Caldolysin, a highly active protease from an extremely Thermophilic Bacterium

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Proteases comprise a significant proportion of those proteins which have been subject to detailed characterisation (amino acid sequence and high resolution crystallographic analysis). The extent of research interest in proteolytic enzymes reflects both their historical status, and the practical advantages of proteases as research subjects (available in quantity, extracellular etc.) widely occurring.

Since 1952, a variety of proteases from thermophilic organisms have been isolated and characterised. In particular, the extracellular protease (Thermolysin) from *Bacillus thermoproteolyticus* has been subjected to numerous detailed kinetic[1] and structural studies[2]. However, to date, there have been no extensive studies on proteolytic enzymes from extreme thermophiles, although a number of reports of their existence have appeared[3,4]. The work reported here on Caldolysin, the extracellular protease from the extreme thermophile, *Thermus aquaticus* strain T-351[5-8], thus appears to be the first detailed characterisation of a protease from an extremely thermophilic (caldoactive) bacterium.

OCCURRENCE AND PURIFICATION

Thermus aquaticus strain T-351, an aerobic, nonmotile, non-sporulating, gram-negative rod, was initially isolated from hot alkaline pools in the Rotorua thermal area and has since been shown to actively grow *in vivo* at temperatures up to 100°C. Caldolysin is present at a concentration of up to 2 mg 1^{-1} in the supernatant of cultures grown at 75°C.

Purification of Caldolysin is carried out by a sequence involving ammonium sulphate precipitation, DEAE-cellulose ion exchange, Sephadex SP-C25 ion exchange, affinity chromatography on carbobenzoxy-D-phenylalanine-tetraethylenetetramine-Sepharose 4B, and gel filtration chromatography with Sephadex G75. The yield of fully purified enzyme is routinely of the order of 50 per cent.

With a specific activity of 21,000 Proteolytic Units per mg (PU mg^{-1}) at 75°C (using casein as substrate), purified Caldolysin is one of the most active protease known (Table 1). This is probably due in part to denaturation of protein substrates at high temperatures and a resulting increase in their susceptibility to proteolytic attack. Highly active mesophilic proteases such as *Bacillus subtilis* neutral protease [10] denature when heated above about 60°C, thus precluding the attainment of as high an activity as Caldolysin.

Table 1. The specific activities of thermophilic and mesophilic proteases.

Protease	Temperature (°C)	Specific Activity (PU mg^{-1})^a	Reference
Caldolysin	75	21,000	-
Tok 3 Protease	75	38,450	-b
Thermolysin	35	12,930	-c
B. subtilis neutral protease	30	13,600	10
Papain	30	2,550	10
Chymotrypsin	30	1,813	10
Trypsin	30	1,653	10
Pronase	40	1,250	11

a. 1 PU = 1 μg tyrosine released per minute from casein under specified assay conditions.
b. Saravani, Cowan, Daniel and Morgan, unpublished results.
c. Calculated from data provided in Matsubara[9].

PROPERTIES

Proteases can normally be designated as being Acid, Serine, Thiol, or Metal-chelator-sensitive types[12]. Inhibitor sensitivity is one of the primary classification tools (Table 2). Further sub-classification is determined by pH optimum and other characteristics.

Since treatment with 25mM o-phenanthroline (two hours, 75°C) did not significantly reduce the activity of Caldolysin (Table 2), it appeared that no zinc ion was present in the molecule (c.f., in Thermolysin, a catalytic zinc ion was rapidly chelated by 0.2mM o-phenanthroline at 25°C [13]). However, analysis of purified Caldolysin by atomic absorption spectroscopy indicated that both before and after dialysis against the chelating agent EDTA zinc was present in a stoiciometry of approximately one atom per protein molecule. Furthermore, the zinc was retained against EDTA dialysis even after Caldolysin was inactivated by autoclaving, suggesting an inaccessible location for the zinc ion within the protease. It is not yet known whether the zinc has either a stabilising or activity-related role.

The response of Caldolysin to EDTA and EGTA (Table 2) suggested that calcium was implicated in either the activity or stability of the enzyme. However pretreatment of Caldolysin with EDTA did not impair catalytic function, but activity was then rapidly and irreversibly lost after heating to 75°C. Calcium is thus present as a molecular stabilising agent.

