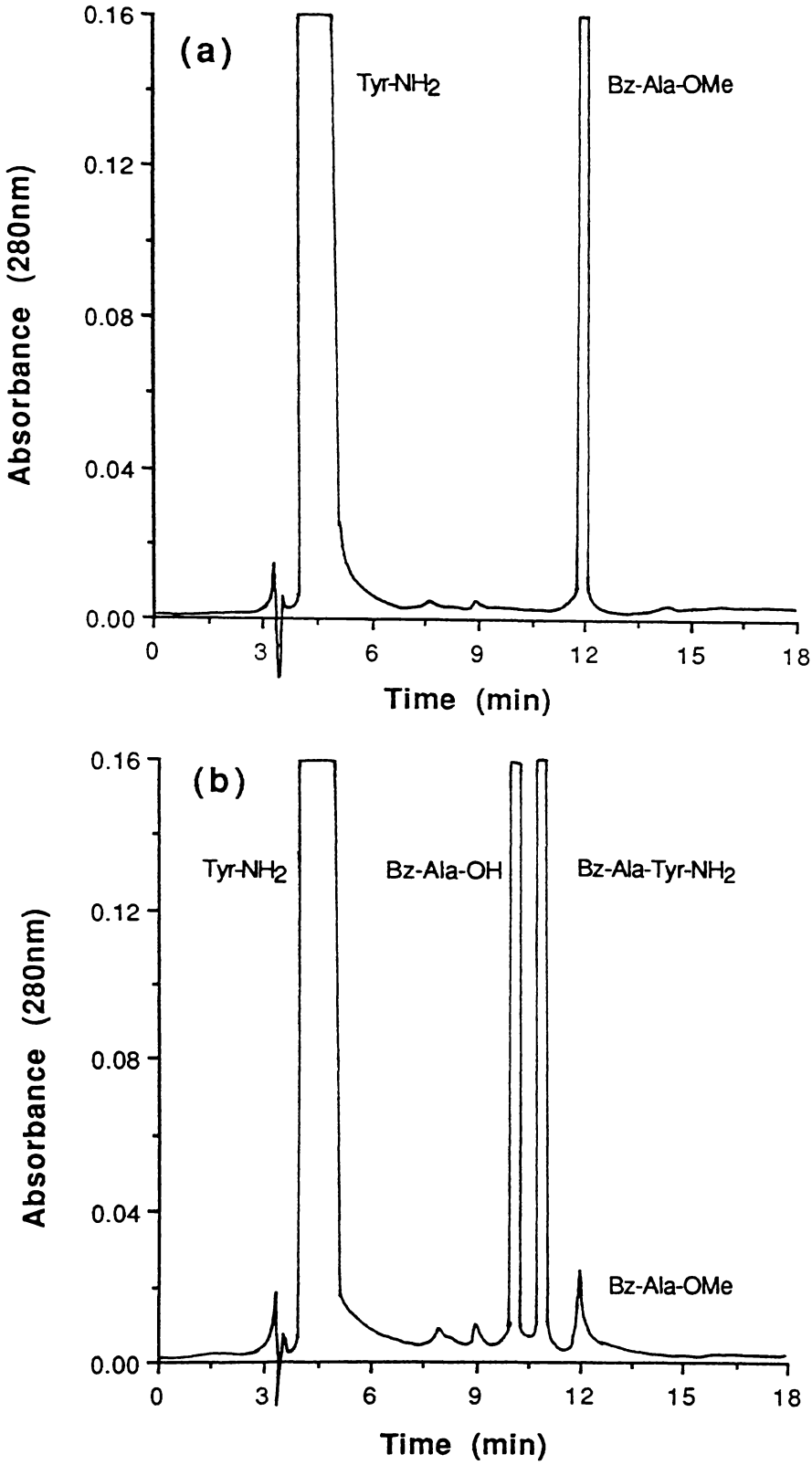


Fig 4.3 HPLC Traces Showing Bz-Ala-Tyr-NH_2 Synthesis.

Taken from the Time course experiment illustrated in Fig 4.11. HPLC conditions as described in section 4.2.2. (a) No enzyme addition and (b) 21 h after enzyme addition.

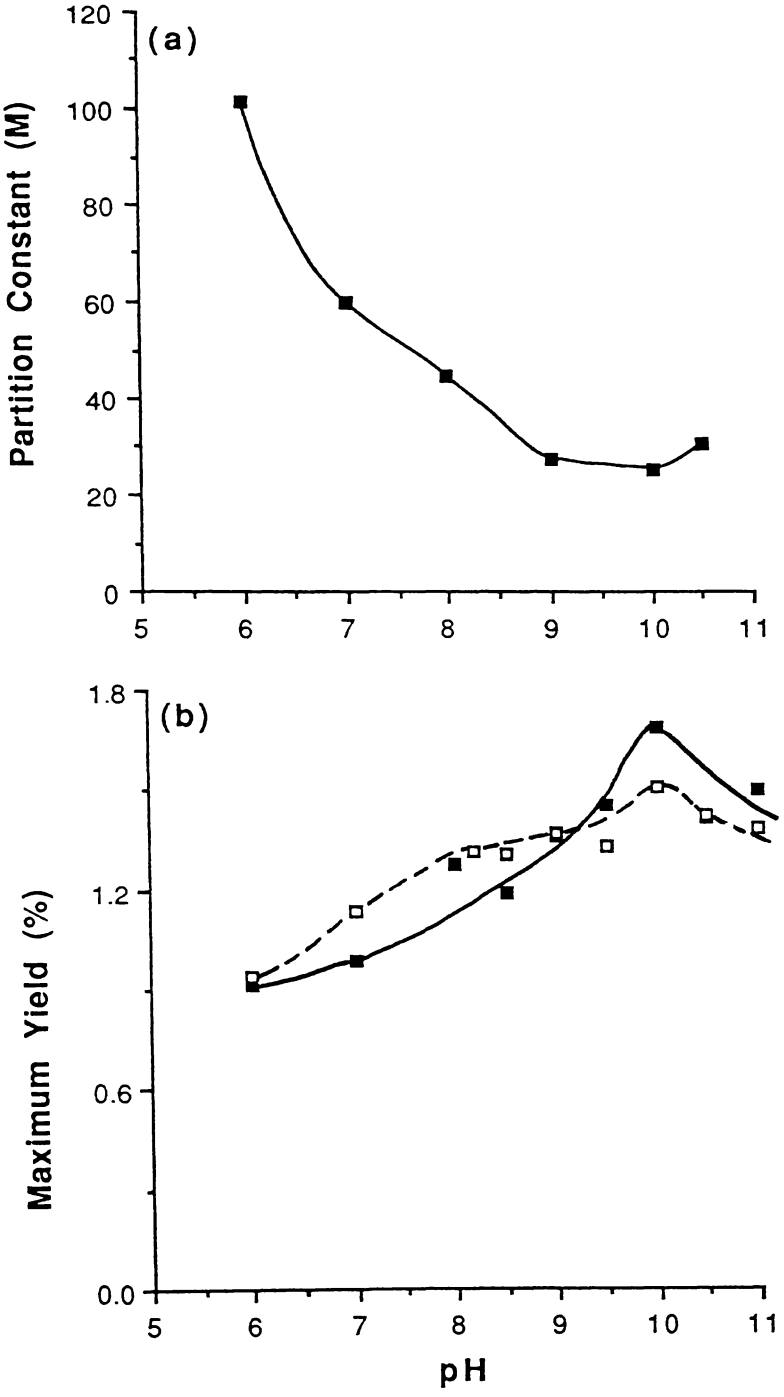


Fig 4.4 The Effect of the Reaction pH on Bz-Ala-Tyr-NH_2 Synthesis.

(a) The effect on the partition constant with free *Thermus* strain Rt41A proteinase.
(b) The effect on the maximum yield with free [-■-] and immobilised [- □ -] *Thermus* strain Rt41A proteinase.
The reaction volume consisted of 50% v/v DMF in 50 mM Hepes (pH 6,7,8) or 50 mM Caps (pH 8.5-11) buffer containing 5 mM $CaCl_2$. Other details are as in section 4.2.1.

These results are as expected. For deacylation of the acyl-enzyme intermediate the nucleophile must be uncharged. At pH values above the pK_b of the substrate it will become increasingly deprotonated as the pH increases, therefore maximum yields will be achieved (Kasche, 1986). The substrate will be approximately 98% deprotonated at 2 pH units above the pK_b. At pH values above the pH optimum the yield decreases, most likely due to the increasing rate of deacylation by the OH ions present. The pK_b for tyrosine amide is 7.7 (Fersht *et al.*, 1973), therefore maximal deprotonation of the reactive amino group will occur above this pH value. The high partition constant observed in the pH range 6-8 reflects the high protonation of the nucleophile at more acidic pH values. The pH optimum is also comparable to chymotrypsin catalysed peptide synthesis (Morihara and Oka, 1977). All further synthetic reactions were carried out at pH 10.

4.3.2.2 Effect of the Solvent Concentration

Although some enzymes have been found to catalyse peptide bonds under aqueous or near aqueous conditions (Cramer and Horvath, 1989a; Bratovanova *et al.*, 1988) in most cases the addition of a co-solvent improves the yields. Both water-miscible (e.g Kise and Shirato, 1988) and water-immiscible solvents (e.g Nakanishi *et al.*, 1986) have been utilised. The initial optimisation of synthesis in this case was restricted to DMF, a water-miscible solvent.

The effect of the DMF concentration on the partition constant is shown in Fig. 4.5a. Increasing the solvent concentration up to 40% increases the partition constant. There is little change in the partition constant between 40 and 75% solvent, whereas increasing the concentration from 80 to 90% caused a dramatic drop in the partition constant.

The initial increase in partition constant at low solvent concentrations is unexpected, in that, if any effect is seen one would expect a decrease in the partition constant due to reduced hydrolysis of the acyl-enzyme complex. In most cases the addition of an organic solvent decreases the activity of enzymes (Khmelnitsky *et al.*, 1991). In this case the rate of aminolysis decreases faster than the rate of hydrolysis, hence the increase in the partition constant in the range 10-40% solvent. This effect (an increase then a

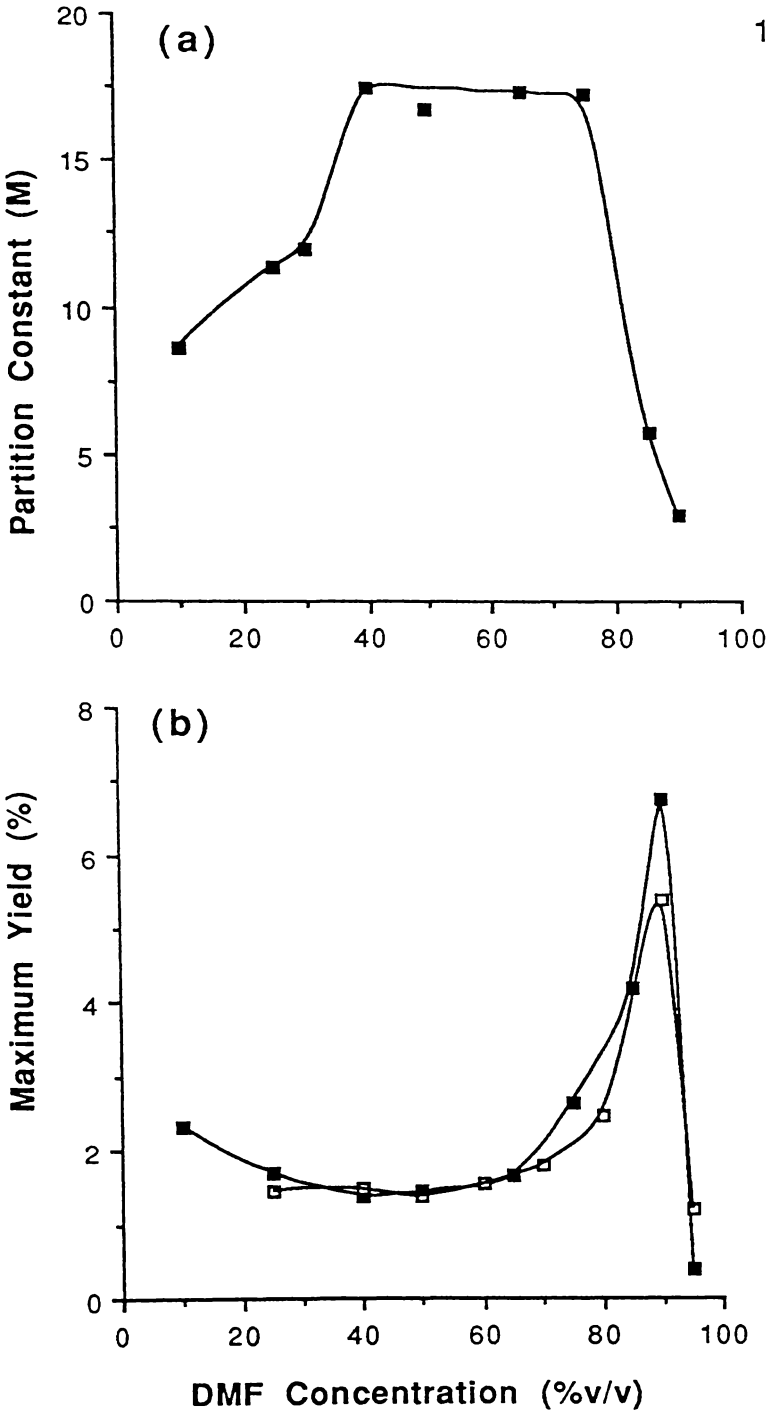


Fig 4.5 The Effect of the DMF Concentration on the Bz-Ala-Tyr-NH_2 Synthesis.

