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Thermostable Proteases

R.M. DANIEL[1] *, H.S. TOOGOOD[1] AND P.L. BERGQUIST[2,3]

[1] *Thermophile Research Unit, The University of Waikato, Private Bag 3105, Hamilton 2001, New Zealand;* [2] *Macquarie University, North Ryde, New South Wales 2109, Australia and* [3] *Centre for Gene Technology, University of Auckland, Private Bag 92019, Auckland, New Zealand*

Introduction

Proteases have a long commercial history, with many early biotechnology patents dealing with protease applications. They are still the most widely used of any enzyme type. They have also figured largely in the earliest studies of enzyme physiology, structure and mechanism. Gastric digestion has been the subject of scientific studies since the work of Reaumur and Spallanzoni in the 1700s, and the discovery of pepsin by Wassman in 1839 (Neidleman, 1989). The crystallisation of pepsin (Northrup, 1930) closely followed that of urease (Sumner, 1926). The serine proteases are possibly one of the most thoroughly studied classes in enzymology, and their mechanism of action one of the best understood. More recently, the serine protease subtilisin was one of the earliest subjects for enzyme engineering (eg Bryan *et al.*, 1986; Katz and Kossiakoff, 1986; Wells and Estell, 1988), partly because of its commercial importance but also because the state of the knowledge base of serine proteases in general and subtilisin in particular was good enough to greatly enhance the chances of success (Wells and Estell, 1988). Over the last 15 years or so there has been increasing interest in proteases as their intracellular importance in metabolic and regulatory processes, as opposed to their role in digestion and nutrition, becomes apparent. There have been a number of recent books and reviews on proteases (e.g. Kalisz, 1988; Beynon and Bond, 1989; Outtrup and Boyce, 1990; Rawlings and Barrett, 1994a,b).

Given the prominence of proteases in fundamental studies and in industry, it is not surprising that thermostable proteases from thermophiles and hyperthermophiles are attracting increasing interest. Such stable proteases are often more attractive research subjects than their mesophilic counterparts (Daniel, 1986), especially when the stability includes resistance to autolysis (Daniel *et al.*, 1982). The advantages of stable enzymes in industrial situations has been well discussed (Doig, 1974; Ljungdahl and Sherod, 1976; Daniel, Cowan and Morgan, 1981; Sonnleitner and Fiechter, 1983;

* Corresponding Author

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Daniel, Morgan and Martin, 1985; Weigel and Ljungdahl, 1986; Ng and Kenealy, 1986; Bergquist *et al.*, 1987; Ward and Moo-Young, 1988; Peek *et al.*, 1992b; Coolbear, Daniel and Morgan, 1992). Many of the organisms growing at high temperatures are apparently novel, in the sense of being only distantly related to known mesophilic organisms. Furthermore those growing at the highest temperatures make up a large part of a third kingdom of life, the Archaea (Woese, 1987). As such these organisms seem likely to be of particular interest. Their taxonomy has already shed light upon the origins and early evolution of life (Woese, 1987; Achenbach-Richter *et al.*, 1987), and novel metabolic pathways have been found (e.g. Danson, 1989). Biochemically, their growth at extremes of temperature and at high salt concentrations is leading to a better understanding of the limits of metabolic processes and biochemical structures. In particular, the Archaea are the only organisms with growth optima above 90°C, and there are only a very few bacteria with optima above 80°C. Future studies of protein stability at the upper limits of temperature are therefore likely to focus on Archaeal proteins (Daniel, Bragger and Morgan, 1989), and the most stable proteins are likely to arise in thermophilic or hyperthermophilic Archaea.

This review aims to focus specifically on thermostable proteases, updating and expanding the very brief review by Cowan, Daniel and Morgan (1985). It deals with proteases which are heat stable or which are produced by organisms growing at high temperatures (optimum temperature for growth above 70°C). Very roughly, proteases have been included if their half life is greater than 1 h at 65°C or greater than 10 min at 75°C, or, in a few cases, if such a half life can be very clearly inferred from 'temperature optima' data. (In fact, while specific processes using proteases will indeed have a temperature optimum, the enzyme itself will not. These so-called 'temperature optima' depend on the length of the enzyme assay, the temperature coefficient (activation energy) of the enzyme, and its stability, as well as on the degree of substrate stabilisation, and are therefore of little use in comparing protease stability outside specific applications.) The use of these criteria for inclusion has had the effect, for example, of including some proteases secreted by *Bacillus stearothermophilus* and excluding others. Given the wide temperature range for the growth of this group, this is perhaps not surprising. The stability limit has been relaxed somewhat for a few proteases that seem of particular interest. Most of the proteases reviewed here are extracellular, reflecting both the historical focus of protease research and the higher stability of extracellular enzymes.

Protease stability

Although we will focus here on thermal stability it is well accepted that resistance to heat confers resistance to many denaturing and chaotropic agents. This arises because the increase in the net free energy of stabilisation which leads to enhanced thermal stability is caused by an increase in the interactions which stabilise proteins against denaturation by most other agents.

There are three mechanisms by which heat causes activity loss in proteases. Firstly, the application of heat results in the conformational unfolding (denaturation) of proteases. Secondly, heat enhances autolysis, that is, the self-digestion of proteases. It should be noted that this mechanism of activity loss applies only to proteases and

not to other proteins. Finally, at high temperatures other irreversible covalent modifications, such as deamidation, may occur (Ahern and Klibanov, 1985).

In general terms, the net free energy of the conformational stabilisation of proteins is the result of a delicate balance between large stabilising and large destabilising forces (e.g. Brandts, 1967). This means that quite small percentage changes in either the stabilising or destabilising forces can result in large changes in the net free energy of stabilisation. This explains how the stability of proteins to thermal denaturation can be changed by quite small changes in the amino acid sequence (e.g. Langridge, 1968), altering a few intramolecular interactions (Perutz and Raidt, 1975), without any obvious structural alterations (Grutter Hawkes and Matthews, 1979; Matthews, 1993). The difference in the free energy of stabilisation between enzymes with a half-life of about 1 h at 50°C, which is what we might regard as normal for enzymes, and those enzymes that are exceptionally stable, having a half-life of the same sort of period at above 90°C, is only a few 10s of kJ/mol. A single salt bridge can probably contribute between 4 and 12 kJ/mol, so a small number of extra salt bridges, for example, can confer this extra degree of stabilisation (Perutz and Raidt, 1975). Small numbers of additional hydrophobic interactions or hydrogen bonds can perform a similar function. There is therefore no reason for there to be systematic structural differences between extremely stable and normal proteins significantly greater than those observed between proteins of similar stability, and such differences have not been found (e.g. Matthews, 1993).

Conformational unfolding is, in principle, a reversible process, although subsequent aggregation of the denatured protein may prevent renaturation. For proteases, heat-driven conformational unfolding is less frequently reversible than is the case for other enzymes. This is because unless heat denaturation happens very rapidly and completely, the rate of the second mechanism causing activity loss, which is autolysis, is greatly enhanced, since the partially or fully unfolded form of the enzyme is a particularly good substrate for the surviving active protease molecules. Sometimes it can be difficult to demonstrate a single band on SDS-PAGE for a pure protease because the standard heat-denaturing step (boiling in SDS) prior to gel application does not occur quickly enough to prevent autolysis and the subsequent appearance on the gel of low molecular weight fragments (Peek *et al.*, 1992a).

In practical terms, these phenomena mean that it is often difficult to investigate the denaturation of proteases free of the influence of autolysis. If one of these two processes is predominant, then kinetic analysis will enable determination of whether activity loss is due to denaturation (1st order) or autolysis (2nd order) (eg Voordouw and Roche, 1974), but results frequently show the two processes occurring together. One approach to this problem is to immobilise the protease to prevent autolysis (Wilson, Peek and Daniel, 1994). For some proteases it has been found that low levels of Ca^{2+} will prevent autolysis but much higher levels are needed to prevent thermal denaturation (Voordouw, Milo and Roche, 1976; Fassina *et al.*, 1986; Wilson, Peek and Daniel, 1994), so that by manipulating the Ca^{2+} concentration it may be possible to allow the examination of one of these phenomena in the absence of the other.

Despite their stability, the use of proteases for the study of denaturation (conformational unfolding) is thus complicated by autolysis. Although a protease inhibitor can be used to block autolysis, inhibitor binding is also likely to affect stability. For the

same reason, proteases are less than satisfactory subjects for the study of the irreversible mechanisms causing activity loss at high temperatures (see below).

The third form of activity loss for all enzymes at high temperature arises from non-enzymic irreversible reactions (Ahern and Klibanov, 1985), of which the deamidation of labile asparagine residues (Stephenson and Clarke, 1989; Aswad, 1990) may be the most important. Until comparatively recently, in the absence of any evidence that conformational stability had an upper temperature limit, these irreversible reactions seemed likely to place a theoretical upper temperature limit upon protein stability. However, there is now some evidence that these reactions depend upon peptide chain flexibility and are much slower in proteins in their native conformation than in the unfolded (denatured) state (Hensel *et al.*, 1992; Daniel, 1996).

The three forms of temperature-driven activity loss described above are therefore interlinked, and related to the effect of temperature on activity itself. A general relationship between resistance to proteolytic attack and thermal stability has been demonstrated by Daniel *et al.* (1982), and others (Ogasahara *et al.*, 1985; Parsell and Sauer, 1989). Therefore it follows that even below temperatures at which denaturation occurs there is a relationship between resistance to autolysis and thermal stability. At any given temperature, proteases that are more thermostable will be more resistant to autolysis. Autolysis is faster at high temperatures because activity is temperature dependent, and also because, even below temperatures at which denaturation is significant, proteolytic susceptibility rises as denaturing temperatures are approached. Other irreversible losses such as deamidation and peptide bond hydrolysis seem to be dependent to some degree on conformational instability (Hensel, 1992; Daniel, 1996).