The specificity of metal ion binding to Caldolysin was investigated by reconstitution of the apoenzyme (calcium removed by dialysis against EDTA) with solutions of calcium, zinc, strontium, magnesium, cobalt, barium, vanadyl and copper salts. With the exception of the vanadyl ion, (Khoo, Daniel, Cowan and Morgan, unpublished results), in no case was more than 40 per cent of the calcium-conferred stability regenerated, suggesting that none of the other metal ions could fully satisfy the structural requirements of the calcium binding sites. VO^{2+}, however, was able to provide almost 100 per cent of the calcium-conferred stability. Reconstitution of solutions of the apoenzyme with 10mM calcium, 10mM, zinc and a mixture of both calcium and zinc (each 10mM) yielded half-lives at 90°C of 59 min, 6 min and 24 min respectively. These results indicate that zinc is capable of competing for the calcium sites (N.B. crystal ionic radii: Ca - 0.99A°; Zn -0.74A°), but in doing so, provides little molecular stabilisation.

The lack of response of Caldolysin to the specific inhibitors of the serine, thiol and acid protease classes (Table 2) implies that it cannot be assigned to these groups.

The classification of Caldolysin as a metal-chelator-sensitive lytic protease [12] was established by the discovery that the enzyme was capable of lysing bacterial cells. Caldolysin was shown to lyse 16 of the 18 strains of Gram-negative bacteria tested, but was unable to completely lyse any of 12 strains of Gram-

Table 2. The effect of inhibitors on the activity of Caldolysin.

Class	Inhibitor	Concentration	% Inhibition of protease activity
Serine-protease inhibitor	Phenylmethyl sulphonyl fluoride	2.0mM	14%
		0.5mM	0
Thiol-enzyme inhibitor	p-Chloro mercuri-benzoate	5.0mM	0
Acid-protease inhibitor	Diazo-norleucine methyl ester	0.015mM	0
Trypsin inhibitor	Soybean trypsin inhibitor	1.0 mg ml^{-1}	0
Metal chelator	EDTA	10.0mM	68%
		1.0mM	40%
		0.1mM	0
Calcium-specific chelator	EGTA[a]	10.0mM	45%
		1.0mM	18%
Zinc-specific chelator	o-phen-anthr-oline	25.0mM	0

a. Ethylene glycol-bis-(β -amino ethyl ether) N, N'-tetra acetic acid.

positive bacteria, (four Gram-positive strains were partially lysed). This marked specificity for Gram-negative organisms is uncharacteristic of most lytic enzymes since the outer layers of lipoprotein and lipopolysaccharide of Gram-negative cell walls normally prevents access of lytic enzymes to the underlying peptidoglycan[14].

In a comparison (Table 3) of some of the physical and biochemical characteristics of Caldolysin with those of Thermolysin and the lytic proteases from *Sorangium* and *Myxobacter* (the proteases which most closely resemble Caldolysin), it is evident that no general similarity exists. However, there are some points of equivalence:

* the lytic and proteolytic specificities of Caldolysin and α-lytic protease are comparable;
* both Caldolysin and *Myxobacter* AL-1 lytic protease possess the unusual characteristic of a minimum substrate size limit (both will hydrolyse pentaglycine and tetraglycine but not triglycine or diglycine);
* the metal ion requirements and inhibitor responses of Caldolysin and *Myxobacter* AL-1 lytic protease are similar.

Neither the lytic proteases nor Thermolysin possess the degree of thermostability shown by Caldolysin (see below). Furthermore, Caldoysin demonstrates an unusually high carbohydrate content.

THERMOSTABILITY

Thermostability data for Caldolysin, apocaldolysin and a variety of proteases from thermophilic and mesophilic sources are presented in Table 4. Not only does Caldolysin possess a greater degree of thermal stability than any other characterised protease from a thermophilic organism, but the stability of the apoenzyme, although greatly reduced, is still of the same order as that of many proteases from mesophilic organisms. Calcium stabilisation, such as observed in Caldolysin, is a feature of many of the enzymes listed in Table 4.

In Caldolysin, a large proportion of the total energy of stabilisation of the molecule (about 50 per cent) is derived from the presence of calcium ions[6]. These are presumably located as linking agents in crucial salt-bridges on the molecule surface.

In solution, proteolytic enzymes can lose activity from autolysis, thermal denaturation, or a combination of the two, depending on the incubation temperature. An analysis of the kinetics of activity loss in solutions of Caldolysin has shown that at 90°C and above, thermal denaturation is the major factor, while at lower temperatures, autolysis is responsible. This conclusion is confirmed by the presence of a major discontinuity at about 90°C in the Arrhenius plot for Caldolysin, suggesting that significant structural changes occur at this temperature.

STABILITY TO OTHER REAGENTS

Caldolysin is very stable in the presence of detergents and other denaturing agents (Table 5). Hydrogen-bonding disrupting agents (urea and guanidine hydrochloride) and cystine cleavage reagents (dithiothreitol and mercaptoethanol) result in a substantial destabilisation at 75°C, whereas sodium dodecylsulphate (SDS; an anionic detergent known to dissociate hydrophobic bonds [26]) is less effective. The contribution of disulphide bonds (ca. 6) to the total energy of stabilisation of Caldolysin at 75°C has been calculated to be approximately 30 per cent, a value consistent with the results in Table 5.