(a) The effect on the partition constant, with free *Thermus* strain Rt41A proteinase.
(b) The effect on the maximum yield, with free [■] and immobilised [□] *Thermus* strain Rt41A proteinase. Details as in section 4.2.1.

decrease in the partition constant with increasing solvent concentration) has not been reported before; generally a decrease in the partition constant with increasing solvent concentration is observed (e.g Schellenberger *et al.*, 1991d).

The decrease observed at the highest concentrations is likely to be caused, in part, by a decrease in the hydrolysis of the acyl-enzyme intermediate. Because of the dramatic nature of the change an additional factor may be involved. This could be a solvent effect on the enzyme or the nucleophile, resulting in increased binding of the nucleophile to the S_1' subsite. Kasche *et al.* (1991) has shown that hydrophobic solvents bind to α–chymotrypsin, which produces structural changes in the S_1 and to a greater extent the S_1' subsite of the enzyme. Although DMF is not hydrophobic a similar effect may be occurring here.

The maximum yields for the free and immobilised proteinase show a similar trend to the partition constant in that there is a slight decrease in the maximum yield up till 40%, then little change until 75% at which point the maximum yield increases.

The maximum yields observed between 10-90% v/v DMF directly reflect the effect of increasing solvent concentration on the partition constant. The sharp decrease in the maximum yield at 95% v/v solvent for the free and immobilised proteinase is due to enzyme instability in both cases. This was realised from the fact that significant amounts of Bz-Ala-OMe remained at the end of the reaction and no proteinase activity could be detected. The free proteinase appears to be inactivated in the first 4 min of the reaction at 95% v/v DMF, from the calculation of initial rates, (results not shown). The free proteinase is also inactivated at 90% v/v DMF, but the rate of inactivation is slow enough to allow the reaction to go almost to completion. The immobilised proteinase, however, appears to be more stable at 95% v/v DMF compared to the free proteinase and results in a higher yield (Fig. 4.5b).

Because of the unusual effect of the DMF concentration on the partition constant, the effect of the acetonitrile concentration on the partition constant and maximal yield was also investigated with the free proteinase (see Fig. 4.6a,b). Acetonitrile has near identical effects on the partition constant and maximum yields as found for DMF. This suggests that the observed effect is

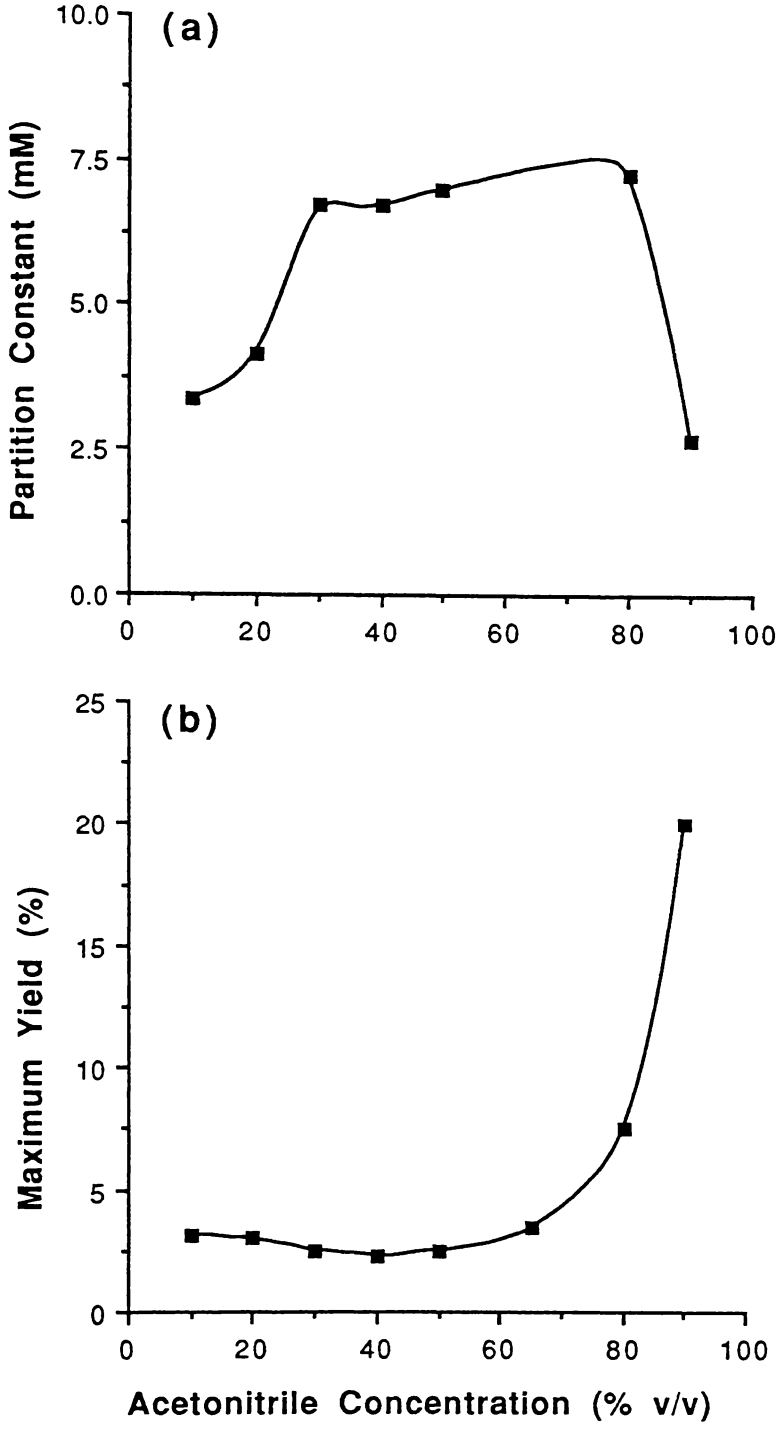


Fig 4.6 The Effect of the Acetonitrile Concentration on Bz-Ala-Tyr-NH_2 Synthesis.

(a) The effect on the partition constant
(b) The effect on the maximum yield with free *Thermus* strain Rt41A proteinase.
Details as in section 4.2.1.

not due to DMF *per se*, but a general effect that this type of solvent has on the proteinase itself. At all solvent concentrations lower partition constants are observed with acetonitrile and at all solvent concentrations the maximum yields were higher. The reason for the increased yields with acetonitrile is not clear. Acetonitrile has a slightly higher log P value (partitioning of solvent between water and octanol) than DMF (-0.33 compared to -1.0, Laane *et al.,* 1987), which may be responsible for the increased yield. However, solvents that are generally thought of as more useful for enzymatic synthetic reactions have log P values of >4, therefore the increase in log P in this instance is relatively low. Alternatively, acetonitrile may increase the binding of the nucleophile, possibly by causing conformational changes in the proteinase. Kasche and coworkers (1991) have shown that the binding of organic solvent molecules can influence the P_1'-P_2' stereo and sequence specificity of α-chymotrypsin catalysed peptide synthesis, as mentioned earlier. A similar affect could be occurring here. It is difficult, however, to draw conclusions without testing other solvents.

4.3.2.3 Effect of the Ionic Strength

The effect of ionic strength on the synthesis of Bz-Ala-Tyr-NH_2 is shown in Fig. 4.7a,b. The partition constant decreases up to an ionic strength of 0.5 (NaCl) and is not affected by any further increases in ionic strength. The maximum yield, however, increases linearly with ionic strength.

Increased yields are normally observed with increasing solvent concentration when equal charges on the enzyme and nucleophile are involved (Kasche, 1986). As the nucleophile should be deprotonated at the pH used here (section 4.3.4.1) and, tyrosine has no additional charged functional groups the reacting nucleophile should be uncharged.

The cause of the different effect ionic strength has on the partition constant as compared to the maximal yield is not known. It appears that the factors that are affecting the initial rates are different from those affecting the maximum yield, or a combination of effects are occurring.

The effect of electrostatic effects on α–chymotrypsin catalysed acyl transfer has been reported by Schellenberger *et al.* (1991a,b). The effect of ionic

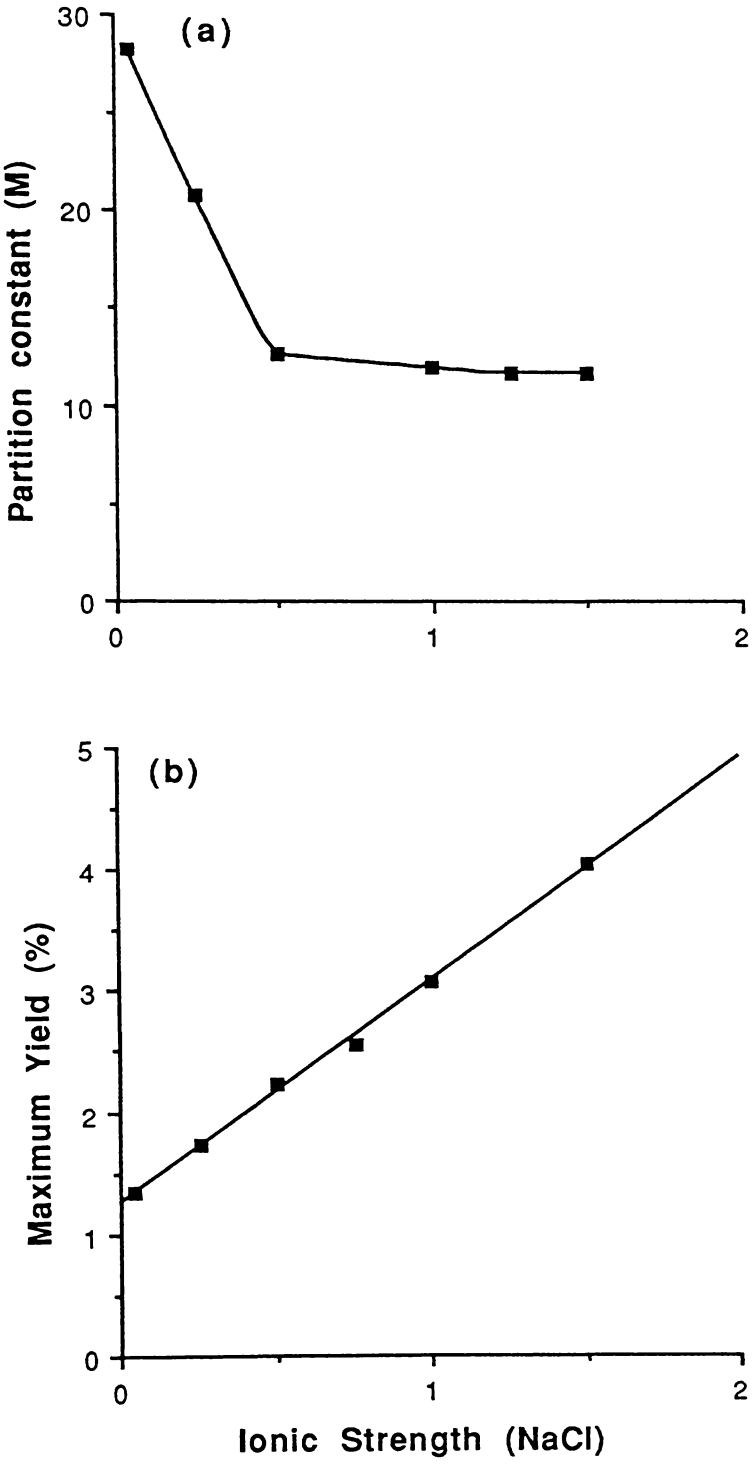


Fig 4.7 The Effect of Ionic Strength on Bz-Ala-Tyr-NH_2 Synthesis.