EFFECT OF CALCIUM

The stability of enzymes from extreme thermophiles is normally intrinsic, dependent upon the primary, secondary, and tertiary structure of the enzyme rather than on stabilising 'factors', but for some enzymes Ca^{2+} plays a major stabilising role. The stable protease ('caldolysin') from *Thermus* strain T-351, for example, appears to be dependent upon six Ca^{2+} ions for its high stability (see *Table 1*) (Cowan and Daniel, 1982a; Khoo *et al.*, 1984). Apocaldolysin has a half life of 40 min at 55°C, comparable with those of proteases from mesophiles. It has been suggested that the enhanced stability of thermitase, compared with other members of the subtilisin family of proteases, is also due to Ca^{2+} (Frommel and Sander, 1989). Because of this stabilising effect of Ca^{2+}, it may protect against irreversible reactions such as deamidation because of the possible linkage of these with flexibility (Aswad, 1990). In practical terms, the effect of Ca^{2+} is often inferred from the effect of EDTA on activity, which may leave open the question of whether any 'inhibition' observed is due to reduced activity or reduced stability. In the case of thermostable proteases, this can often be most simply resolved by reducing the temperature of the assay by 50°C or so to a temperature where even a Ca^{2+}-depleted protease is likely to be stable. In most cases the 'inhibition' disappears, indicating an effect of Ca^{2+} on stability rather than activity. Kinetic analysis of activity loss at different temperatures can usually then be used to determine whether, or at what temperature, Ca^{2+} affects thermal denaturation or autolysis.

Table 1. Properties of some thermostable proteases

Source (enzyme Name)	MW (kDA)	pI	pH_{opt}	Stability	References
Serine					
Arthrobacter aureus	22	10	7.0	'T_{opt}' = 70°C	Michotey and Blanco (1994)
Bacillus sp. AH-101	29–30	9.2	10–13[3]	60% left 10 min 80°C	Takami, Akiba and Horikoshi (1989), 1990), Fujiwara, Masui and Imanaka (1993)
Bacillus st. Ak.1	36.9	4.0	7.5	$t_{1/2}$ = 19 min, 90°C	Peek *et al.* (1993)
Bacillus st. B18' (AprM)	28–30	–	12–13	$t_{1/2}$ = 75 min, 70°C	Fujiwara, Masui and Imanaka (1993)
Bacillus st. K-295G-7 I	28	9.22	9.0	'T_{opt}' = 65°C, 30 min assay	Park *et al.* (1987)
II	29.5	9.45	9.0	'T_{opt}' = 65°C, 30 min assay	Park *et al.* (1987)
Bacillus stearothermophilus F1	20–33.5	–	9.0	$t_{1/2}$ =4 h 85°C	Rahman *et al.* (1994)
B. stearothermophilus RM-67 II	19.95	–	8.0	20% left 30 min 65°C	Chopra and Mathur (1985)
B. subtilis NCIM No. 64	28	–	9.7	No act. loss 1 h 70°C + Ca^{2+}	Kembhavi, Kulkarni and Pant (1993)
B. subtilis NRRL B3411 I	28.2–32.5	–	10.0	~75% left, 30 min 65°C	Keay, Moser and Wildi (1970)
B. thermoruber BT_2^T	39	5.3	9.0	60% left 30 min 70°C	Manachini, Fortini and Parini (1988)
B. thuringiensis var. *kurstaki* HD-255	34	9.0	8.5–9	88% left >7 h 60°C	Kunitate, Okamoto and Ohmori (1989
Desulfurococcus st. $Tok_{12}S_1$ (Archaelysin)	52	8.7	7.2	$t_{1/2}$ = 70–79 min, 95°C	Cowan *et al.* (1987)
Malbranchea pulchella var. *sulfurea* (Thermomycolin)	32.7	6.0	8.5	$t_{1/2}$ = 1.8 h 73°C + Ca^{2+}	Gaucher and Stevenson (1976)
Pyrobaculum aerophilum (Aerolysin)	–	–	neu-alk	'T_{opt}' = 100–130°C	Volkl *et al.* (1994)
Pyrococcus furiosus DSM 3638 (S66)	66	–	7.0	–	Blumentals, Robinson and Kelly (1990)
(S102)[5]	$102_{\text{(Dimer ?)}}$	–	7.0	$t_{1/2}$ = 33 h, 98°C(S66 & S102)	Connaris, Cowan and Sharp (1991)
(Pyrolysin)	65–140[6]	–	6.5–10.5	$t_{1/2}$ = 20 min, 105°C	Eggen *et al.* (1990)
Staphylothermus marinus DSM 3639	30–300[2]	–	$9.0_{\text{(Phosphate)}}$	$t_{1/2}$ = 5 h, 90°C	Klingeberg, Hashwa and Antranikian (1991)
Streptomyces rectus Protease B var. *thermoproteolyticus*	21.5	9.5	10.7[4]	65–70% left 10 min, 80°C	Mizusawa and Yoshida (1972), Mizusawa, Ichishima and Yoshida (1964)
Sulfolobus solfataricus MT-4 (ATCC 49155) Protease I	118	5.6	6.5–8[3]	$t_{1/2}$ = 5.7 h, 92°C	Burlini *et al.* (1992)
Protease II	32	–	7.0[3]	Inactivated at 60°C, 15 min	Fusi *et al.* (1991)
Thermoactinomyces vulgaris (Thermitase)	28.4	9.1	8.5	'T_{opt}' = 85°C	Gusek and Kinsella (1987), Frommel and Hohne (1981)
Thermobacteroides proteolyticus DSM 5265	30–300[2]	–	9.5	$t_{1/2}$ = 1.5 h, 90°C	Klingeberg, Hashwa and Antranikian (1991)
Thermococcus st. AN1	30–300[2]	–	7.0	$t_{1/2}$ = 30 h, 90°C	Klingeberg, Hashwa and Antranikian (1991)

$t_{1/2}$, half life
[1] assay con
[2] possibly r
[3] depends c
[4] shoulder
[5] membersl
[6] from a cr
–, data not

Ca^{2+} protects many stable proteases against both thermal denaturation and autolysis. In at least some proteases different concentrations of Ca^{2+} are required to protect against autolysis and against thermal denaturation. For the protease from *Thermus* strain Rt41A, a comparison of the effects of Ca^{2+} on free and immobilised enzyme enabled the demonstration that only ~10μM $CaCl_2$ was needed to stabilise the protease against thermal denaturation, but about 5 mM $CaCl_2$ was required to protect against autolysis (Wilson, Peek and Daniel, 1994). This is consistent with the results for thermolysin, where the first, high affinity, binding site protects against thermal denaturation, and the fourth, lower affinity, binding site protects against autolysis (Voordouw, Milo and Roche, 1976; Fassina *et al.*, 1986). It should be noted that thermolysin is a metalloprotease, but those from *Thermus* are serine proteases: in neither case does the Ca^{2+} affect activity directly.

Effect of temperature on protease activity

In general terms, there is no good evidence to suggest that enzymes from thermophiles or hyperthermophiles have higher specific activities than those from mesophiles, *in vivo*. However, given the wide range of specific activities within isoenzyme/isofunctional enzyme groupings, a small systematic difference may be difficult to detect. Certainly, the difficulty in ascertaining the 'evolved' or 'natural' growth temperature of many thermophiles will complicate any such comparison. Many strains of *Thermus*, for example, are isolated from boiling springs, but grow optimally in the laboratory at 75°C in media greatly different from any they will encounter naturally. Is the 'real' specific activity that at 100°C or that at 75°C?

However, stable proteases from thermophiles do indeed display quite substantially higher specific activities than proteases from their mesophilic counterparts at their respective optimum growth temperatures (Cowan, Daniel and Morgan, 1985, 1987a): but, as shown by Cowan, Daniel and Morgan (1987a), this high activity is conferred by the increasing susceptibility of the protein substrate to proteolytic attack as the temperature is raised. The same effect gives rise to temperature coefficient (Q_{10}) values substantially higher than 2. Both the high specific activity and the apparent high activation energies are decreased to normal values if a small synthetic substrate is used instead of a mesophilic protein. The inference is that these effects are entirely caused by the increasing susceptibility of the substrate rather than being intrinsic to the enzyme. This high specific activity has clear commercial (and research) potential, given that almost all commercial substrates of proteases are mesophilic proteins. However it does require that use of such proteases takes place at a high enough temperature to take advantage of this effect.

At lower temperatures, all the available evidence suggests that the decrease in activity of proteases with temperature is essentially that which would be expected from the activation energy even in the case of thermophilic proteases cooled to below 0°C (Toogood and Daniel, unpublished results). That is, even with enzymes from the most extreme thermophiles, activity slows, but does not cease, even at temperatures below zero. Studies on a β-glucosidase and a glutamate dehydrogenase from extreme thermophiles showed an essentially linear Arrhenius plot over the whole of the temperature range, –80°C to +90°C, over which activity was measured (More, Petach and Daniel, 1994). Needless to say, rather sensitive assay techniques may be needed

to measure the enzyme activity at the lower end of this temperature range, reduced as it is by up to eight orders of magnitude

Properties of thermostable proteases

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

Since the mid 1980s, a large number of thermostable proteases have been characterised from eubacteria, and more recently from hyperthermophilic archaea. This section aims to give an overview of properties of some of the thermostable proteases characterised to date, with some emphasis on thermostability. *Table 1* lists some properties of proteases defined as being thermostable according to the criteria outlined earlier in this review. The substrate specificity of some of these proteases is presented in *Table 2*, although the substrates listed are by no means exhaustive.