Table 3. A comparison of the properties of some proteases.

Property	Myxobacter AL-1 protease [1,15,16]	Sorangium proteases α [1,17]	β [1]	Thermolysin [9,12]	Caldolysin
Molecular weight	13,500	19,778	19,000	37,500	20,500
Carbohydrate content	1.3%	?	?	0	12%
Cystine residues	2	3	2	0	4-6
Esterase activity	No	Yes	?	No	No
Lytic activity	Predominantly Gram-positive	Gram-Negative	Yes, specificity unknown	No	Gram-negative
Elastase activity	?	Yes	?	Yes	Yes
Specificity	Small a.a. on either side of splitting point	Small aliphatic a.a. on C side of splitting point	Lysine on amino side of splitting point	Hydrophobic or bulky a.a. on amino side of splitting point	Small aliphatic a.a. on either side of splitting point
Size limit in active site	4 residues	No	?	No	4 residues
Metal ion cofactors	possibly Zn	None	1 Zn	1 Zn, 4 Ca	1 Zn, Ca
Inhibitors	EDTA, citrate, phosphate (10^{-2}M)	DIFP	?	EDTA,o-phenanthroline	EDTA, EGTA

Properties similar in all of the above proteases: pI values, 8-10, pH optima, 7.5-9.0.

Table 4. The thermostability of Proteases from Thermophilic and Mesophilic organisms.

Enzyme name	Source [a]	Half-life (hours)	at: T (°C)	Ref
Caldolysin	Thermus T-351 (E)	0.5 30 193	95 80 75	
Protease	Bacillus caldolyticus (E)	8	80	3
Thermolysin	B. thermoproteolyticus (T)	1.0	80	9
Lytic protease	Micromonospora vulgaris (T)	0.5-0.75	80	18
Thermomycolase	Malbranchea pulchella (T)	2	73	19
Neutral protease	B. stearothermophilus (T)	5	65	20
Alkaline protease	Aspergillus sydowi (M)	0.1	55	21
Bromelain	Ananas comosus (M)	0.3	55	22
Neutral protease	B. subtilis (M)	0.25	61	23
Lytic protease AL-1	Myxobacter (M)	5.7	50	15
Pepsin C	Porcine (M)	1.2	25	24
Apocaldolysin	Thermus T-351 (E)	1.0 5.5	50 35	

a. (E) - extreme thermophile; (T) - thermophile; (M) -mesophile. Conditions of incubation (pH, ionic strength, etc) vary between the examples. However, conditions are normally chosen to maximise thermostability. Calcium is present, where required, in all cases except apocaldolysin.

Table 5. The effect of denaturing agents on the stability of Caldolysin.

Denaturing agent added	Activity loss at 18°C	Half-life at 75°C
None	40% after 30 weeks	193 hours
1% SDS	none after 18 hours	5 hours
6M guanidine HCl	25% after 31 hours	59 min
8M urea	none after 67 hours	53 min
8M urea + 120mM mercaptoethanol	none after 72 hours	26 min
10mM dithiothreitol	16% after 8 hours	5.3 hours

It has been suggested [26] that the acceleration of oxidative processes in the high temperature environments of thermophilic organisms should provide selective pressures to decrease the level of sulphur amino acids and hence cystine residues within the proteins of these organisms. This proposal is not supported by the results presented.

We are currently investigating several other proteases from extreme thermophiles. These differ considerably from one another and the only common features we have been able to distinguish so far are high specific activity and thermostability. It seems likely that the proteases from extremely thermophilic bacteria will display the same variation as proteases from mesophiles, both in enzyme characteristics and in the mechanisms responsible for thermostability. The stabilisation of thermophilic proteins has been investigated in some detail in recent years [27] and it has been shown that no one single mechanism is responsible.

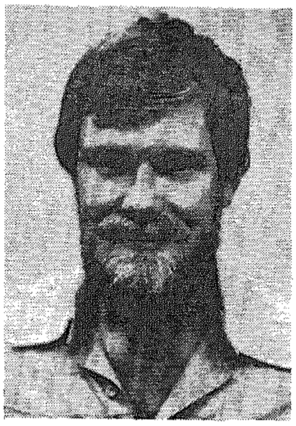
The major contributing factors to the thermostability of Caldolysin are calcium binding and disulphide bonding. However, these mechanisms are by no means characteristic of all thermophilic enzymes [27,28].

The thermostability, detergent stability and high activity of Caldolysin may render this enzyme potentially valuable in industrial applications. The advantages of operating enzymatic hydrolysis processes at high temperatures have been previously outlined[29].