(a) The effect on the partition constant.
(b) The effect on the maximum yield with free *Thermus* strain Rt41A proteinase.
The solvent concentration was 50% v/v DMF. Other details as in section 4.2.1.

strength on positively and negatively charged and uncharged nucleophiles was investigated. With respect to uncharged nucleophiles a decrease in the partition constant with increasing ionic strength (KCl) was observed. These results are consistent with those observed here. The authors described the influence of salts in this case to be due to the salt dependence of the activity coefficients of the uncharged nucleophiles by a salting out/salting in effect. The increase in the activity coefficient may be due to the decrease in the pK of the nucleophile with increasing ionic strength. Alternatively, salt-dependent conformational changes of the enzyme may also be occurring causing increased nucleophile binding. Yet another possibility is that the increasing salt concentration may be decreasing the water activity, thereby increasing yields in a similar manner to increasing the solvent concentration. There is no evidence to suggest which of these effects is causing the increase in peptide yield.

4.3.2.4 Effect of Increasing the Nucleophile Concentration.

Increasing the concentration of the nucleophile in relation to the acyl component has been shown to increase peptide synthesis yields (e.g. Shin and Takahashi, 1989). As the solubility of Tyr-NH_2 is limited to approximately 500 mM in 50% DMF, the concentration of Bz-Ala-OMe was reduced to 10 mM, allowing high Tyr-NH_2/Bz-Ala-OMe ratios to be obtained. However, the initial rates decreased introducing large errors when calculating the ratio of aminolysis to hydrolysis. Therefore, for this experiment the ratio of $[P_3]/[P_2]$ (equation 2) was used to investigate the effect of the nucleophile concentration on the partition constant. The low maximum yields observed and the excess of nucleophile, meant that the nucleophile concentration was essentially constant. This was therefore, a valid means, for calculating the ratio of $[P_3]/[P_2]$. The ratio of aminolysis to hydrolysis is linearly dependent on the nucleophile concentration (Fig. 4.8a). The slope of the plot is equal to $1/p$, the partition constant is therefore independent of the nucleophile concentration. This suggests that there is no hydrolysis of the acyl-enzyme-nucleophile complex, i.e $k_5 << k_4$ (see equation (3) and Fig. 4.1). The maximum yields are also linearly dependent on the nucleophile concentration for the free proteinase, whereas, the immobilised proteinase is linear initially then the rate of increase in yield with increasing nucleophile concentration appears to decreases (see Fig. 4.8b). This could be due to diffusional limitations of the substrate or products.

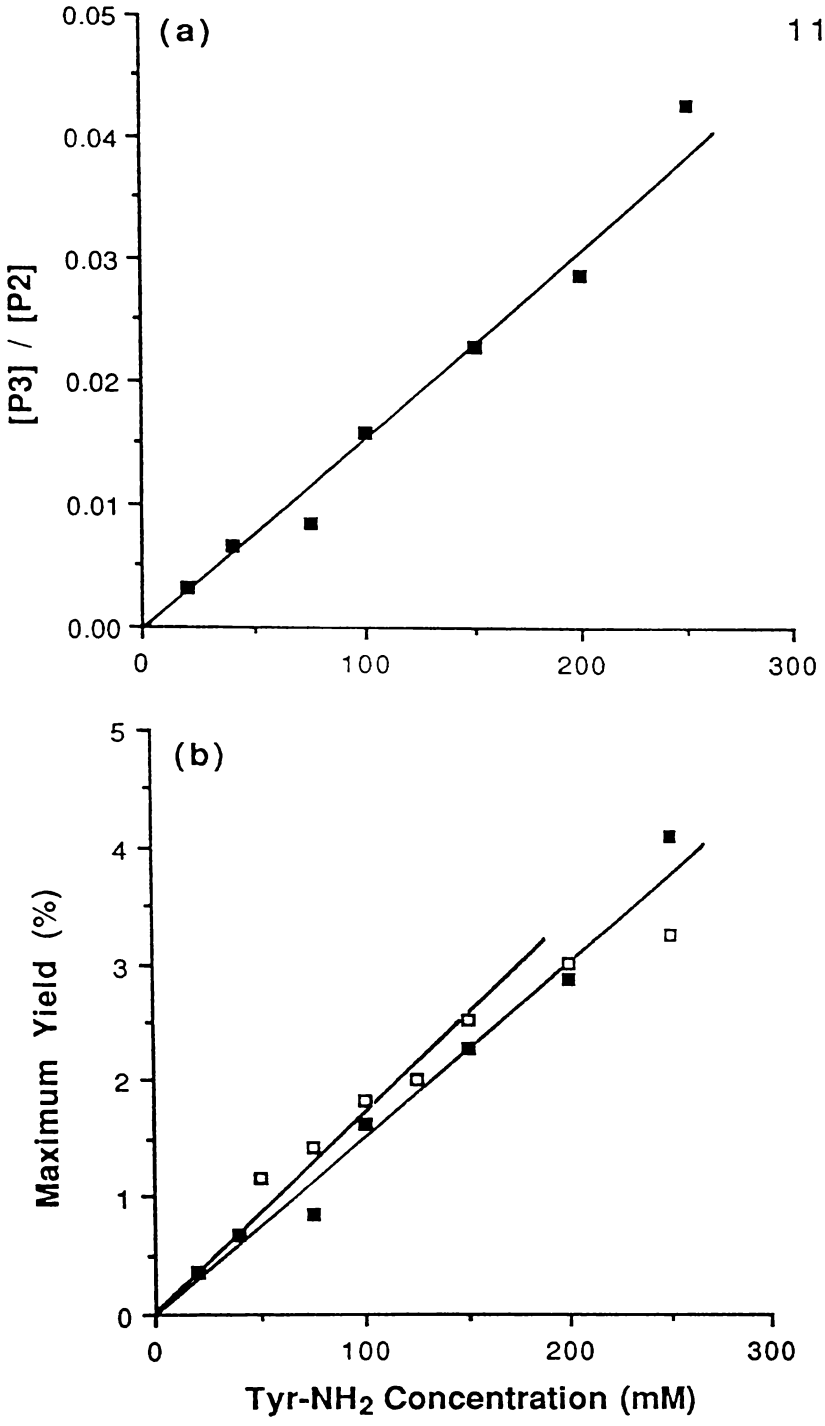


Fig 4.8 The Effect of the Nucleophile Concentration of Bz-Ala-Tyr-NH_2 Synthesis.

(a) The effect on the ratio of[P3]/[P2] with free *Thermus* strain Rt41A proteinase.
(b) The effect on the maximal yield with free [■] and immobilised [□] *Thermus* strain Rt41A proteinase.
The solvent concentration was 50% v/v, and the Bz-Ala-OMe concentration was 10 mM. Other details as in section 4.2.1.

4.3.2.5 Effect of Temperature.

Increasing the temperature affects the partition constant for Bz-Ala-Tyr-NH_2 synthesis as is seen in Fig. 4.9a. Optimal partitioning of aminolysis and hydrolysis occurs at about 40 °C, the partition constant is greater at the two other temperatures. Similar results have been reported by Schellenberger *et al.*, (1991c). At two different acetonitrile concentrations optimal partitioning was observed for trypsin at 30 °C, the mid-point of the temperature range (20-40 °C).

The maximum yields for the synthesis of Bz-Ala-Tyr-NH_2 by free and immobilised Rt41A proteinase, at various temperatures, are illustrated in Fig. 4.9b. A decrease with increasing temperature is observed. Relatively few studies have investigated the effect of this parameter on kinetically controlled peptide synthesis (e.g Nilsson and Mosbach, 1984; Reichmann and Kasche, 1985), but it has been found that generally the yields decrease with increasing temperature. Kasche (1986) predicted, from the effect that temperature would have on the maximum yield equation, that this would be the case. Results were also presented to support this prediction.

The partition constant for the reaction at 20 °C is higher than expected when compared to the maximal yield found at the same temperature. Repeat experiments have shown that this is a real observation. There is no evidence to suggest why the disparity in the partition constant and maximal yield is observed. It may be that the hydrolysis and aminolysis reactions are differentially affected with time at 20 °C.

Although the solvent stability of the free and immobilised proteinase has already been investigated (section 3.3.5.5) the stability of the free proteinase under the conditions used in the peptide synthesis reactions was determined. The results of this investigation are illustrated in Table 4.2. The half-life of the proteinase in 25 % v/v DMF is 21 h at 20 and 40 °C and 13 h at 60 °C. The percent activity remaining at the time the maximal yields were determined were approximately 50% at 20 and 40 °C and 25% at 60 °C. At 80 °C the half-life of the proteinase is only 1 h; the reaction must be sufficiently rapid to compensate for this, as all of the acyl substrate is utilised. Stability of the