SERINE PROTEASES

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

Bacillus *serine proteases*

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

The *Bacillus* serine proteases are principally alkalophilic, with pH optima typically >8.0. *Bacillus* sp. no. AH-101 produces an extremely alkalophilic protease with a pH optimum between 10 and 13 (Takami, Akiba and Horikoshi, 1989). It is moderately thermostable, and most readily hydrolyses insoluble fibrous proteins such as elastin and keratin (Takami, Akiba and Horikoshi, 1990). It has recently been cloned and

B. thermoproteolyticus (Thermolysin)		Tyr^{16}-Leu^{17}, His^{10}-Leu^{11}	Morihara (1974)
Chloroflexus aurantiacus J-10-fl	–	Ala^{14}-Leu^{15}, Gly^{23}-Phe^{24}	Watanabe *et al.* (1993)
Sulfolobus solfataricus DSM 1616	Ala-pNA, Leu-pNA, Arg-pNA, Phe-pNA	–	Hanner, Redl and Stoffler (1990)
Sulfolobus solfataricus MT-4 (ATCC 49155)			
(Carboxypeptidase)	Bz-Gly-Lys, CBZ-Tyr-ethyl ester	–	Fusi *et al.* (1991), Colombo *et al.* (1992)
Talaromyces duponti AP-1 (Aminopeptidase)	Leu-pNA	–	Chaupis and Zuber (1970)
Thermomicrobium st. KN-22	–	Phe^{24}-Phe^{25}, Gly^{23}-Phe^{24}, His^{10}-Leu^{11}, Tyr^{16}-Leu^{17}	Murao *et al.* (1991)
Thermus aquaticus st. YT-1 (Aminopeptidase T)	Gly-Phe-Ala, Leu-2-NA, Leu-4-NA, Val-Tyr-Val	–	Minagawa *et al.* (1988)
(CPase *Taq*)	Cbz-Phe-Tyr-NH_2, Cbz-Gly-Phe-NH_2, Cbz-Gly-Leu-NH_2	–	Lee *et al.* (1992)
Aspartic			
Bacillus st. MN-32 (Kumamolysin)	–	Leu^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}	Murao *et al.* (1993)
Bacillus st. Wai21a	–	Leu^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}, Gln^{4}-His^{5}, His^{5}-Leu^{6}, Phe^{24}-Phe^{25}	Prescott, Peek and Daniel (1995)
Bacillus st. Wp22.A1	–	Val^{2}-Asn^{3}, His^{5}-Leu^{6}, Ley^{15}-Tyr^{16}, Phe^{25}-Tyr^{26}	Toogood, Prescott and Daniel (1995)
Sulfolobus acidocaldarus (Thermopsin)	Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu	Leu^{11}-Val^{12}, Leu^{15}-Tyr^{16}, Phe^{24}-Phe^{25}, Phe^{25}-Tyr^{26}, Tyr^{26}-Thr^{27}	Fusek, Lin and Tang (1990) Lin and Tang (1990)
Cysteine			
Bacillus stearothermophilus 1503	Leu-Gly-Gly, $(Gly)_3$, $(Gly)_4$	–	O'Brien and Campbell (1957)
Sulfolobus solfataricus MT-4 (ATCC 49155)			
Protease III	Bz-Arg-pNA	–	Fusi *et al.* (1991)

–, data not available.
The list of substrates is not complete.
succ = sucanyl; CBZ = benzoyloxycarbonyl; pNA = π-nitroanilide; ONp = *p*-nitrophenyl ester; Bz = benzoyl; pip = piperazine; Nh-Np = 2-naphthylamide; FAGLA = Furylacryloyl-Gly-Leu-amide.

expressed in *B. subtilis*, and shows high sequence homology to the alkaline subtilisin-like proteases (Takami *et al.*, 1992).

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

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

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

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

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

Thermus *serine proteases*

The first *Thermus* serine protease to be characterised was caldolysin, an extracellular serine protease from *Thermus aquaticus* st. T-351 (Cowan and Daniel, 1982a). The original data on caldolysin suggested that it was a metallo-protease, but later studies showed that it is actually a serine protease (Cowan, Daniel and Morgan, 1987c). It has a molecular mass of 21 kDa, a pH optimum of 8.0, and is specific for small aliphatic amino acids on either side of the sissile bond (Cowan and Daniel, 1982a). Caldolysin is a very thermostable protease, with a half life of 228 min at 85°C (Cowan, Daniel and Morgan, 1987b). It binds a total of six Ca^{2+} ions per molecule, but has two high affinity binding sites ($Ka = 7.5 \times 10^{5} M^{-1}$) and four lower affinity sites ($Ka = 2.8 \times 10^{4} M^{-1}$) for

Ca^{2+} (Khoo *et al.*, 1984). The contribution to the free energy of stabilisation of caldolysin made by the six calcium ions is about 170 kJ mol^{-1}. However, the four weakly bound calcium ions contribute about 95 kJ mol^{-1}, which is almost identical to that made by the four calcium ions in both thermolysin and the *B. subtilis* neutral protease. It is possible that the higher thermostability of caldolysin is due to the additional two calcium ions. An interesting finding is that lanthanide ions confer a greater thermostability on apo-caldolysin than Ca^{2+}, increasing its thermostability from 1 h at 95°C with Ca^{2+} to more than 4 h with La^{2+}. It was shown that the relative ability of various divalent cations to stabilise the enzyme was dependent on their ionic radius and directionality (Khoo *et al.*, 1984). When immobilised on Sepharose 4B and CM-cellulose supports, caldolysin had an increased thermostability, but a decreased activity, perhaps due to steric hinderance (Cowan and Daniel, 1982b).

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

Thermus st. Rt41A produces a thermostable extracellular serine protease (Peek *et al.*, 1992a) that has been recently been cloned and expressed in *E. coli* (Munro *et al.*, 1995). It cleaves a high proportion of the bonds of the oxidised insulin B chain. It has a higher specificity for substrates containing small aliphatic or aromatic amino acids at the P1 position. Like aqualysin I, Rt41A protease contains four cysteine residues and has two disulphide bonds. It is stabilised against thermal denaturation at 10μM Ca^{2+}, and against autolysis at 5mM Ca^{2+} (Wilson, Peek and Daniel, 1994). Immobilised onto controlled pore glass (CPG) beads (Wilson, Peek and Daniel, 1994), the enzyme had a lowered pH optimum for the substrates azocasein and Succ-Ala-Ala-Pro-Phe-pNA, a similar effect to that found for caldolysin immobilised onto CPG beads (Cowan and Daniel, 1982b). The immobilised protease showed a dramatic increase in the thermostability at 70°C in the presence of 10μM Ca^{2+}, compared to the free enzyme at similar levels of calcium, with half lives of 110 h and 5 h respectively (Wilson, Peek and Daniel, 1994). The protease was capable of synthesising Bz-Ala-Tyr-NH_2 with a yield of 26% in the presence of 90% dimethylformamide at 40°C (Wilson, Daniel and Peek, 1994; Peek *et al.*, 1992c).

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

at 80°C and 100°C respectively, although Ca^{2+} ions enhanced stability (Freeman *et al.*, 1993).

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

Serine proteases from Archaea

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

Pyrococcus furiosus DSM 3638 produces a number of intracellular proteases of different molecular weights (Blumentals, Robinson and Kelly, 1990; Eggen *et al.*, 1990; Connaris, Cowan and Sharp, 1991; Snowden, Blumentals and Kelly, 1992). Blumentals, Robinson and Kelly (1990) described two of these proteases (S66 and S102) which they found to be SDS-resistant. The proteases were extremely thermostable, as an extract containing both S66 and S102 had a half-life of 33 h at 98°C. S66 was slightly inhibited by EDTA at 37°C, but as inhibition at high temperature was not investigated, it is not known if Ca^{2+} is required for thermostability. Immunoblot analysis showed that S66 and S102 are unrelated (Blumentals, Robinson and Kelly, 1990). Connaris, Cowan and Sharp (1991) identified 13 active proteases, including one metallo protease, produced by the same *Pyrococcus* sp., in contrast to the five identified by Blumentals, Robinson and Kelly (1990). They saw no resistance to SDS by any of the proteases, though they did identify a 66 kDa protease which may correspond to S66. A few of these proteases were stabilised by Ca^{2+}. The 66-kDa protease had half lives of 117 and 15 min at 105°C in the presence and absence of Ca^{2+} respectively (Connaris, Cowan and Sharp, 1991). Eggen *et al.* (1990) described the properties of an enzyme named 'pyrolysin' from the same *Pyrococcus* sp. However pyrolysin had multiple activity bands on SDS/PAGE, and the enzyme had a broad pH optimum of about 6.5–10.5. Eggen *et al.* (1990) contended that the multiple proteolytic bands of pyrolysin are processed to give a final 66-kDa protease, but investigations by others do not support this conclusion (Blumentals, Robinson and Kelly, 1990; Connaris, Cowan and Sharp, 1991). Thus, it appears that *P. furiosus* produces a heterogeneity of highly thermostable intracellular proteases of molecular weights ranging from 66 to 135 kDa, with the exact relationships between them not certain.

The thermoacidophilic archaebacterium *Sulfolobus solfataricus* ATCC 49155 produces six intracellular proteases, two of which are serine proteases (Fusi *et al.*, 1991). One of these, protease I, is extremely thermostable, having a half life of 342

min at 92°C (Burlini *et al.*, 1992). Ca^{2+} did not significantly affect the enzyme, but Mn^{2+} appeared to activate it. It is unstable in the presence of high salt, but is stabilised by Triton X–100. Interestingly, it lacks cysteine and tyrosine residues. It has a narrow substrate specificity, and amino acid residues close to, but not directly involved in the scissile peptide bond appeared to be crucial in substrate recognition.

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

Recently further work has been conducted on the protease from *Thermococcus stetteri* (Klingeberg *et al.*, 1995). This single polypeptide protease has a molecular weight of 68 kDa. It has a half-life of 2.5 h at 100°C and is SDS-resistant. Metal ions such as Ca^{2+}, Co^{2+} and Mg^{2+} did not have any significant effect on activity, but activity was stimulated (170%) by 0.5M NaCl. This requirement for NaCl is not surprising as *T. stetteri* was grown in a medium containing 0.43M NaCl. The protease has a narrow substrate specificity, cleaving only n-protected basic or hydrophobic (phe or tyr) p-nitroanilides and p-nitrophenol esters. It also has transferase activity (Klingeberg *et al.*, 1995).

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

Other thermostable serine proteases

Streptomyces rectus var. *proteolyticus* produces two serine proteases (A and B) that differ only in their amide content (Mizusawa and Yoshida, 1972). Protease B was found to retain 60–70% of its activity after a 10-min incubation at 80°C (Mizusawa,

Ichishima and Yoshida, 1964). It cleaved oxidised insulin B chain most readily at Phe^{24}-Phe^{25}, Leu^{15}-Tyr^{16} and Leu^{11}-Val^{12}, and proangiotensin at Tyr^{4}-Ile^{5} (Matsue, Majima and Ichishima, 1982).