REFERENCES

1. H. Matsubara, and H. Feder, in *The Enzymes,* Vol. 3, (P.D. Boyer, ed.) Academic Press, New York, 721-795 (1971).
2. R.S. Roche, and G. Voorduow, *Crit. Rev. Biochem. 5,* 1 (1978).
3. U.J. Heinen, and W. Heinen, *Arch. Mikrobiol. 82,* 1 (1972).
4. W. Sidler, G. Frank and H. Zuber, *Experientia* (Basel) *33*,800, (1977).
5. D.A. Cowan, D. Phil. Thesis, University of Waikato, Hamilton, New Zealand (1980).
6. D.A. Cowan, and R.M. Daniel, *Biochim. Biophys. Acta* in press (1982).
7. D.A. Cowan, and R.M. Daniel, *J. Biochem. Biophys. Methods. 6*,31 (1982).
8. D.A. Cowan, and R.M. Daniel, *Biotechnol. Bioeng.* in press (1982).
9. H. Matsubara, in *Methods in Enzymology,* Vol. 19, (G.E. Perlmann, and L. Lorand, eds.) Academic Press, New York, 642-651 (1970).
10. K.T. Yasunobu, and J. McConn, in *Methods in Enzymology,* Vol. 19, (G.E. Perlmann, and L. Lorand, eds.) Academic Press New York, 569-575 (1970).
11. Y. Narahashi, in *Methods in Enzymology,* Vol. 19, (G.E. Perlmann, and L. Lorand,. eds.) Academic Press, New York, 651-664 (1970).
12. K. Morihara, *Adv. Enzymol. 41,* 179 (1974).
13. S.A. Latt, B. Holmquist, and B.L. Vallee, *Biochem. Biophys. Res. Comm. 37,* 333 (1969).
14. J.M. Ghuysen, *Bacteriol. Rev. 32.* 425 (1968).
15. R.L. Jackson, and G.R. Matsueda, in *Methods in Enzymology,* Vol. 19, (G.E. Perlmann, and L. Lorand, eds.) Academic Press, New York, 591-599 (1970).
16. J.C. Ensign, and R.S. Wolfe, *J. Bacteriol. 90,* 395 (1965).
17. D.R. Whitaker, in *Methods in Enzymology,* Vol. 19, (G.E. Perlmann, and L. Lorand, eds.) Academic Press, New York, 599-613 (1970).
18. I.G. Golovina, E.P. Guzhova, T.I. Bogdanova, and L.G. Loginova, *Mikrobiologiya 42,* 620 (1973).
19. G.M. Gaucher, and K.J. Stevenson, in *Methods in Enzymology,* Vol. 45, (L. Lorand,. ed.) Academic Press, New York 415-433 (1976).
20. R.T. O'Brien, and L.L. Campbell, *Arch. Biochem. Biophys. 70,* 432 (1957).
21. G. Danno, and S. Yoshimura, *Agric. Biol. Chem. (Tokyo) 31*,1151 (1967).
22. M. El-Gharbawi, and J.R. Whitaker, *Biochemistry 2,* 476 (1963).
23. J.D. McConn, D. Tsuru, and K.T. Yasunobu, *J. Biol. Chem. 239,* 3706 (1964).
24. A.P. Ryle, and R.R. Porter, *Biochem. J. 73,* 75 (1959).
25. K. Weber, and D.J. Kuter, *J. Biol. Chem. 246, 4504 (1971).*
26 *J.D. Hocking, and J.I. Harris, in Experientia Suppl.*

26 (H. Zuber., ed.) Birkhauser Verlag, Basel. 121-133 (1976).
27. R.E. Amelunxen, and A.L. Murdock, *Crit. Rev. Microbiol. 6*, 343 (1978).
28 D.A. Cowan, and R.M. Daniel, unpublished results.
29 R.M. Daniel, D.A. Cowan, and H.W. Morgan, *Chemistry in New Zealand 45*, 94 (1981).



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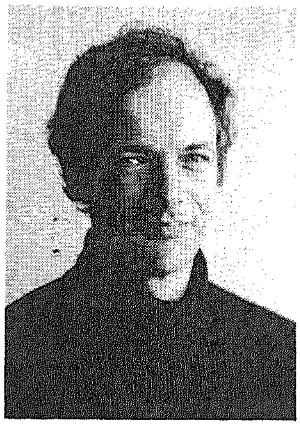

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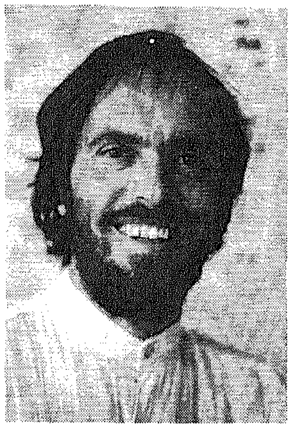

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