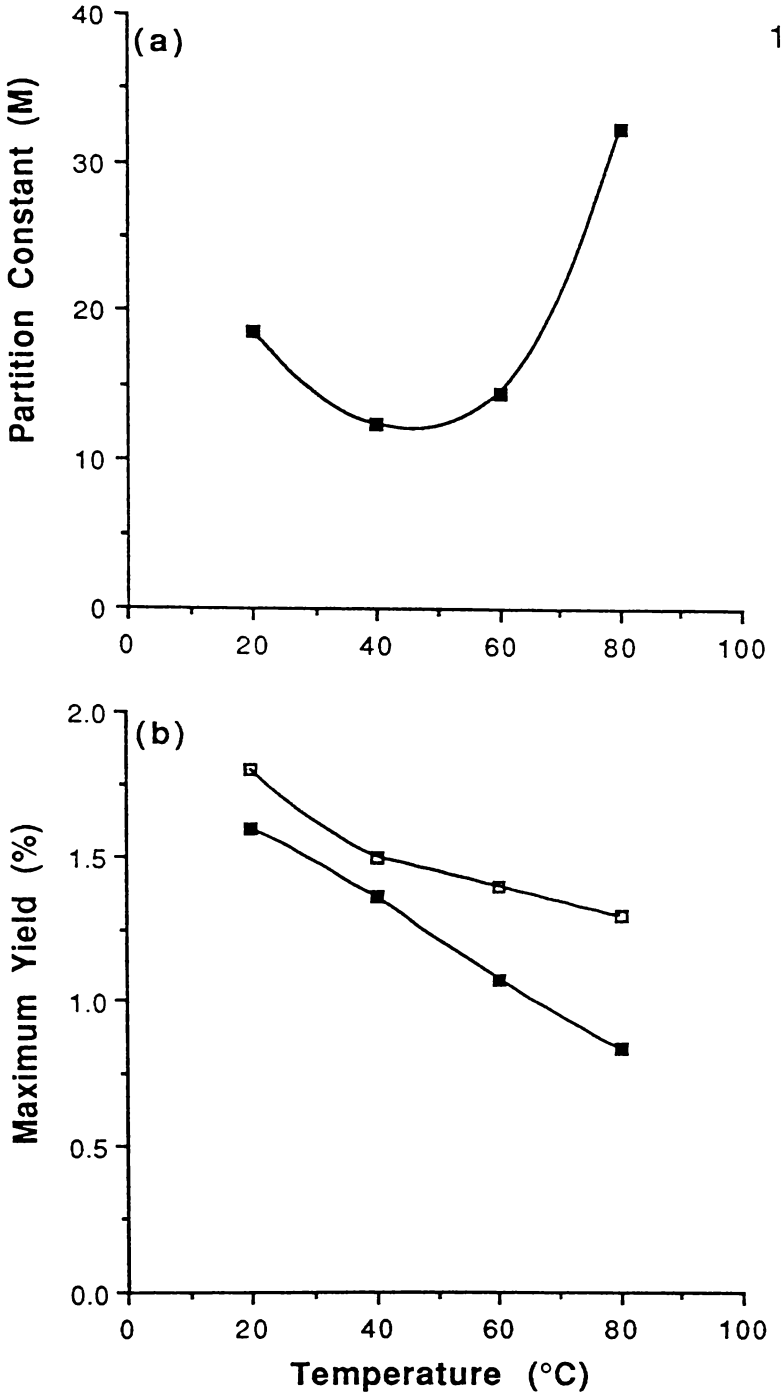


Fig 4.9 The Effect on Temperature on Bz-Ala-Tyr-NH_2 Synthesis

(a) The effect on the partition constant with free *Thermus* strain Rt41A proteinase
(b) The effect on the maximum yield with free [■] and immobilised [□] *Thermus* strain Rt41A proteinase . The solvent concetration was 25% v/v DMF. Other conditions as in section 4.2.1.

Table 4.2 Thermostability of Free *Thermus* strain Rt41A Proteinase in the Presence of DMF and Peptide Synthesis Reaction Conditions[a].

Values are quoted as half-lives (h).

Temp (°C)	% Solvent (v/v) 25	50	90
20	21	ND	8.5
40	21	21	3
60	13	13	<0.2
80	1	0.5	0.1

[a] 25 mM Bz-Ala-OMe, 100 mM Tyr-NH_2, 50 mM Caps (pH 10), 5 mM $CaCl_2$, 40 µg of proteinase/ml

enzyme does not, therefore, affect the maximum yield results presented above.

The half-lives at 50% v/v DMF are similar to those at 25% v/v DMF except that at 80 °C the half-life is is reduced to 0.5 h. In the presence of 90% v/v DMF the stability of the proteinase drops dramatically, especially with increasing temperature. Although the proteinase activity is decreased at 90% v/v DMF the rate of the reaction is sufficiently high to utilise most of the Bz-Ala-OMe substrate at 20 and 40 °C. Because of the short half-life of the proteinase at 60° and 80 °C, it is essential to use lower temperatures when using 90% v/v DMF.

4.3.2.6 Effect of Bz-Ala-OMe Concentration

The effect of altering the concentration of the acyl donor was not investigated in detail, three concentrations (82, 164, and 246 mM) of Bz-Ala-OMe were combined with 615 mM Tyr-NH_2. It should be noted that at this concentration the Tyr-NH_2 is not completely soluble. The Bz-Ala-OMe concentrations above were chosen as it was determined that the K_m hydrolysis of Bz-Ala-OMe, i.e. in

the absence of Tyr-NH_2, is 184 mM (Fig. 4.10). The yield of peptide (mM produced) increased with increasing Bz-Ala-OMe concentration as is expected (see Table 4.3). Conversely, the % yield (compared to the starting concentration of ester) decreased slightly. No advantage was gained, in terms of percent yield, by using higher acyl donor concentrations than 82 mM.

4.3.2.7 Time Course under Semi-optimised Conditions

A combination of the parameters investigated during optimisation of synthesis were utilised to produce Bz-Ala-Tyr-NH_2, the results of which are shown in Fig. 4.11. The reaction conditions used were 90% v/v DMF, 82 mM Bz-Ala-OMe, 615 mM Tyr-NH_2 at 40 °C and at pH 10. The time course illustrates the increase in the products, Bz-Ala-OH and Bz-Ala-Tyr-NH_2 with the simultaneous utilisation of the substrate, Bz-Ala-OMe. The maximum yield observed in this reaction was 26%. This a 35 fold increase from that observed for Rt41A proteinase synthesis of Bz-Ala-Tyr-NH_2 in the initial substrate screen (section 4.3.1). The time course also illustrates that hydrolysis of the peptide product does not occur to any significant extent. This is illustrated by Bz-Ala-Tyr-NH_2 production continuing to rise up to the point of substrate exhaustion and is characteristic of case (ii) synthesis (see section 1.3.3). This indicates that the expected maximum yield for the reaction can be calculated from the initial rates of aminolysis and hydrolysis by utilising the relationship in equation (4):

$$[\text{R-CONH-R'}]_{max} = \frac{[\text{R-COOX}]_o\,[\text{R'-NH}_2]_o}{[H_2O]/(k_4/k_3)_{app} + [\text{R'-NH}_2]_o} \qquad (4)$$

where

$$\frac{k_4}{k_3} = \frac{V_A}{V_H} \cdot \frac{[H_2O]}{[N]} \qquad (5)$$

From equation (5) (k_4/k_3) was calculated to be 5.68. Inserting this into equation (4) gave a predicted maximum yield of 31.8 mM. This is higher than the observed maximum of 21.6 mM. The cause of the difference between these two values is not certain. Although the proteinase loses activity during the reaction period (see section 3.3.5.3) this should not affect the yield as the

Table 4.3 Effect of Bz-Ala-OMe Concentration on Bz-Ala-Tyr-NH_2 Synthesis with *Thermus* strain Rt41A Proteinase[a]

Ester concn (mM)	Yield (mM)	Yield (% of Ester)
82	8.8	9.0
164	15	7.5
246	20	6.6

[a] 50% v/v DMF, 40 °C

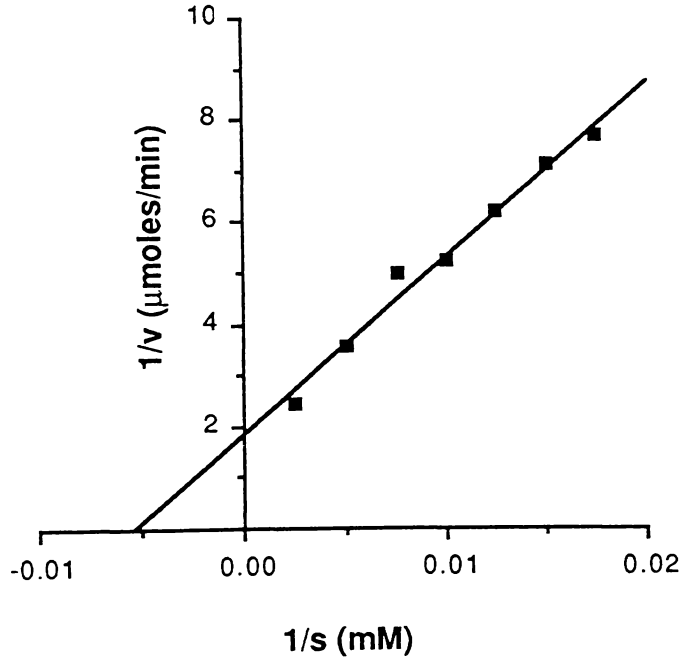


Fig. 4.10 Lineweaver Burke Plot for Bz-Ala-OMe Hydrolysis.

The solvent concentration was 50% v/v DMF, 250 azocasein units of free *Thermus* strain Rt41A proteinase/ml was added. hydrolysis was carried out in the absence of Tyr-NH_2. Other details as in section 4.2.1.

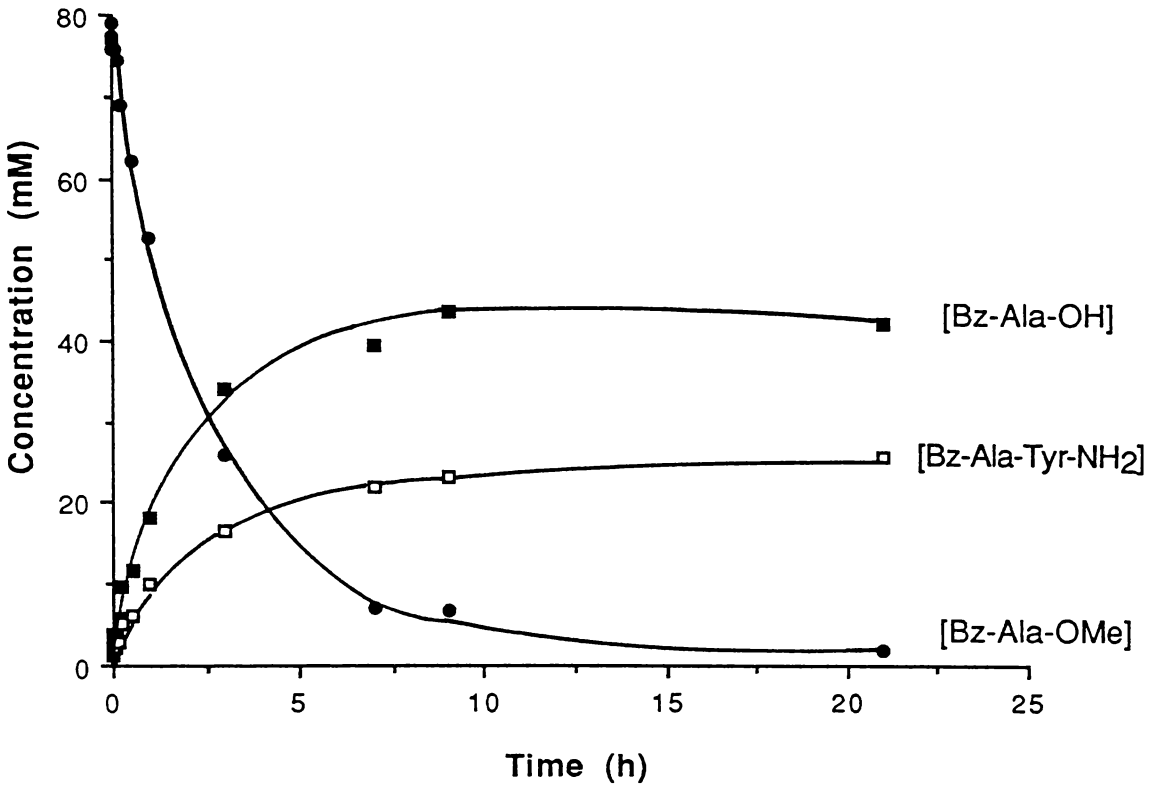


Fig 4.11 The Effect of Time on the Synthesis of Bz-Ala-Tyr-NH_2 with Free *Thermus* strain Rt41A Proteinase.

The solvent concentration was 90% v/v DMF and the incubation temperature was 40 °C. Substrate concentrations were 82 mM and 615 mM for Bz-Ala-OMe and Tyr-NH_2 respectively. Other details are as in section 4.2.1.

reaction has gone to completion. It is possible that during enzyme denaturation the partition constant is altered, favouring the hydrolysis reaction as opposed to aminolysis.

The k_4/k_3 value of a number of proteinases has been compared by Kasche (1986). The value given for Rt41A proteinase above does not compare favourably with these values, in most cases it is at least 2 orders of magnitude less than those quoted by Kasche. However, this does not necessarily discount the use of Rt41A proteinase for peptide synthesis, as will be discussed in subsequent sections, yields can be increased further by changing the solvent and the type of nucleophile.