Thermitase, from *Thermoactinomyces vulgaris*, is a thermostable alkaline serine protease (Hausdorf, Kruger and Hohne, 1980) which has a high sequence and structural similarity to the subtilisin-like serine proteases (Meloun *et al.*, 1985). It contains a cysteine residue near the active site that is apparently essential for activity. Thus, it is postulated to belong to the SH-dependent subtilisin subgroup of proteases (Meloun *et al.*, 1985). Thermitase binds three Ca^{2+} ions per enzyme molecule. One of these ions binds weakly to the enzyme causing an increase in both thermostability and activity. The other two Ca^{2+} ions bind more strongly, and have only small effects on thermostability and activity (Frommel and Hohne, 1981).

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

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

METALLO-PROTEASES

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

There are basically two groups of metallo-proteases (see Beynon and Bond, 1989). The first group consists of thermolysin-like proteases, while the second group of proteases are carboxypeptidase A-like. These groups differ in their three-dimensional structure, but have very similar active site configurations.

Thermolysin

Thermolysin, from *Bacillus thermoproteolyticus* (Endo, 1962), has been extensively investigated (eg. Titani *et al.*, 1972; Voordouw, Milo and Roche, 1976; Dahlquist, Long and Bigbee, 1976; Holmes and Matthews, 1982). It has a molecular weight of 34.5 kDa and a pH optimum of 7.2. Its amino acid sequence has been determined (Titani *et al.*, 1972), and three dimensional structure refined to a high resolution

(Matthews *et al.*, 1972a,b; Holmes and Matthews, 1982). Sequence and structural studies have shown that metallo-proteases from *B. subtilis, B. stearothermophilus* and *B. cereus* are closely related to thermolysin (Yang, Ferrari and Henner, 1984; Sidler *et al.*, 1986a, b; Paupit *et al.*, 1988). The eukaryotic protease carboxypeptidase A shows little structural and sequence similarity to thermolysin, but the active site structures and mode of binding of dipeptide inhibitors are quite similar (Kester and Matthews, 1977).

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

An incubation of thermolysin in the presence of EDTA results in a rapid inactivation of the enzyme due to autolysis (Fontana, 1988). Early work suggested that the two weakest bound Ca^{2+} ions were those bound to the double Ca^{2+} binding site (Voordouw and Roche, 1974; Roche and Voordouw, 1978). However, Weaver *et al.* (1976) showed that the ranking of the binding affinities of the Ca^{2+} ions are $Ca(1) >> Ca(3) > Ca(4) \geq Ca(2)$. EDTA titration of the two weakest-bound Ca^{2+} ions (Ca(2) then Ca(4)) showed that Ca(2) had no significant effect on thermostability, but the chelation of Ca(4) resulted in a fast and selective autolysis within the residues 190–205 (Weaver *et al.*, 1976; Fassina *et al.*, 1986). This polypeptide region corresponds to the binding site of Ca(4), which is within the conformationally most extended region of thermolysin (Fontana, 1988).

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

It is interesting to note that the metallo-protease from *B. subtilis* strain NRRLB3411 has a similar amino acid composition to thermolysin, as well as structural and functional similarities, but has a much lower thermostability (Pangburn *et al.*, 1976). A comparison of the amino acid sequences of these proteases showed that Asp^{200} in thermolysin was replaced by proline in *B. subtilis* protease, and three other residues in the region of Glu^{190}-Asp^{200} in thermolysin are absent in the *B. subtilis* protease

(Pangburn *et al.*, 1976). This region corresponds to the Ca(4) binding site in thermolysin, which suggests that the lower thermostability could be due to a lack of a Ca^{2+} ion binding to a region homologous to the Ca(4) region in thermolysin. Also, Asp^{57}, Asp^{59} and Gln^{61} of the Ca(3) binding site of thermolysin have been replaced by Ser, Thr and Thr respectively in this protease (Sidler *et al.*, 1986b). This could account for the observation that *B. subtilis* strain NRRLB3411 protease binds fewer Ca^{2+} ions than thermolysin (Levy *et al.*, 1975).

Other Bacillus *metallo-proteases*

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

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

The EA.1 protease differs in its amino acid sequence from YP-T protease by only one amino acid (Val^{61} of EA.1 —> Gly^{61} of YP-T; Saul *et al.*, 1995), but is almost twice as thermostable as YP-T at 85°C. Sequence alignments between these proteases and thermolysin show that residue 61 in EA.1 and YP-T corresponds to Ala^{58} in thermolysin, a residue within the binding pocket of calcium(3) (Matthews, Weaver and Kester, 1974). Preliminary molecular modelling studies, based on the structure of thermolysin, suggest the higher thermostability of EA.1 may be due to extra hydrophobic interactions between the isopropyl group of Val 61 and the aromatic ring of Tyr^{30} (Tyr^{27} in thermolysin). However, as both EA.1 and YP-T contain three more amino acids than thermolysin (Ser^{26}-Tyr^{27}-Tyr^{28}) that lie close to the proposed calcium (3) binding region, the actual interactions occuring are difficult to predict (Saul *et al.*, 1995).

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

B. cereus DSM 3101 produces a metallo-protease which has a high sequence homology to thermolysin, but is less thermostable (Sidler *et al.*, 1986a; Paupit *et al.*, 1988). It is a zinc-containing enzyme, and is stabilised by Ca^{2+} ions. It retained about 60% of its activity after an incubation of 20 min at 70°C in the presence of Ca^{2+} (Sidler *et al.*, 1986a). Its amino acid sequence is very similar to that of thermolysin, with fewer amino acid changes in the Ca^{2+} binding regions than in the *B. subtilis* NRRLB3411 protease. The crystal structure of the protease has been refined to 3.0Å resolution (Paupit *et al.*, 1988). When compared to that of thermolysin, it appears that the enhanced thermostability of thermolysin is not due to additional salt bridges, but is more likely to be due to extra hydrogen bonding interactions and the amino acid differences between them in the region of the double Ca^{2+} binding site (Paupit *et al.*, 1988). Sidler *et al.* (1986b) proposed that the difference in thermostability was due to effects such as lower hydrophobicity in the β-pleated sheets and lower bulkiness of the α-helical regions as compared to thermolysin.

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

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

Eijsink (1991) changed Ala^{69} of NprT to proline, the residue present in thermolysin at this position. This resulted in an increase in thermostability of 5.5°C of the mutant NprT (shifting of the half-life of 30 min at ~69°C to ~75°C), as opposed to the native NprT. This accounts for about 40% of the difference in thermostability between native NprT and thermolysin. It has been proposed that Pro^{69} has increased the thermostability of NprT by decreasing the flexibility of a surface loop, possibly resulting in a decrease in autolysis at that site (Eijsink, 1991). However, others (see Matthews, 1993) have suggested that the stabilising effect of proline substitutions often arises from an increase in the free energy of the unfolded state. Other mutations of NprT such as the substitution of Gly^{144} to Ala (Takagi and Imanaka, 1989), have resulted in a slightly increased thermostability and catalytic rate.

B. stearothermophilus st. MK232 produces a metallo-protease (NprM) that has a

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

NprM has also undergone site-directed mutagenesis to increase the stability at high temperatures (Kubo *et al.*, 1992). Instead of substituting amino acids at sites that could possibly be important for conformational stability, they substituted an amino acid at an autolytic cleavage site (Tyr^{93} to Gly and Ser). The mutant enzymes had lower specific activities, but were less rapidly inactivated at high temperatures because autolysis was reduced. This suggests that, for some proteases, engineering autolytic resistance as opposed to increasing conformational stability may be a useful method of enhancing stability. Interestingly, the mutant enzymes were more effective catalysts in the peptide synthesis reaction, producing more aspartame (50°C, 16 h) than the native enzyme (Kubo *et al.*, 1992). The decrease in specific activity appears to have been offset by the increased stability.

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

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

Two other thermostable metallo proteases have been characterised from *Bacillus* sp. The first is a zinc-containing protease isolated from *Bacillus brevis* 7882 (Paberit *et al.*, 1984). It has a broad substrate specificity, hydrolysing many of the bonds of oxidised insulin B chain including Leu^{17}- Val^{18} and Phe^{24}- Phe^{25}, and a half life of at least 1 h at 80°C which is dependent on Ca^{2+} ions (Paberit *et al.*, 1984). *B. stearothermophilus* KP1236 produces a metallo-protease which shares some antigenic determinants with thermolysin (Takii *et al.*, 1987). Activity was inhibited by *o*-phenanthroline, which could be recovered by the addition of either Zn^{2+}, Co^{2+} or Mn^{2+}. It was also thermostable, with no activity loss detected after an incubation of 10 min at 80°C.

Other metallo-proteases

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

The carboxypeptidase showed no loss of activity after 15 min at 85°C (Villa *et al.*, 1993), but thermostability was dependent on Zn^{2+} ions rather than Ca^{2+} ions. Zn^{2+} was also required for activity. Co^{2+} could substitute for zinc, but was not as effective. The activation free energies, enthalpies and entropies dropped when Zn^{2+} was removed. Thermal inactivation was found to be due to denaturation rather than autolysis under all conditions tested (i.e., with or without Zn^{2+}). Another interesting feature of this enzyme was its relatively low activation energy of 31.0 kJ/mol (Colombo *et al.*, 1992) which resulted in significant activity being detectable at room temperature.

Sulfolobus solfataricus strain DSM 1616 produces a tetrameric intracellular aminopeptidase of molecular weight 320 kDa (Hanner, Redl and Stoffler, 1990). Its substrate specificity is for peptides containing Ala, Leu and Phe, similar to that of other known aminopeptidases (e.g. Roncari, Stoll and Zuber, 1976). It was completely inhibited by EDTA, and activity was restored by Co^{2+} or Mn^{2+} but not by Ca^{2+}, Mg^{2+} or Zn^{2+}. Co^{2+} also significantly increased the thermostability of the enzyme. The aminopeptidase exhibited a 'temperature optimum' of 75°C (six minute assay), although the organism grows optimally around 85°C.