4.3.2.8 Effect of the Nucleophile Blocking Group

A number of workers (e.g. Gololobov *et al.,* 1990; Morihara and Oka, 1981 and Aso, 1989) have shown that the C-terminal blocking group of the nucleophile can influence the yield of peptide synthesis. An investigation of the effect of four blocking groups, viz. X = $-NH_2$, -OBu, -ßNA and -pNA, on the synthesis of Bz-Ala-Tyr-X was undertaken with free Rt41A proteinase. The results illustrate that the C-terminal blocking group has a dramatic influence in the yield of peptide (see Table 4.4). No reaction was observed with Tyr-OBu, but increased yields of 6 and >7.5 fold were observed when Tyr-ßNA and Tyr-pNA respectively were utilised instead of Tyr-NH_2.

The non-reactivity of Tyr-OBu is not surprising when the esterase activity of the proteinase is considered. The ester group could well be cleaved from the substrate rendering it ineffective as a nucleophile. α-chymotrypsin also has less activity when amino acid amides are replaced with amino acid esters (Oka and Morihara , 1978), whereas amino acid esters are more efficient nucleophiles than amides in trypsin catalysed peptide synthesis (Tsuzuki *et al.*, 1980). This may be a reflection of the differing esterase activities or secondary specificities of the enzymes.

The increase in yield with the Tyr-ßNA and Tyr-pNA nucleophiles is most likely due to the higher specificity for -ßNA and -pNA, than amides, in the S_2' binding site of Rt41A proteinase. Similar results were observed by Gololobov

Table 4.4 Effect of the Nucleophile Blocking Group and the Acyl Activating Group on the Formation of the Ala-Tyr bond.[a]

Nucleophile	% Yield with Bz-Ala-OMe	Ester	% Yield with Tyr-pNA
Tyr-NH_2	8	Bz-Ala-OMe	61
Tyr-OBu	0	Ac-Ala-ONap	58
Tyr-ßNA	48	Ac-Ala-Ala-OMe	63
Tyr-pNA	61		

[a] 90% v/v DMF , 40 °C

et al. (1990), with α–chymotrypsin catalysed synthesis, when comparing amino acid ß-naphthylamides with amino acid amides. The authors concluded that hydrophobic interactions with the corresponding sub-site in the proteinase may favour the binding of some nucleophiles to the acyl-enzyme intermediate than others.

4.3.2.9 Effect of the Acyl Donor Structure

In this study the reactivity of 3 acyl donors were compared with Tyr-pNA as the nucleophile (see Table 4.4) and the free proteinase as the catalyst. The product yields observed were all high, no significant difference was observed with the different acyl donors. This may reflect a lack of secondary specificity of the free proteinase in the S_2 subsite. Schellenberger *et al.* (1990) concluded from more extensive experiments of this nature, with α–chymotrypsin, that the acyl donor had a smaller influence on the partition constant than variations in the nucleophile. However, Voyushina *et al.* (1985,) observed increased yields, in elastase-catalysed synthesis, by using dipeptide esters as acyl donors as opposed to amino acid esters. More trials are needed for conclusive results in this area with Rt41A proteinase.

Table 4.5 Effect of the Reaction Solvent on Bz-Ala-Tyr-NH_2 Synthesis[a]

	% Yield
Acetonitrile	3.0
Acetone	1.5
Amyl Alcohol[b]	1.0
DMF	1.5
Dimethylsulphoxide	2.3
1,4 Dioxan	1.6
Ethyl acetate[b]	3.0
Glycerol	2.2

[a] Solvent concentration = 50% v/v DMF, other details as in section 4.2.1.
[b] water-immiscible solvents

4.3.2.10 Effect of Different Organic Co-solvents

Peptide synthesis in 2 water-immiscible and 6 water-miscible solvents were compared (see Table 4.5). Regardless of the co-solvent utilised, there is little variation in the yield of Bz-Ala-Tyr-NH_2, except for acetonitrile and ethyl acetate where the yield was doubled. A 3 fold increase in yield was also observed at 90% v/v acetonitrile compared to 90% v/v DMF (see Figs. 4.5b and 4.6b).

Many authors have investigated the effect the organic solvent on peptide synthesis yields (Aldercreutz *et al.*, 1990; West and Wong, 1986; Cerovsky and Martinek, 1989a). Both water-miscible and -immiscible solvents have been utilised. The advantages of using either water-miscible or -immiscible solvents has been discussed briefly elsewhere (section 1.3.4.3). Although only two water-immiscible solvents were investigated , the results do not indicate any advantage for their use in the synthesis of Bz-Ala-Tyr-NH_2 compared to water-miscible solvents.

Acetonitrile appears to be effective in increasing the yields only when Tyr-NH_2 is the nucleophile, no increase in yield was observed with Tyr-pNA in 90% v/v acetonitrile compared to 90% v/v DMF (results not shown).

Acetonitrile has also been found to increase yields compared to DMF in proteinase K catalysed synthesis reactions (Cerovsky and Martinek, 1989b). The reason given for this was loss of enzyme activity in the presence of DMF. However, less than 50% of the ester substrate had been converted in the presence of DMF whereas no ester remained in the acetonitrile reaction yet, the concentration of the hydrolysis product formed was similar in both cases. This suggests that the partition constant is decreased in the presence of acetonitrile .

4.3.3 Synthesis of the Ala-Tyr Bond, Optimised Conditions

From the above optimisation experiments the optimal conditions for synthesising Bz-Ala-Tyr-NH_2 were found to be 90% v/v DMF, pH 10.0, 40 °C, 80 mM Bz-Ala-OMe, 615 mM Tyr-NH_2. The peptide yield was 26% of the original peptide concentration. Acetonitrile was found to be a better solvent than DMF at 50% v/v solvent. Synthesis in 90% v/v acetonitrile gave similar yields (20%) to those found in DMF above except lower substrate concentrations (25 mM and 100 mM respectively) could be used. However, at the higher substrate concentrations acetonitrile could not be utilised due to the insolubility of the Tyr-NH_2.

As Tyr-ßNA and Tyr-pNA were better nucleophiles than Tyr-NH_2 peptide synthesis with Tyr-ßNA, under the above optimal conditions, was investigated; the yields in DMF and acetonitrile were compared. In DMF quantitative yields were observed; no Bz-Ala-OMe or Bz-Ala-OH peaks were detected by HPLC analysis. It was therefore assumed that all of the Bz-Ala-OMe substrate had been converted to the peptide product. The Tyr-ßNA concentration could possibly be reduced and quantitative yields still produced as not all of the Tyr-ßNA went into solution. The peptide yield in acetonitrile was only 26%.

The results and those described in 4.3.2.10 suggest that the solvent can have a significant effect on the peptide yield but depends on the substrate utilised. That is, acetonitrile, compared to DMF increased yields when Tyr-NH_2 was

the nucleophile but had no effect on the yield with Tyr-pNA as the nucleophile and decreased the yield with Tyr-ßNA as the nucleophile.

4.3.4 Substrate Specificity

After optimisation of Bz-Ala-Tyr-NH_2 synthesis a rescreen of the substrates used in section 4.3.1 was carried out to determine yields and whether additional peptides, other than the 5 original dipeptides observed could be produced. Leu-pNA and Val-pNA were also included in the nucleophiles to be tested. The amino acid nucleophile concentration was increased to 100 mM and the reaction mixture contained 90% v/v DMF. Yields were determined after incubation at 40 °C for 20 h.

The results in Table 4.6 illustrate that the best yields are obtained with either Ac-Phe-OEt or Bz-Ala-OMe as the acyl donor. Tyr-NH_2, Phe-NH_2, Ala-NH_2 and Trp-NH_2 were all accepted as nucleophiles, however, no activity was found with the His or Glu amides. This indicates that positively and negatively charged nucleophiles cannot be accommodated in at the S_1' subsite. This is generally consistent with the observed bond specificity against the insulin B chain (section 2.1.4.2).

Of the reactive amide nucleophiles no one nucleophile consistently gave better yields. Instead, there also seems to be a "pairing" effect of the nucleophiles with the acyl donors. For example, when using Ac-Phe-OEt as the acyl donor the best nucleophiles are Phe-NH_2 and Tyr-NH_2, whereas with Bz-Ala-OMe Trp-NH_2 and Phe-NH_2 gave better yields. Schellenberger *et al.* (1990) found similar results with α–chymotrypsin, in that the effect of the acyl donor on the partition constant depended on the nucleophile used.

Leu-pNA and Val-pNA generally proved to have greater nucleophilicity than the amides subsites. This is most probably reflects better binding of the pNA group to the S_2' subsite rather than increased specificity for the P_1' amino acid as was discussed in section 4.3.2.8.

These results are consistent with the esterase activity of Rt41A proteinase against Cbz-amino acid p-Nitrophenyl esters (Peek, unpublished results, see Table 2.5). The authors found that the enzyme had a preference for aromatic

and aliphatic amino acids in the P_1 site, the order of reactivity was Tyr> Ala> Trp, Leu, Phe.

Of the reactive nucleophiles Tyr, Phe, Ala, Leu, and Val are all found in the P_1' site of peptide bonds in oxidised insulin B chain that are cleaved by Rt41A proteinase (Peek, unpublished results). The results show that the subsite specificity for peptide synthesis is the same as the hydrolytic subsite specificity. This phenomenon has also been observed for a number of proteinases used in kinetically controlled synthesis and indicates the importance of subsite interactions in determining peptide yield (see Fruton, 1982).

Table 4.6 Percent Yield of Dipeptides, Synthesised with Free *Thermus* strain Rt41A Proteinase, from Amino Acid Esters with Amino acid Amides or p-Nitroanilides[a].

Yield is calculated as a percentage of the original ester concentration.

	Acyl Donor			
Nucleophile	Ac-Phe-OEt	Ac-Tyr-OEt	Ac-Trp-OEt	Bz-Ala-OMe
Ala-NH_2	4.8	1.0	2.5	2.0
Trp-NH_2	3.4	0.5	ND	8.0
Phe-NH_2	7.0	1.4	1.4	8.0
Tyr-NH_2	7.0	ND	1.0	3.0
Leu-pNA	15	4.2	1.6	6.0
Val-pNA	16	5.6	6.0	15

[a] Reactions carried out in 90% v/v DMF at 40 °C, acyl donor concentrations = 25 mM, nucleophile concentrations = 100 mM, other conditions as in section 4.2.1.
Other nucleophiles tested but unreactive are His-NH_2 and Glu-NH_2.

4.3.5 Activity of Immobilised *Thermus* strain Rt41A Proteinase Column

4.3.5.1 Hydrolysis of SucAAPFpNA

The void volume of the column was determined (using the SucAAPFpNA substrate) to be 2.45 ml (~80% of total volume). This substrate was utilised as it meant the activity of the column could also be determined simultaneously. At a flow rate of 0.25 ml/ min 100% conversion of the SucAAPFpNA substrate was observed. The SucAAPFpNA substrate was used subsequent to this as a measure of the retention of proteolytic activity of the column after peptide synthesis trials. For this a flow rate of 1.5 ml was used, at which conversion of the substrate was reduced to 78%. The activity of the column was measured after the first 2 solvent trials and then after every subsequent trial. Initially the substrate conversion dropped to 70% and then to 60%, after which no further loss in activity was observed.

4.3.5.2 Synthesis of Bz-Ala-Tyr-NH_2

It was shown that by reducing the flow rate from 0.7 to 0.03 ml/ min that complete conversion of the Bz-Ala-OMe to Bz-Ala-OH and Bz-Ala-Tyr-NH_2 could be achieved. It should be noted that the activity of the column had been reduced to 60% of its original activity at this stage (see above). The percent yield was similar to that observed for the free and immobilised enzyme (1.5%), in reaction vials, with the same solvent and substrate concentrations.

No comparative experiments were carried out to try and increase this percent yield . This was due to a number of technical problems, which could not be overcome in the time available. The Eldex meter pumps were not able to maintain a consistent flow rate when the back pressure of the column built up. No other pumps were available that would pump under pressure at the low flow rates required. The back pressure of the column was caused by precipitation of a white solid on the column frits. This precipitate was assumed to be $CaCl_2$. Although periodically sonicating the frits and reducing the $CaCl_2$ from 5 to 1 mM alleviated this problem to a certain extent, reliable flow rates could not be achieved. This precipitation problem was also observed by

Nakanishi and Matsuno (1990), at 40 °C but not at 25 °C. The buffer used by these authors also had $CaCl_2$ present at a concentration of 5 mM.

The stability of the immobilised proteinase was checked under the reactions conditions used here, except reaction vials were used. The immobilised proteinase was shown to be stable for at least 24 h in the presence of both 25 and 50% v/v DMF. The loss of enzyme activity of the column, therefore, was possibly due to the constant flow of the solvent, as the total number of hours of column usage was less than 24 h. It is likely that a particular population of the enzyme was removed, due to the heterogeneous nature of immobilised enzymes (see section 3.3.5.4), hence no further loss was observed once the activity had dropped to 60% of the original. The high pressures to which the enzyme was exposed (2500 psi) may also be responsible for some loss in activity.

CHAPTER 5

FINAL DISCUSSION

Proteinases are widely utilised in industry today and have become, in economic terms, one of the most important groups of industrial enzymes. The use of immobilised enzymes has proved useful in industry, enabling the enzyme to be easily recovered and reused and, in some cases increased operational stability is observed. In the case of proteases there is also the added benefit of eliminating autodigestion of the enzyme. Immobilisation of enzymes can alter the conditions under which the enzyme functions optimally including pH shifts and changes in the affinity of the enzyme for particular substrates. Although many proteinases from mesophilic organisms have been immobilised, only two proteinases from moderate thermophiles (thermolysin and *Thermomonospora fusca* YX proteinase) and one proteinase from an extreme thermophile (caldolysin) have been immobilised. This is surprising considering the inherent stability of the proteinases from thermophilic and extremely thermophilic organisms, and hence their potential for use in industry. Immobilisation of these enzymes could further increase their stability. This inherent stability, not only to temperature but also to denaturing agents and organic solvents, may prove useful in the synthesis of peptides. An area of research that is expanding rapidly at the present time.

The investigation presented here had two aims. The first was to immobilise Rt41A proteinase covalently to a solid support and compare the activity and stability of the free and immobilised proteinase under various conditions. The second aim was to investigate the potential use of both the free and immobilised proteinase in the synthesis of peptides.

There are numerous methods that can be utilised to immobilise enzymes as described in section 1.2. The difficulty lies in choosing a suitable method for immobilisation which, often depends on the intended use of the enzyme. Rt41A proteinase was immobilised to two solid supports (Chapter 2): Polymer carrier VA-Epoxy Biosynth and controlled pore glass. Although both supports had similar binding efficiencies, CPG was utilised in subsequent investigations as it was known to be thermostable, is robust and is also resistant to microbial attack. The latter point enabled long term storage of the immobilised proteinase and hence continuity of the enzyme preparation used

throughout all experiments. The fact that controlled pore glass is available in various pore sizes was also an advantage. Rt41A proteinase is routinely assayed with azocasein which has a high molecular weight, therefore diffusional limitations within the pores can cause an under estimation of the activity bound during the immobilisation process. Therefore, a pore size could be chosen to reduce these limitations. There are other methods of immobilising enzymes to controlled pore glass. These have been reviewed by Weetall (1976) and include isothiocyanate, carbodiimide and triazine coupling to alkylamine glass compared to the glutaraldehyde coupling used here. Arylamine glass and glass that has not been pretreated can also be utilised. No attempt was made to investigate the use of these techniques and is an area that could be further examined. Other factors that could be investigated is the effect of immobilising the proteinase in the presence of an inhibitor or a substrate. The presence of an inhibitor will reduce autolysis during immobilisation. Immobilisation in the presence of a substrate has been shown to change the kinetic parameters of the immobilised enzyme including a decrease in the K_m and increased stability when immobilising trypsin (Royer and Uy, 1973). Weetall and Van (1976) reported similar results with the same enzyme; an increase in the specific activity and V_{max} and a decrease in the K_m was observed. The authors suggested that the presence of the substrate in saturating conditions induces a conformational change which freezes or traps the enzyme in this configuration while being immobilised. A small molecular weight substrate would have to be utilised in these types of experiments.

The immobilisation of the proteinase enabled the effect of calcium on the thermostability of the proteinase to be studied in more detail. It can be difficult in some instances to determine whether or not the loss of activity of a proteinase at a particular temperature is caused by autolysis or thermal denaturation. Immobilisation eliminates autolysis, hence any effect of temperature and/or the calcium concentration on the stability will be due to thermal denaturation. From this fact, and the results presented in section 3.3.5.4, it is suggested that generally, high concentrations of calcium stabilise the proteinase against autolysis and low concentrations of calcium stabilise against thermal denaturation. Studies on thermolysin (Dalquist *et al.*, 1976, Voorduow *et al.*, 1976; Fassina *et al.*, 1986) have yielded similar results. These experiments were based on the affinity of the four calcium binding sites for calcium and analysis to determine which sites were responsible for

stabilising against autolysis and thermal denaturation. It would be interesting to carry out calcium binding studies with Rt41A proteinase to confirm the findings mentioned above.

The high pH optimum of both the free and immobilised proteinase observed here is also interesting. It is likely that the buffer utilised to determine the pH optimum of the proteinases, in this case, had a stabilising affect on the enzyme, or buffers used in previous determinations inhibited the activity of the proteinase. More work could be carried out in this area, investigating a wider range of buffers.

The free and immobilised proteinase were both more stable in DMF than heptanol at 25 and 50% v/v solvent. Conversely at 90% v/v solvent the proteinases were more stable in heptanol. However, immobilised Rt41A proteinase was more stable than the free proteinase, especially at high solvent concentrations and at high temperatures. It is generally accepted that proteins are more stable, at high solvent concentrations, in water-immiscible than water-miscible solvents. The reason for this is that water-miscible solvents are able to disrupt the water from the hydration shell surrounding the enzyme. Water-immiscible solvents are not soluble enough to disturb this hydration shell, which must remain intact for retention of the native state of the protein (Khmelnitsky *et al.*, 1991). Therefore, at lower heptanol concentrations the stability of Rt41A proteinase, compared to that in DMF, is less because of denaturation at the solvent/aqueous interface. The lower the heptanol concentration the greater this affect is due to the increased surface area of the interface. A more systematic study of stability in both water-miscible and immiscible solvents would be useful to confirm the findings presented here.

In Chapter 4 the potential use of the proteinase in peptide synthesis was investigated. A number of peptides were synthesised successfully and the optimisation of Bz-Ala-Tyr-NH_2 was undertaken. Quantitative yields were observed under optimised conditions with Tyr-ßNA as the nucleophile instead of the Tyr-NH_2 nucleophile used initially. While optimising the synthesis of Bz-Ala-Tyr-NH_2 some of the effects observed were difficult to explain. The first is the initial increase in the partition constant a low solvent concentrations. Trials with more solvents, particularly water-immiscible, may help in explaining this.

Also investigating the effect of the solvent concentration on the relative binding of both the acyl component and the nucleophile may prove useful.

Further investigation into the effect of the ionic strength on synthesis is also required, the difference in the effect on the partition constant and maximum yield is unusual. A number of experiments could be carried out in this area including, the effect of a divalent instead of a monovalent cation, the use of positively and negatively charged nucleophiles instead of uncharged, the use of different co-solvents (water-miscible and -immiscible) and the use of different acyl components and nucleophiles.

The effect of temperature on peptide synthesis is also an interesting aspect that could be explored more extensively. The use of a larger range of temperatures, especially at the lower end of the scale, may prove interesting. In addition the use of different substrates and/or different organic solvents may provide useful results.

Although a large range of substrates were compared in this investigation, there are many more that could be utilised, especially in relation to the effect of the nucleophile blocking group and the "pairing" effect that was observed. Of the amide nucleophiles compared in Table 4.6 it would be interesting to repeat the screen with the corresponding amino acid p-nitroanilides and ß-naphthylamides. Another interesting aspect of the substrate specificity study would be to compare the effect of different solvents on the yields of the different peptides formed. It has been found by Klibanov (1991) that the specificity (substrate, regioselectivity, and enantioselectivity) of an enzyme can be significantly altered simply by changing the solvent. The use of di-, tri- and tetra-peptide nucleophiles is another aspect that could be explored.

There are also many other thermophilic proteinases that could be investigated for use in peptide synthesis, both kinetically and thermodynamically controlled, including caldolysin (from *Thermus* strain T-351), caldolase (from *Thermus* strain Tok3), and archaelysin (from *Desulfurococcus* strain $Tok_{12}S_1$). The increased temperatures that these proteinases are active at, compared to mesophilic proteases, may be of more use in thermodynamically controlled synthesis reactions, where temperature may enhance yields. Synthesis reactions need not be confined to peptide synthesis, proteases have also been utilised to synthesise esters and carry

out transesterification reactions (Kise *et al.*, 1990). Preliminary experiments of peptide synthesis with Rt41A proteinase in ethanol have yielded unidentifiable peaks which are likely to be caused by a transesterification reaction with the -OMe of the acyl component.

Continuous synthesis of peptides with the immobilised proteinase was observed. The system utilised was not optimised but showed potential providing the problem of the precipitation of the $CaCl_2$ can be eliminated. If the rate of reaction could be increased, possibly by the use of different substrates or a different solvent system, the problem of maintaining a constant flow rate could also be eliminated. The use of the Tyr-pNA substrate would certainly give better yields than those observed with Tyr-NH_2, whether an increase in rate would also be observed is unknown.

In conclusion the aims of this study were achieved. Rt41A proteinase was successfully immobilised and the comparison of the free and immobilised proteinase provided some interesting results as discussed in Chapter 3. The proteinase can also be utilised to produce dipeptides in quantitative amounts and one tripeptide also was produced. Proteinases from extreme thermophiles may be advantageous in this area in some cases as they cannot hydrolyse these small peptides once they are formed as discussed in chapter 2. However, more work is required to optimise peptide synthesis before these thermostable proteinases can compete with those proteinases that are currently used.

APPENDIX 1

SPECIFICATIONS OF CONTROLLED PORE GLASS BEADS

Table A1.1 Specifications of CPG Beads[a].
All Beads were 80-120 Mesh size (125-177 μm)

Pore Size (nm)	Pore Dist[b] (± %)	Pore Vol. (ml/g)	Surf Area (m^2/g)	Density (g/ml)
15	7.3	0.49	119.5	0.46
22.6	6.2	0.85	94	0.45
50		<1	70	0.30
105	8.9	0.88	22.7	0.35
187	9.2	0.95	11	0.32

[a] All beads were purchased from Sigma, except the 50 nm pore size beads which were purchased from Pierce.