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

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

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

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

The fungus *Aspergillus oryzae* produces a thermostable metallo-protease (NpII) with a low molecular mass of 19.0 kDa (Nakadai, Nasuno and Iguchi, 1973; Tatsumi *et al.*, 1994). It requires Zn^{2+} for activity, but shares no significant sequence similarity with any known protein. Tatsumi *et al.* (1994) investigated reversible and irreversible activity loss up to 100°C. At these temperatures, apo-NpII underwent reversible denaturation, while holo-NpII was irreversibly inactivated under the same conditions, with this inactivation peaking around 75°C. This was presumably because the reversible denaturation of the active form of the enzyme (holo-NpII) was accompanied by autolysis. At temperatures higher than 75°C, irreversible inactivation of the holoenzyme decreased, probably because rapid thermal denaturation allowed less autolysis to occur.

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

ASPARTIC PROTEASES

The aspartic proteases, sometimes known as acid proteases, are characterised as

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

Bacillus *aspartic proteases*

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

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

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

Other thermostable aspartic proteases

Thermopsin, from *Sulfolobus acidocaldarius*, is an extracellular, extremely thermostable aspartic protease with unusual properties (Lin and Tang, 1990, Lin and Tang, 1995). It has a molecular weight of about 48 kDa, and a pH optimum of about 2. It is only non-specifically modified by DAN and EPNP, but is sensitive to pepstatin (Fusek, Lin and Tang, 1990). It bears no sequence similarity to any known aspartic protease, and is missing the characteristic active site Asp-Thr-Gly sequence found in almost all other aspartic proteases. Thus, it may represent a new sub-class of aspartic proteases. It has a half life of more than 48 h at 80°C, making it the most thermostable aspartic protease characterised (Lin and Tang, 1990). It cleaves the oxidised insulin B chain mainly between the characteristic Leu^{15}-Tyr^{16} and Phe^{25}-Tyr^{26} residues (Fusek, Lin and Tang, 1990).

Aspergillus niger F2078 produces a mildly thermostable protease with a pH optimum of 4.0 (Singh, Ghosh and Ghosh, 1994). It suffers only about a 10% loss of

activity after an incubation of 1 h at 60°C. It was not inhibited by EDTA, suggesting that Ca^{2+} may not be required for stability.

CYSTEINE PROTEASES

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

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

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

PROTEOSOMES

Proteosomes, or intracellular high molecular mass proteolytic multi-subunit enzyme complexes, are ubiquitous in eukaryotes, but are also found in the archaeum *Thermoplasma acidophilum* (Zwickl *et al.*, 1992). Puhler *et al.* (1994) screened a large number of archaea from all the major lineages and some eubacteria, but only found proteosomes in *Thermoplasma* strains. These complexes are unusual as they can typically catalyse peptide bond cleavage on the carboxyl side of basic, hydrophobic and acidic amino acid residues, though the *Thermoplasma* proteosome has a more restricted substrate specificity (Rivett, 1993). The multicatalytic activities seen in the eukaryotic proteosome (Orlowski, Cardoso and Michaud, 1993) are believed to be located at independent sites within the proteosome, as different activities responded differently to a variety of activators and inhibitors. High levels of Ca^{2+} (500 mM) stimulate the activity of the *Thermoplasma* proteosome against Suc-Ala-Ala-Phe-NMec sixteen-fold, and metal chelators such as EDTA and EGTA, but not *o*-phenantholine inhibit this activity strongly (Dahlmann *et al.*, 1989). This suggests that this activity was dependent on Ca^{2+}, not a heavy metal ion. Activity with the above peptide was also sensitive to diisopropylfluorophosphate, which led to the assumption that at least part of the proteosome is a serine protease.

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

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

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

OTHER THERMOSTABLE PROTEASES

A number of thermostable proteases have been described that have either not been classified, or do not apparently belong to any of the classes. The protease from the deep sea thermophilic methanogen *Methanococcus jannaschii* is extremely thermophilic and barophilic, with activity being detectable up to at least 131°C (Michels and Clark, 1995). Activity was increased over 3-fold at 130°C and thermostability was increased by the addition of 5OMPa pressure. The protease was highly specific for leucine at the P_1 position, and showed some esterase activity at lower temperatures (Michels and Clark, 1995).

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

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

Thermoactinomyces sp. HS682 produces a thermostable alkaline protease of molecular weight 25 kDa (Tsuchiya *et al.*, 1991). The enzyme was stable for 60 minutes at 65°C at pH 11.0.

Thermoactinomyces vulgaris (strain A 60) protease has a pH optimum of 9.0 and a half-life of 1 h at 85°C (Desai and Dhala, 1969). A protease with similar properties was produced by *Thermomonospora fusca* (A20). It had a pH optimum of 8.5 and a half-life of 1 h at 80°C. These latter two proteases were able to lyse a number of Gram (+) bacteria and *E. coli* (Desai and Dhala, 1969).

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

Molecular genetics of thermostable proteases

PROTEASES FROM *THERMUS* SPP.

Alkaline serine proteinases have been described from several *Thermus* isolates including *Thermus ruber* (Skjenstad *et al.*, 1992; Souter, Sharp and Marks, 1992) and *Thermus* strains T351 (Cowan and Daniel, 1982a), Rt_6 (Cowan, Daniel and Morgan, 1987b), Ok_6 (Jones, Morgan and Daniel, 1988), Tok_3 (Saravani *et al.*, 1989), ITI243 (Aevarsson, Holst and Kristjansson, 1991), IS-15 (Bjarnason *et al.*, 1992), and Rt41A (Peek *et al.*, 1992a). Only a few of the serine proteases from *Thermus* have been expressed in *E. coli* – e.g. aqualysin I (Kwon *et al.*, 1988) and Rt41A protease (Munro *et al.*, 1995). *Thermus aquaticus* st. YT-1 also produces thermostable metalloproteases such as CPase *Taq* that have been cloned and sequenced (Lee *et al.*, 1994a).

Alignment of the peptide sequences from members of the subtilisins and subtilisin-like serine proteases has revealed considerable homology between all of the sequences. Generally, the greatest homology is found in the regions involved in substrate catalysis (Asp32, His64 and Ser221; Wells and Estell, 1988) and to a lesser extent, in substrate specificity. *Thermus* sp. strain Rt41A produces an extracellular alkaline serine protease that has a half-life of 13.5 h at 80°C, and a pH optimum of 8.0. The protease gene was identified using primers designed to amplify the region between two highly conserved amino acid motifs present in subtilisin-like alkaline serine proteases using the polymerase chain reaction (PCR, see *Figure 1*). The PCR product was used to identify an *Apa* I fragment containing the gene (*apr*A), which was sequenced using standard dideoxy-procedures. The amino acid sequence deduced from the Rt41A gene sequence contained a region identical to that obtained by amino-terminal sequencing of purified mature Rt41A protease (Munro *et al.*, 1995). Comparison of the entire derived peptide sequence with other subtilisin-like serine proteases revealed significant homology, particularly with Aqualysin I (71% identity) and with Exoprotease A from *Vibrio alginolyticus* (61% identity).

*apr*A was amplified by the PCR and the products inserted into the vector pGEX-KG (Guan and Dixon, 1991) as an in-frame fusion to glutathione-S-transferase. Two different fusions were produced, pNZ1997 and pNZ1989, which differed in that the signal peptide of *apr*A was deleted in pNZ1989. Cell extracts prepared from induced cultures containing pNZ1997 displayed no detectable proteolytic activity when assayed with azocasein. However, incubation at 85°C showed that proteolytic activity increased as time progressed. With pNZ1997, the cell extracts had to be incubated at 85°C for at least 1 h before active protease could be detected. Activation was temperature-dependent, requiring a 4-h incubation at 70°C or a 5-min incubation at 100°C, in order to produce mature active protease. However, the enzyme produced by pNZ1989, which lacked the Rt41A signal peptide, required only a 20-min incubation at 85°C before peak proteolytic activity was detected (Munro *et al.*, 1995).

It should be noted that expression of Rt41A protease was successful only when it was produced as a protein fusion to glutathione-S-transferase. Normally, thermostable enzymes fused to GST are inactive at their optimal temperature because, at elevated temperatures, the mesophilic GST portion of the fusion denatures and causes the protein to precipitate. With fusions produced by the vector pGEX-KG, it is normal to remove the GST portion by cleavage with thrombin. In the case of Rt41A protease, the

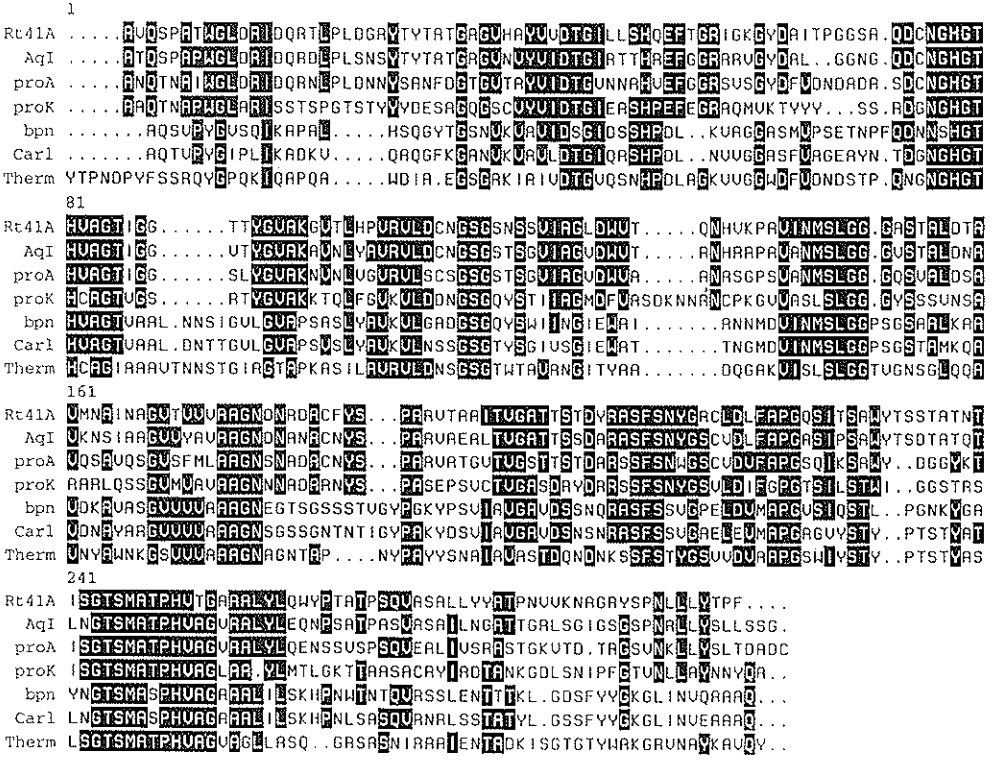

Figure 1. Comparison of the deduced mature peptide sequence of the Rt41A protease with subtilisins and subtilisin-like proteases compiled using the program PILEUP (Wisconsin Computer Group). Sequences used were: Aqualysin I, Aq1; Exoprotease A, proA; Protease K, protK; subtilisin BPN', bpn; subtilisin Carlsberg, Carl; and Thermitase, Therm. Amino acids are shaded where there is agreement in four or more sequences.