[b] Pore distribution

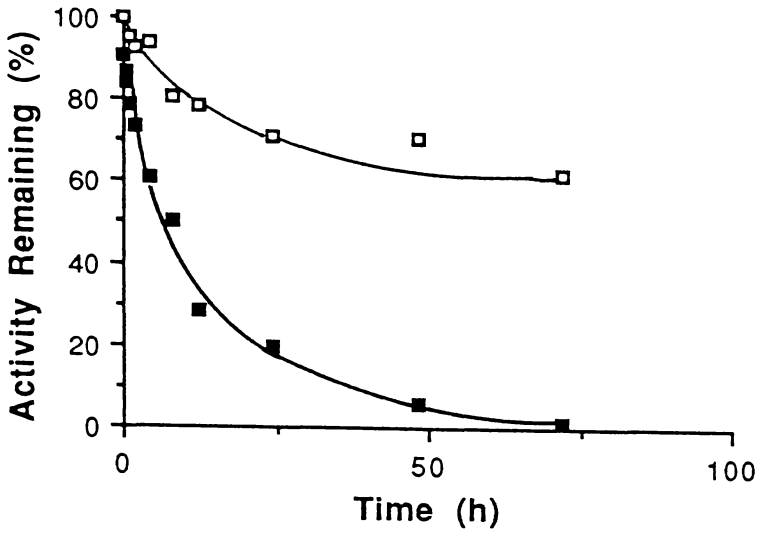
APPENDIX 2

Thermal Denaturation Curves of Free and Immobilised *Thermus* strain Rt41A Proteinase

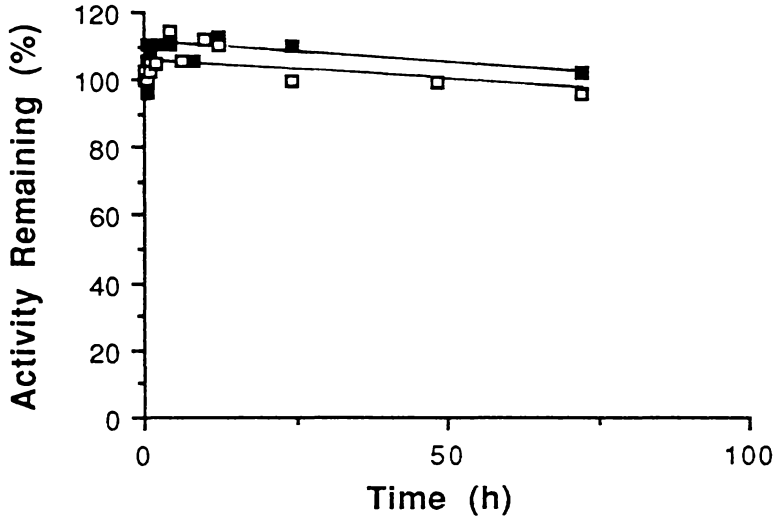
Fig A2.1-A2.3 The Effect of Temperature (50, 65, 70 °C) and the $CaCl_2$ Concentration on the Thermostability of Free and Immobilised *Thermus* strain Rt41A Proteinase.

The thermostability of the free [■] and immobilised [□] proteinase was determined in the absence (a) and presence of 10 μM (b) or 5 mM (c) $CaCl_2$.

Fig A2.1 50 °C (a) 0 $CaCl_2$



(b) 10 μM $CaCl_2$



(c) 5 mM $CaCl_2$

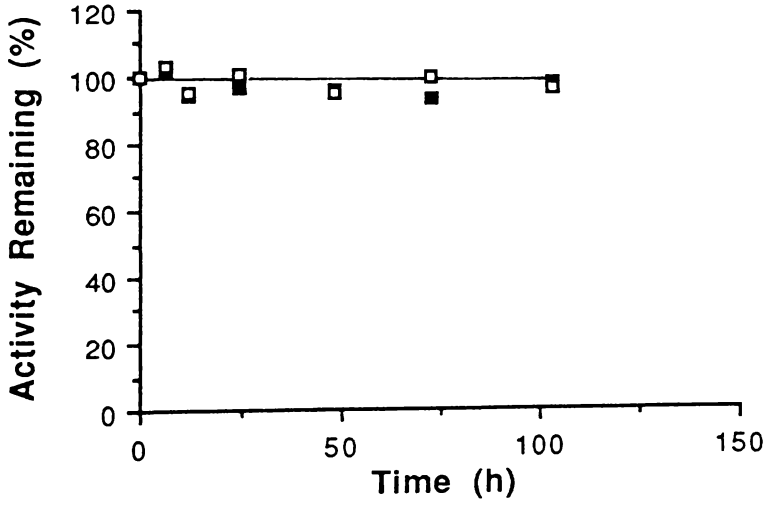
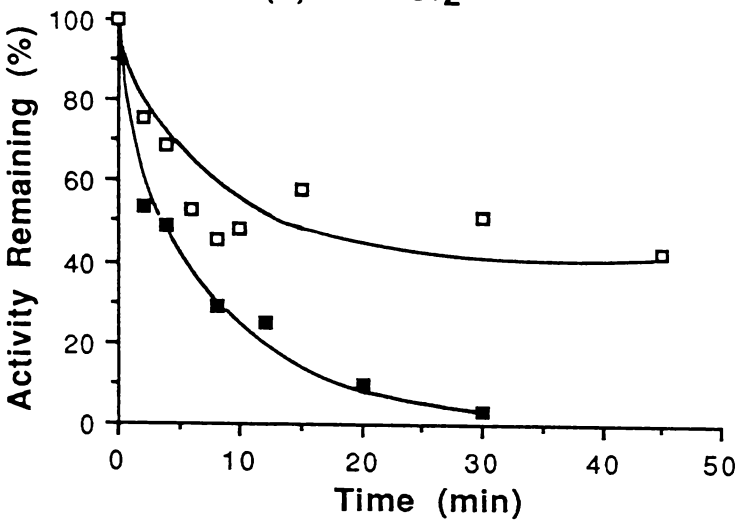
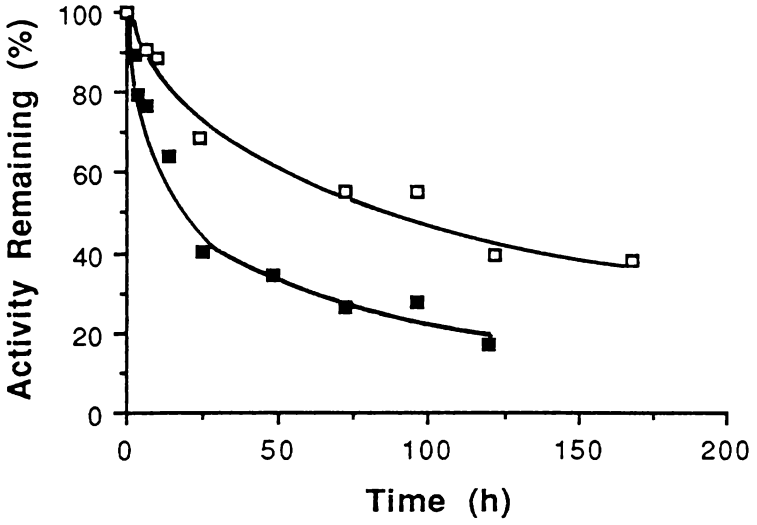


Fig A.2.2 65 °C (a) 0 $CaCl_2$



(b) 10 μM $CaCl_2$



(c) 5 mM $CaCl_2$

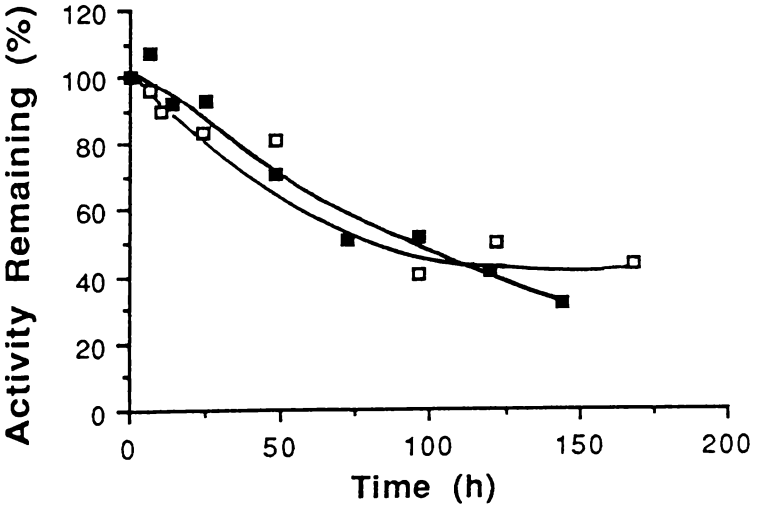
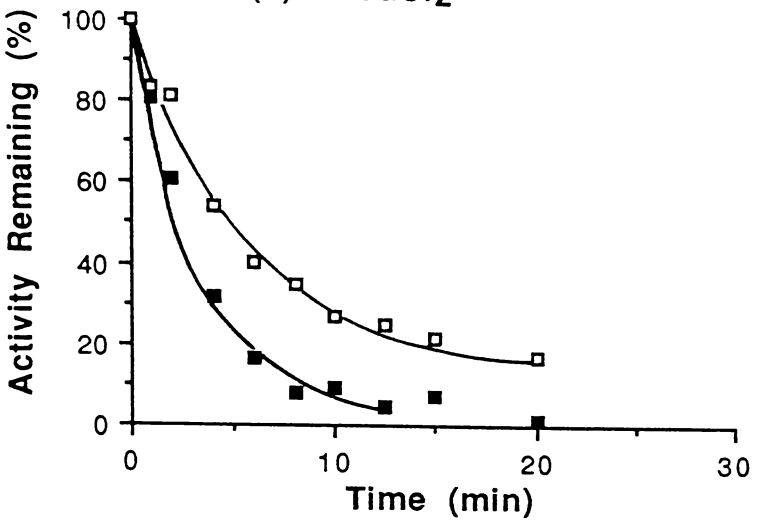
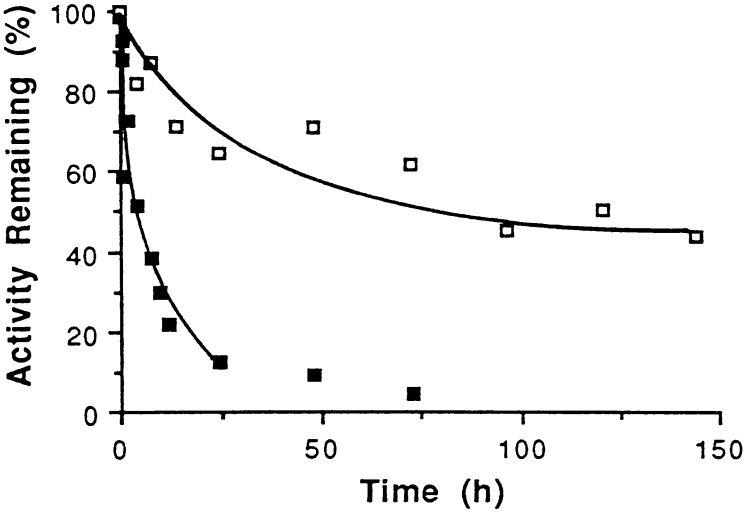


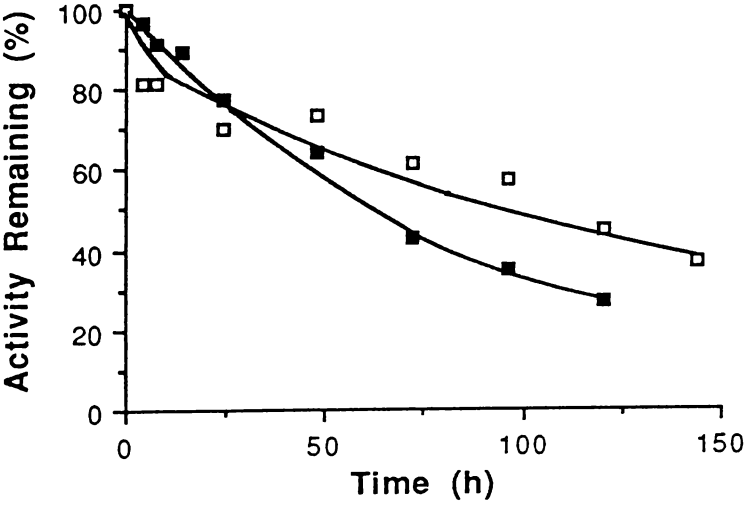
Fig A2.3 70 °C (a) 0 $CaCl_2$



(b) 10 μM $CaCl_2$



(c) 5 mM $CaCl_2$



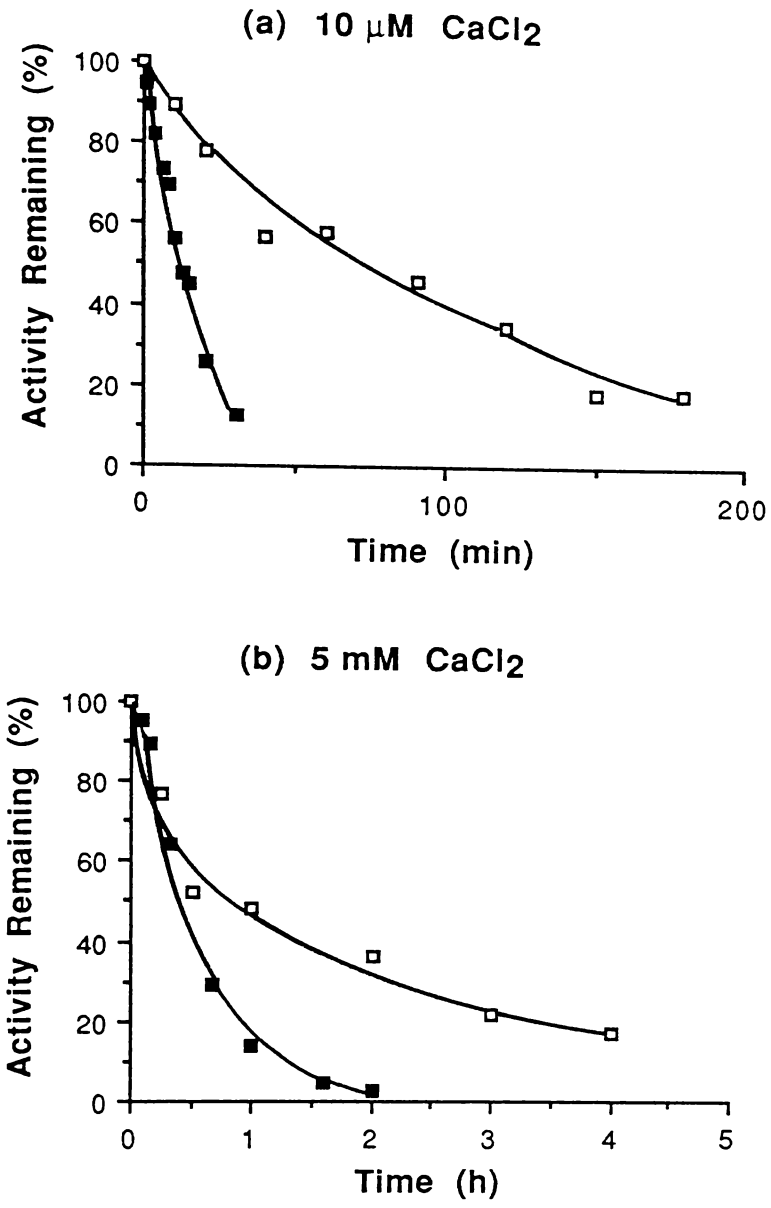


Fig A2.4 The Effect of the $CaCl_2$ Concentration on the Thermostability of Free and Immobilised Rt41A Proteinase at 90 °C.

The thermostability of the free (■) and immobilised (□) proteinase was determined in the presence of (a) 10 μM or (b) 5 mM $CaCl_2$.

APPENDIX 3

GRADIENT PROFILES FOR AMINO ACID ANALYSIS

The gradient former utilised in conjunction with amino acid analysis allows the generation of eleven types of gradient profiles as illustrated below.

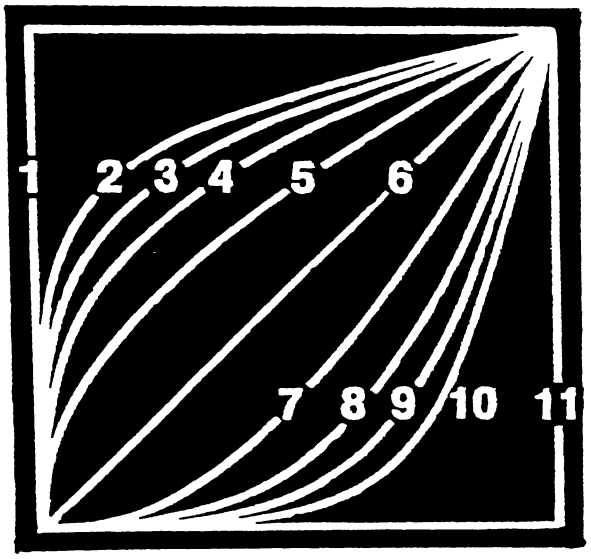


Fig A3.1 Gradient Profile Curves

The calculation of these curves is based on the following equations.

$$\%A = \%A_{start} + (\%A_{end} - \%A_{start}) \frac{(t - t_0)^{(n-5)}}{(t_1 - t_0)}$$

for n = 6 to 10

$$\%A = \%A_{start} + (\%A_{end} - \%A_{start} \frac{(t - t_0)^{(1/7-n)}}{(t_1 - t_0)}$$

for n = 2 to 5

Where: n is the curve number

% A is the percentage flow rate of pump A.

t is the elapsed time from inject

t_0 is the elapsed time from the beginning of the segment

t_1 is the elapsed time from the end of the segment

$\%A_{end}$ is the end point value of pump A at the end of the segment

$\%A_{start}$ is the start point value of pump A at the begining of the segment.

APPENDIX 4

CORRELATION OF AMINO ACID ANALYSIS TO HPLC ANALYSIS

Product peaks (Bz-Ala-Tyr-NH_2) were collected from successive experiments, the final concentration of the sample was unknown. Various dilutions of the Bz-Ala-Tyr-NH_2 sample were applied to the reverse phase C_{18} as described in section?. The peaks were collected and the peak areas from these were plot against the dilution factor (Fig A4.1). The collected peaks were then amino acid analysed and the peak area observed plotted against the dilution factor (Fig A4.2). The concentration of Bz-Ala-Tyr-NH_2 in the original solution was also calculated.

As both sets of results gave good correlations a factor could be determined to calculate the concentration of the Bz-Ala-Tyr-NH_2 synthesised from the peak areas observed in HPLC analysis.

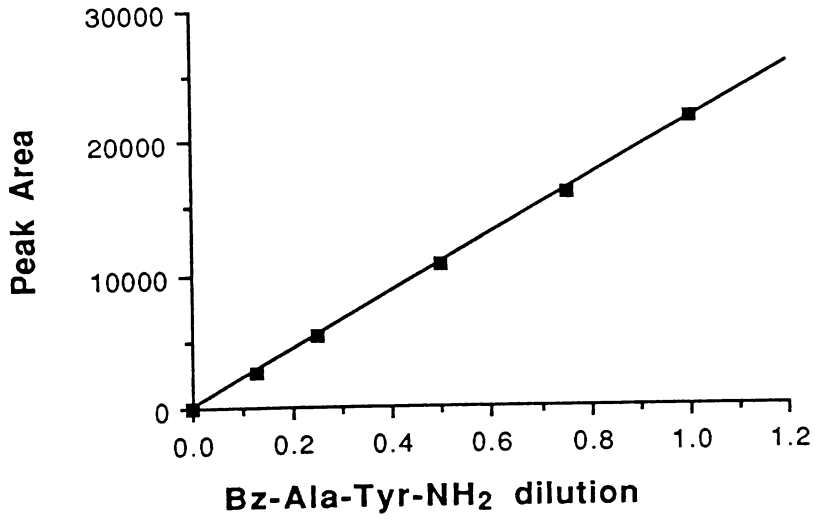


Fig A4.1 HPLC Standard Curve Bz-Ala-Tyr-NH_2.

Dilutions of Bz-Ala-Tyr-NH_2, of unknown concentration, were injected on to a reverse phase C_{18} column (section 4.2.2).

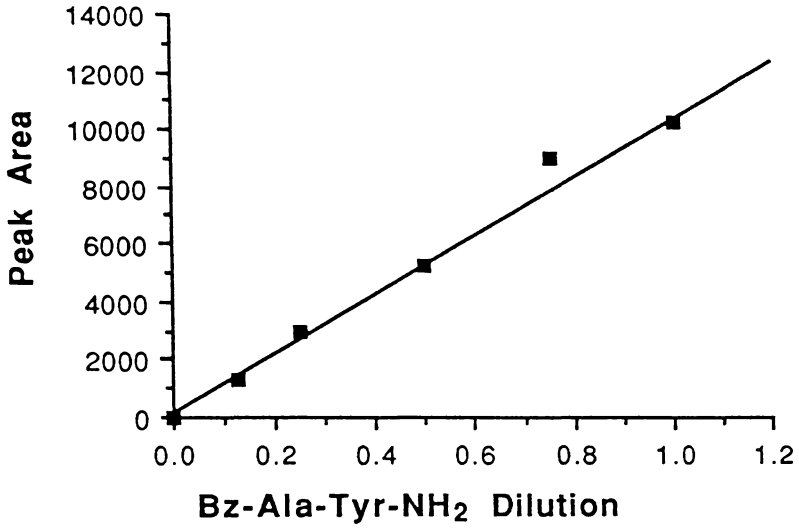


Fig A4.2 Amino Acid Analysis Standard Curve of Bz-Ala-Tyr-NH_2.

The peaks were collected from HPLC analysis (Fig A4.1) and then subjected to amino acid analysis. Details as described in section 4.2.3

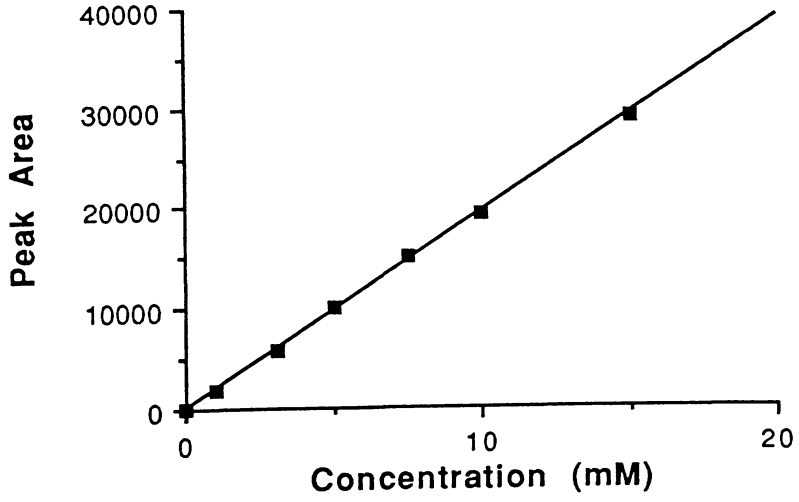


Fig A4.3 HPLC Standard Curve for Bz-Ala-OMe

60 µl of Bz-Ala-OMe standards were injected onto the HPLC and the peak area recorded.

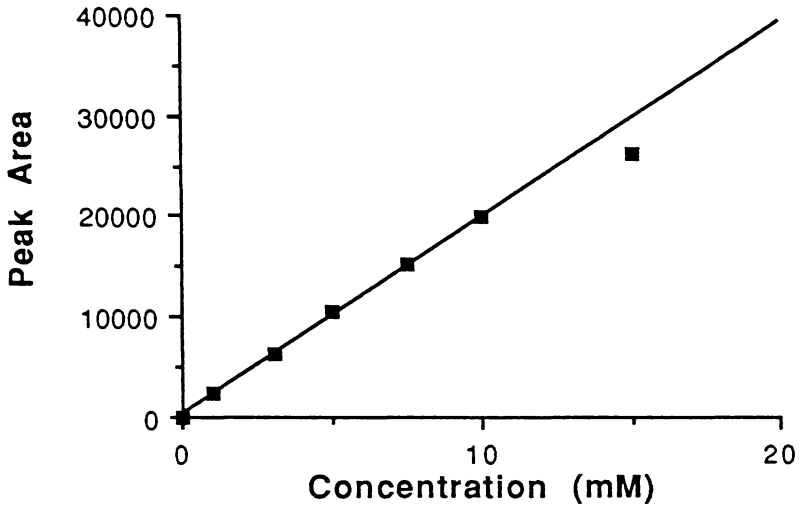


Fig A4.4 HPLC Standard Curve for Bz-Ala-OH

60 µl of Bz-Ala-OH standards injected onto HPLC and the peak area recorded.

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