GST is removed along with the pre-pro-region during activation. As a result, a one-step purification process using glutathione-agarose can be employed to produce exceptionally pure enzyme. A heat treatment step similar to that used for Aqualysin I was required for the activation of the protease from the protein fusion (Munro *et al.*, 1995).

Assays of the activation of the Rt41A protease showed that there was a temperature-dependent lag period where no protease activity was detectable. Removal of the putative signal peptide virtually eliminated this lag period, suggesting that the signal peptide somehow interferes with the processing of the pro-enzyme to the mature enzyme. Activation of subtilases has been shown to be an intramolecular process with the pro-region responsible for correct folding of the molecule (Jany, Lederer and Meyer, 1986; Ohta and Inouye, 1990). Possibly, the signal peptide, which is normally removed before processing in the native organism (Fujishige *et al.*, 1992; Terada *et al.*, 1990), prevents the processing of the pro-Rt41A to the mature enzyme. One possible explanation is that the signal peptide could be causing the expressed protein to fold incorrectly and therefore inhibit access of the protease to the pro-enzyme processing site.

The serine protease Aqualysin I from *Thermus aquaticus* has been the subject of several attempts for cloning into *E. coli*. Kwon *et al.* (1988) used oligonucleotide probes based on the amino-terminal sequence of purified Aqualysin I from *Thermus aquaticus* YT-1 to isolate and clone a 1.1 kb *Pst*I fragment that contained the sequence of the mature enzyme. The nucleotide sequence showed that the fragment contained a single open reading frame without a stop codon, and by inference from other Gram-negative bacteria, they concluded that the enzyme was synthesised with three domains, an amino-terminal leader involved in inner membrane transport, the protease and a carboxy-terminal domain that was required for extracellular secretion. The mature enzyme was not expressed in *E. coli*.

Terada *et al.* (1990) showed that Aqualysin I is produced as a large precursor, and that the mature enzyme lacking the amino-terminal pre-pro-sequence accumulates in the membrane fraction of *E. coli*. Treatment of the membrane fraction at 65°C released the active enzyme into the soluble fraction. The entire gene was cloned under a controlled promoter since it was anticipated that direct expression of the intact gene product would be lethal to the cell. The gene was cloned in two parts and then assembled under the control of an inducible promoter. The sequence of the complete gene showed that the amino terminus of the deduced protein sequence had the characteristics of a signal peptide sequence, and an *E. coli*-like promoter sequence could be identified at the 5' end in the non-coding area. This promoter may have allowed constitutive expression of aqualysin and effectively made the intact gene unclonable. Aqualysin appears to be unusual in that there seem to be four domains in the precursor structure, and both amino- and carboxy-terminal processing takes place to give the mature enzyme of M_r = 28 000. The four domains consist of an amino-terminal signal peptide, an amino-terminal pro-sequence, a protease domain and a carboxy-terminal pro-sequence. Lee *et al.* (1991) showed that deletions in the amino-terminal sequence produced by restriction enzyme digestion and religation of the gene eliminated proteolytic activity and decreased the stability of the recombinant enzyme in *E. coli*. These experiments suggested that the amino-terminal pro-sequence is necessary in the production of the active enzyme. In other experiments (Lee *et al.*

1994b), it was shown that the carboxy-terminal pro-sequence was not essential for the production of active Aqualysin I and that the amino-terminal pro-region did not need to be covalently associated with the mature enzyme peptide sequence to allow the folding of the enzyme to give an active conformation. The function of the amino-pro-sequence is reminscent of a molecular chaperone (Lee, Ohta and Matsuzawa, 1992).

In an *E. coli system,* mature Aqualysin I is synthesized and processed to the mature enzyme but it is not secreted. Development of host-vector systems suitable for plasmid transformation of *Thermus thermophilus* (Koyama, Okamoto and Furakawa, 1989) allowed an examination of the expression and secretion of Aqualysin I in an extreme thermophile. The gene was cloned in two halves into a vector derived from the *T. thermophilus* plasmid pTT8 and correctly processed aqualysin I was found to be secreted into the medium; no intracellular proteolytic activity could be detected (Touhara *et al.*, 1991). The necessity of the *C*-terminal pro-sequence for secretion in *T. thermophilus* was demonstrated by Lee, Ohta and Matsuzawa (1992) who showed that transformants carrying plasmids with deletions in the *C*-terminal pro-sequence of more than five amino acids were toxic to the host cells. Their results suggest that the *C*-terminal pro-sequence is important for the extracellular secretion of the enzyme in the *Thermus* host and may be a necessary component for translocation of the precursor enzyme across the outer cell membrane of the bacterium.

In an interesting approach derived from this work, Takagi *et al.* (1990) have used site-directed mutagenesis to introduce cysteine substitutions to allow the formation of a disulphide bond in subtilisin E, using the information obtained on the location of the two cysteine residues in Aqualysin I. The mutant subtilisin carrying cysteine residues at positions 61 and 98 appeared to form a disulphide bond spontaneously in the *E. coli* expression system, and the half-life and optimal temperature of enzyme action was increased significantly over the wild type (Takagi *et al.*, 1990). These results suggested that it was possible to enhance the thermostability of subtilisin without changing the catalytic efficiency of the enzyme.

Lee *et al.* (1994a) reported the isolation, sequencing and expression of a carboxypeptidase from *Thermus aquaticus* YT-1 (CPase *Taq*). This thermostable metallo-carboxypeptidase was initiated from a GTG start codon and was not expressed in *Escherichia coli*. Conversion of the GTG to ATG in an expression vector carrying the *tac* promoter and a suitable ribosome binding site allowed the production of substantial amounts of the enzyme, which was shown to bind 1 mole of zinc ion per mole of enzyme protein. The CPase *Taq* had the consensus active site motif for zinc-dependent aminopeptidases, but comparison of the overall sequence showed no obvious sequence similarity with other metallo-peptidases (Lee *et al.*, 1994a).

GENES AND PROTEINS FROM THERMOPHILIC STRAINS OF *BACILLUS*

Thermophilic bacilli have been the subject of attention in biotechnology because of their relative ease of isolation and culture and their nutritional diversity. Although there is still confusion over the taxonomy of many isolates, *Bacillus stearothermophilus* is the most frequently used culture. This organism is generally easy to grow, though some strains show instability in continuous culture (Burke and Tempest, 1990) and other strains grow poorly on minimal medium.

We have used PCR to identify an internal fragment from a serine protease gene from

a thermophilic *Bacillus* sp designated strain Ak.1 (MacIver *et al.*, 1994). The primers used were originally designed to amplify a similar region from *Thermus* sp. Rt41A, an organism with a high G + C ratio (Munro *et al.*, 1995). However, the redundancy designed into these primers has allowed their use in amplifying the protease gene fragments from the *Bacillus* species even though the region sequenced has a much lower G + C ratio. The success in amplifying this region is explained by the high degree of homology which exists between all serine proteases around the active site histidine and serine residues.

Phylogenetic analysis employing the SSU rRNA sequence of *Bacillus* sp. Ak.1 showed that the bacterium is a member of the *Bacilli* rRNA group 5. This group includes *B. stearothermophilus* and other thermophilic *Bacilli*. The most closely related organism for which SSU rRNA data is available is *Bacillus thermoglucosidasius* (Ash *et al.*, 1991).

The gene for a serine protease from the thermophilic *Bacillus* species Ak.1 was identified by PCR amplification and the complete gene was cloned by isolation of suitably-sized restriction fragments from Southern blots using the PCR fragment as a probe. Two additional, distinct PCR products were also obtained, which were shown to have been derived from other serine protease genes present in *Bacillus* Ak.1. The three recombinants were sequenced to produce sufficient information to construct a continuous sequence of the inserted DNA. A comparison was made of the sequence with the EMBL and GenBank databases, and all three sequences showed homology to serine proteases. One was highly homologous to an intracellular serine protease from *Bacillus subtilis* and another had 43% identity with the *Bacillus licheniformis* subtilisin Carlsberg peptide sequence and 41% identity with the *Bacillus subtilis epr* gene. Further studies were performed with this latter gene and complete sequence analysis showed an open reading frame of 1206bp coding for a polypeptide of 401 amino acids.

The polypeptide was shown to be an extracellular serine protease with a signal sequence and pro-sequence. The mature protease possessed homology to the subtilisin-like serine proteases from a number of *Bacillus* species, with 61% homology to thermitase, a serine protease from *Thermoactinomyces vulgaris*. Another feature of the mature protease is the occurrence of two cysteine residues at positions 258 and 260 of the amino acid sequence. The formation of two disulphide bonds has been shown in Aqualysin I (Matsuzawa *et al.*, 1988), in Protease K (Jany, Lederer and Meyer, 1986) and in *Thermus* sp. Rt41A (Peek *et al.*, 1992a; Munro *et al.*, 1995). The cysteine residues in AK.1 protease are separated by only one amino acid and it would seem unlikely that an effective disulphide bond would be formed between them. It should be noted that thermitase contains only one cysteine residue, while the subtilisins (Carlsberg, BPN, amylosacchariticus, etc.) contain no cysteine residues.

The gene for AK.1 protease was expressed in *E. coli* in the expression vector pJLA602 and as a fusion with the a-peptide of the *lacZ* gene in the cloning vector pGEM5. The enzyme bound to a bacitracin column and this method provided a simple, one-step method for producing the protease purified to near-homogeneity.

The gene for an extremely alkaline thermophilic serine protease has been isolated from *Bacillus* sp. strain B18' (*aprM*). The expressed enzyme had a 'temperature optimum' of 85°C and a pH optimum of 13 (Masui *et al.*, 1994). Comparison of the sequence with other alkaline serine proteases showed that there was high homology,

but the enzyme contained fewer negatively-charged residues than other *Bacillus* alkaline serine proteases. Specifically, AprM has about the same number of positively-charged amino acids, but there is a preponderance of Arg residues compared to Lys. It was suggested that the Arg residues are located on the surface of the enzyme and enhance stability by altering surface charge at high pH. Substitution by site-directed mutagenesis of a Thr residue by a Pro in a β-turn structure, based on presumed similarities of serine proteases determined by X-ray crystallographic analysis, increased the temperature stability and temperature optimum of the enzyme. Pro 203 apparently stabilises the enzyme by decreasing the overall entropy of the β-turn structure, thereby forming a more rigid structure (Masui, Fujiwara and Imanaka, 1994).

A gene coding for an alkaline serine protease from the thermophile *Bacillus smithii* has been isolated and sequenced. It showed significant sequence similarity to *Bacillus licheniformis* and *B. liquefaciens* alkaline serine proteases, but details of expression were not reported (Milano *et al.*, 1994).

A number of genes coding for mesophilic *Bacillus* neutral proteases have been cloned and sequenced (Takekawa *et al.*, 1991; Wetmore, Wong and Roche, 1991; Tran, Wu and Wong, 1991; Avakov, Bolotin and Sorokin, 1990; Yang, Ferrari and Henner, 1984; Shimada *et al.*, 1985). From different strains of *Bacillus stearothermophilus* three neutral protease genes have been sequenced and characterized in detail, *npr*T (Takagi *et al.*, 1985) from CU21 (Imanaka *et al.*, 1982), *npr*M (Kubo and Imanaka, 1988) from strain MK232 (Kubo *et al.*, 1988) and *npr*S from TELNE (Nishiyama and Imanaka, 1990). Each of these proteases have closely related DNA and deduced peptide sequences with each other and with the neutral proteinase thermolysin from the *Bacillus thermoproteolyticus* (Titani *et al.*, 1972); the deduced peptide sequence of NprM differing from this protein by only two amino acid substitutions. Another member of the same family of proteases has been isolated from the *Bacillus stearothermophilus* variant, *Bacillus caldolyticus* YP-T(van den Burg *et al.*, 1991), with the latter enzyme showing significant increases in thermostability brought about by only minor changes in the primary structure of the enzyme.

Generally, protease genes from thermophilic *Bacillus* strains have been expressed in *Bacillus subtilis* after conventional isolation from gene libraries and sequencing. We have adopted a different approach, using PCR for gene isolation and transfer to regulatable expression vectors which replicate in *E. coli* (Saul *et al.*, 1995; MacIver *et al.*, 1994). This approach takes advantage of the variety of high copy number cloning vectors for *E. coli* and allows facile manipulation of the cloned genes.

The *Bacillus* strain EA.1 (Coolbear *et al.*, 1991) isolated from Mount Erebus, Antarctica, has an optimal growth temperature of 65°C. Phylogenetic analysis showed that EA.1 is a member of the *Bacilli* rRNA group 5. This group includes *B. stearothermophilus* and other thermophilic *Bacilli*. The most closely related organisms for which SSU rRNA sequence data is available is *Bacillus thermoglucosidasius* (Ash *et al.*, 1991) and *Bacillus* strain AK.1 (McIver *et al.*, 1994). The *N*-terminal sequence of the native mature EA.1 protease (K. Peek, personal communication) indicated that the protease was similar to NprT from *Bacillus stearothermophilus* (Takagi *et al.*, 1985) and Npr from *B. caldolyticus* YP-T (van den Burg *et al.*, 1991). Three PCR primers were designed for amplification of both the complete gene and also a gene truncated to produce an enzyme lacking the pre-pro-region. Restriction

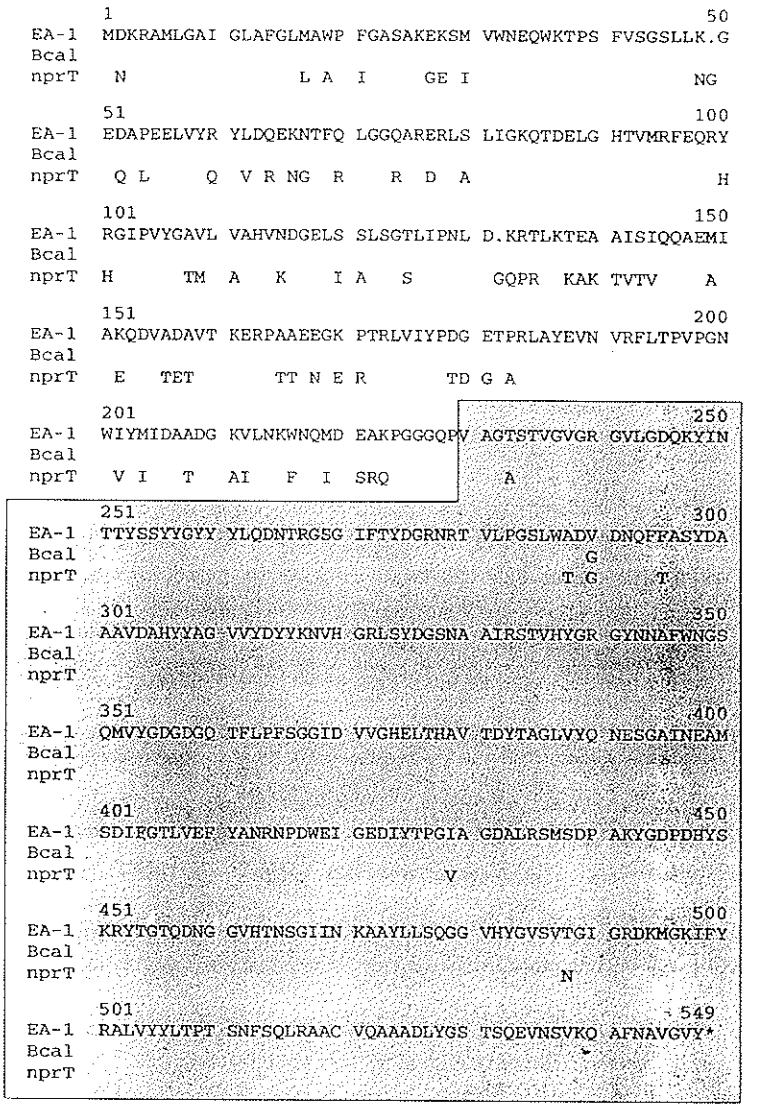

Figure 2. Alignment of the deuced peptide sequence of the neutral protease from Bacillus EA.1 with the Npr from Bacillus caldolyticus YP-T (Bcal) and NprT from Bacillus stearothermophilus (nprT). Only amino acids differing from EA.1 are shown.

sites were also included close to the 5' ends of the primers to facilitate directional insertion into the expression vector, pJLA602 (Schauder *et al.*, 1987). Polymerase chain reactions were performed using pairs of the three primers and the resulting products were digested with *Nco*I and *Sph*I and ligated into the same sites of the expression vector. The primer design allowed optimal positioning of the gene for expression, and in the case of the gene missing the *N*-terminal pre-pro-coding region, an ATG codon was provided also (Saul *et al.*, 1995).

The gene expressed a peptide of 546 amino acids, and homology comparisons with other *Bacillus* neutral proteases showed that EA.1 protease was most similar to YP-T protease. In order to be able to perform direct comparisons between the *B. caldolyticus* YP-T and *Bacillus* EA.1 neutral proteases, the *npr* gene from *B. caldolyticus* YP-T was isolated and cloned in the manner described for EA.1. In the course of our confirmation of the various *B. caldolyticus* YP-T*npr* recombinants, it became apparent that there were a number of discrepancies between our sequence and that published by van den Burg *et al.* (1991). As a consequence, the entire *B. caldolyticus* YP-T gene was resequenced and it was this sequence that was translated to produce the amino acid sequence used for the homology comparisons in *Figure 2*. The two peptides differ from each other by only one amino acid. The EA.1 neutral protease has a lower level of homology with NprT from *B. stearothermophilus* (87% identity) but most of the differences are located in the pre-pro-region and there are only five differences between these two proteins in the mature enzyme portion of the peptide.

PROTEASES FROM ARCHAEA

There is little information in the literature regarding the cloning and expression of proteases from Archaea. Lin and Tang (1990) purified a thermostable aspartic protease (thermopsin) from *Sulfolobus acidocaldarius*. Similar enzymatic activity has been found in other thermoacidophilic Archaea, such as *Sulfolobus solfataricus* and *Thermoplasma acidophilum*, but the genes have not been cloned (Lin, Liu and Tang, 1992). They isolated the gene encoding thermopsin using an oligonucleotide probe designed from the NH-peptide sequence. The nucleotide sequence showed that the gene consisted of 1020 bases giving a deduced peptide sequence in which 41 amino acids preceded the *N*-terminus of the mature enzyme. Most of these residues resembled the composition of a leader sequence and there may be a short pro-sequence. Thermopsin contained a single non-essential cysteine residue and the protein sequence has no apparent similarity to aspartic proteases of the pepsin family, nor to pepstatin-insensitive acid proteases. It may represent a new class of acid protease since it shows no obvious relatedness to any sequences in the databases (Lin and Tang, 1990).

Expression of the thermopsin gene in *E. coli* was examined by Lin, Liu and Tang (1992). Thermopsin expression was only achieved at significant levels as a fusion protein and it appeared that the gene product was degraded in *E. coli*. Fusions of thermopsin to the *C*-terminal end of porcine pepsinogen directed the newly-synthesised thermopsin into inclusion bodies, from which the recombinant thermopsin was refolded from urea. Heat treatment of the refolded protein caused denaturation of the pepsinogen portion of the fusion, which was degraded by the thermopsin. The pepsinogen fusion gave the greatest activity of a number of related fusions to other protease genes such as Procathepsin D and Rhizopuspepsinogen. Low level expression was achieved in a Baculovirus system using a fusion of thermopsin to the bee melittin signal peptide.

The sequence of a gene for an alkaline serine protease from the archeal hyperthermophile *Pyrobaculum aerophilum* was reported by Volkl *et al.* (1994). Computer-based analysis from the deduced amino acid sequence suggested that the enzyme had a similar structure to thermitase, and had some homology to the subtilisins

from bacteria, rather than from archeal or eucaryotic serine proteases. Sequence alignment was used to look for individual sites that contribute to the stability of aerolysin. The most common change was replacement by alanine, particularly at the beginning and end of putative surface helices.

Another moderately thermophilic alkaline serine protease was reported by Kamekura *et al.* (1992) from the archeal halophile *Haloferax volcanii*. The sequence of this enzyme has homologies with aerolysin and other bacterial subtilisins but has a long non-homologous *C*-terminal tail region.

OTHER THERMOSTABLE PROTEASES

The gene for another neutral zinc proteinase with no obvious homology to other metallo-proteases was cloned from the fungus *Aspergillus oryzae* (Tatsumi *et al.*, 1994). The gene was cloned as a cDNA copy into a yeast expression vector as well as into *E. coli*. It was shown to contain an unusually high number of cysteine residues, contributing to the three intramolecular disulphide bonds (Tatsumi *et al.*, 1994). Each of these cysteines was replaced one at a time to alanine by site-directed mutagenesis. Most mutants gave low expression levels, but one (C78A) which was comparable to wild-type had a reduced temperature optimum and thermostability. This suggests a role for the disulphide bonds in the thermostability of the enzyme (Tatsumi *et al.*, 1994).

Applications and potential applications

GENERAL CONSIDERATIONS

The applications of proteases have been well covered in recent reviews (Outtrup and Boyce, 1990; Kalisz 1988) and a brief review by Cowan, Daniel and Morgan in 1985 covered thermostable proteases in particular. We will restrict discussion here to those applications where stable proteases are currently in use, or where the advantages of use at high temperature seem especially marked.

There are a number of general advantages in using enzymes at high temperatures. Temperatures above 70°C are sufficiently high to kill almost all pathogenic bacteria and to greatly reduce the numbers of the bacteria most likely to cause troublesome contamination of food processes. These temperatures will also reduce viscosities by up to 50% compared with those at room temperature, lowering the costs associated with pumping, filtration and centrifugation, or allowing less water to be added during processing. Solubilities and diffusion rates will be higher, and the process is less likely to need cooling. Stable enzymes are also likely to allow the use of organic solvents and detergents, and are resistant to proteolytic attack (Daniel *et al.*, 1982; Daniel, Morgan and Martin, 1985; Owusu and Cowan, 1989; Peek *et al.*, 1992b).

In addition to these general advantages, there are some advantages of high temperature use which are specific to proteases. The major applications of bulk proteases involve the hydrolysis of proteins rather than peptides. This being so, the high specific activity of stable proteases which results from the denaturing of the substrate protein would seem to confer a significant commercial advantage. Associated with this high specific activity at high temperatures are large temperature

coefficients. In other words, when proteins are the substrate, protease activity is often more strongly dependent upon temperature than most enzyme reactions, with Q_{10} values in excess of 3 having been reported above 85°C (Cowan *et al.*, 1987), and values above 2 being relatively common (Cowan, Daniel and Morgan, 1985; Wilson, Peek and Daniel, 1994; Toogood, Prescott and Daniel, 1995). This leads to a very strong temperature-switching effect. For example, Q_{10} values of 1.7 and 3.0 will lead to 12% and 1.2% of the original activity respectively after a temperature decrease from 70°C to 30°C. For a temperature decrease from 95°C to 25°C, and a Q_{10} of 3, the residual activity will be only 0.05% of the original. For those applications where significant residual protease activity in the product is not acceptable, this temperature switching could obviate the need to remove or denature the protease. On the other hand, where a complete absence of protease in the final product is required because of long storage periods or sensitivity of the product, then use of a stable protease could pose serious problems.

An important point to bear in mind, however, especially in view of these high temperature coefficients, is that if thermostable proteases are not used at elevated temperatures, activity will be greatly reduced.

APPLICATIONS

Industry currently seems relatively happy with bulk proteases stable up to about 70°C, with the main reason for this probably being the current low yields of stable proteases, preferred use of temperatures in the range of 30–60°C and the extremely cost competitive nature of this market. So far thermostable proteases have not been produced in large quantities.

Probably the major current use of a stable protease is in the synthesis of the sweetening dipeptide aspartame precursor, N-CBZ-L-Asp-L-Phe methyl ester, by thermolysin (Isowa *et al.*, 1979). One synthetic method employs immobilised thermolysin to condense N-CBZ-L-aspartic acid with *L*-phenylalanine methyl ester at 25°C in water-saturated ethyl acetate (Nakanishi, Takeuchi and Matsuno, 1990). The resistance of the thermostable enzyme to organic solvents is crucial to the process, rather than its use at high temperature.

The only other stable protease actively marketed is a *Thermus* protease, sold as Pretaq by BRL, and used to clean up DNA for amplification in the PCR reaction. Initially developed for the rapid and simple preparation of blood and tissue samples for PCR (McHale, Stapleton and Bergquist, 1991), its use has been extended to cover the preparation of bacterial chromosomal DNA in agarose plugs for pulsed field electrophoresis (Borges and Bergquist, 1992) and as a general substitute for protease K (McHale, Bergquist and Peek, 1993). Success here is due to the relatively cost-insensitive nature of the applications, and possibly the widespread acceptance in molecular biology of thermophile-derived polymerases, such as Taq polymerase, for PCR.

POTENTIAL APPLICATIONS

Because of their high specific activities (see above) and the general advantages of operating enzyme processes at high temperatures, most applications for which proteases are currently used would seem to be potential applications for thermostable

proteases. Some of the recent reviews of protease applications therefore cover this ground (e.g. Kalisz, 1988; Outtrup and Boyce, 1990). We shall focus here on those applications where there is clearly industrial interest or specific application proposals.

Meat tenderising

Wilson *et al.* (1992) have suggested that the ideal meat tenderising enzyme would be active only during the (reasonably defined) cooking period and not during any storage period. This would allow controlled application of the tenderising agent before, or at, the point of sale without risk of over or under tenderising. The thermal stability and high temperature coefficient of thermostable proteases would fit them well for this use. Mechanical and sensory evaluation of three thermostable proteases indicated that good tenderisation and improved taste could be achieved at an appropriate level of application of one of the proteases. This enzyme also had the highest activity against collagen relative to that against meat powder (Wilson *et al.*, 1992).

Cleaning ultrafiltration membranes

The cleaning regime of the ultrafiltration membranes used in the processing of whey has a significant influence on their useful life. While some enzyme preparations for membrane cleaning are already in use, Coolbear *et al.* (1992) have proposed that the use of thermostable proteases at high temperatures (and low viscosities) might reduce the need for strong alkali and/or acid, and thus lengthen the useful life of the membranes. While the combination of an anionic detergent with the *Thermus* protease was an effective cleaning agent at 70°C, cleaning was appreciably slower, and over the 9 minute span of the experiment the water flux only reached about 75% of that obtained using 1% NaOH/1% EDTA (Coolbear *et al.*, 1992).

Cleaning

One of the major bulk uses of protease is in detergent formulations. Although improved stability is desirable for these applications, thermostable proteases are, so far, a long way from being produced in high enough yields to be cost-competitive. While this may eventually be overcome, current trends towards use at 20–40°C (especially in the United States) rather than up to 70°C may present another barrier.

A more attractive option in the cleaning field may be in dishwashing detergent. Enzyme use might allow a less aggressively alkaline pH to be used, and a thermostable protease would be well able to withstand the high detergent concentrations and temperatures above 60°C.

Another possibility may be in laundry detergents for institutional (e.g. hospital) use, where particularly high temperatures are desirable.

Protein recovery/concentration

A number of food industry processes, including the scavenging of waste protein from carcasses, viscosity reduction and the prevention of fouling during the evaporation of protein-containing waste streams (e.g. in the production of fish solubles from

stickwater) are desirably carried out at above 70°C to reduce the risk of microbial contamination of what may eventually be a food component. Proteases stable above 70°C would allow, if necessary, extended hydrolysis periods with a lower risk of significant contamination by potential pathogens. Evaporation of water from these waste streams, which may originate at quite high temperatures, would also be facilitated by proteases sufficiently stable to act while the waste stream is maintained at a high temperature.

Other uses

There are a number of minor applications of proteases where thermostability might be desirable. For example, protein hydrolysates for microbial growth media might be cleaner and cheaper if produced at higher temperatures, and this may also be the case for other protein hydrolysates. Contact lens cleaning using boiling water could allow the effective use at very low concentrations of stable proteases. The ability to function in high concentrations of organic solvents will probably have further applications in peptide synthesis.

The future

Thermostable proteases will continue to attract much research interest, both pure and applied. Our background knowledge of proteases makes them an attractive subject for studies on the effect of temperature on enzyme mechanism. Although much current engineering of proteases has focussed on increasing stability (Wells and Estell, 1988), efforts to manipulate properties have been relatively more successful, and stable proteases would seem a good target for such manipulation. Work to date in this area may well be just the beginning. Our understanding of proteases may also make them a good subject for research designed to investigate the linkage between enzyme activity, stability, and dynamics, despite the complications posed by autolysis.

Finally, we may expect a variety of new and even more thermostable proteases to be found in the Archaea, a relatively unexplored group as far as enzymes are concerned. It may be that they will have unique properties. It should be noted that there is already evidence that some Archaeal proteases have distinctive properties that tend to set them apart from other microbial proteases (Colombo *et al.*, 1992; Lin and Tang, 1990; Fusek, Lin and Tang, 1990). Cysteine proteases, for example, are rare in bacteria, but two have already been found in Archaea (Fusi *et al.*, 1991; Morikawa *et al.*, 1994).

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