Characterization of a chelator-resistant proteinase from *Thermus* strain Rt4A2

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The *Thermus* isolate Rt4A2 was found to produce an extracellular chelator-resistant proteinase. The proteinase was purified to homogeneity by $(NH_4)_2SO_4$ precipitation, cation-exchange chromatography, gel-filtration chromatography, and weak anion-exchange chromatography. The Rt4A2 proteinase was found to have properties typical of an alkaline serine proteinase. It had a pH optimum of 9.0 and was specifically inhibited by phenylmethanesulphonyl fluoride. Its isoelectric point was greater than 10.25. Its molecular-mass was 31.6 kDa as determined by SDS/PAGE. N-terminal sequencing has shown it to have high sequence similarity with other serine proteinases from *Thermus* species. The proteinase hydrolysed a number of substrates including fibrin, casein, haemoglobin, collagen, albumin and the synthetic chromogenic peptide substrate Suc-Ala-Ala-Pro-Phe-NH-Np. The specific activity of the purified proteinase using azocasein as substrate was 313 units/mg. Substrate inhibition was observed above an azocasein concentration of 0.05% (w/v). Esterase activity was directed mainly towards those substrates containing the aliphatic or aromatic residues of alanine, glycine, tryptophan, tyrosine and phenylalanine. Thermostability half-lives of greater than 7 days at 70 °C, 43 h at 80 °C and 90 min at 90 °C were found in the presence of 5 mM $CaCl_2$. At 90 °C increasing the $CaCl_2$ concentration 100-fold (0.5 mM to 50 mM) caused a 4.3-fold increase in the half-life of the enzyme from 30 to 130 min. Half-lives of 19.4 min at 100 °C and 4.4 min at 105 °C were found in the presence of 50 mM $CaCl_2$. The metal chelators EGTA and EDTA reduced the stability at higher temperatures but had no effect on the activity of the proteinase. Activity was not stimulated by common metal activators such as Ca^{2+}, Mg^{2+} and Zn^{2+}.

INTRODUCTION

A number of extracellular proteinases have been characterized from *Thermus* species: caldolysin from *Thermus* strain T351 (Cowan and Daniel, 1982), the proteinase from *Thermus caldophilus* GK24 (Taguchi et al., 1983), aqualysins I and II from *Thermus aquaticus* YT-1 (Matsuzawa et al., 1983), caldolase from *Thermus* strain Tok3A1 (Saravani et al., 1989) and the proteinase from *Thermus* strain Rt4A2 (Peek et al., 1992).

All of these *Thermus* proteinases are of the serine class (inhibited by phenylmethanesulphonyl fluoride) and are stable at temperatures above 70 °C. However, most are found to be unstable at these temperatures in the presence of chelating agents such as EDTA, due to the removal of enzyme-bound Ca^{2+} ions that stabilize the tertiary structure against thermal denaturation. Caldolase and aqualysin I are relatively chelator resistant but caldolase is unstable at low ionic strength.

The extracellular proteinases characterized from thermophilic *Bacillus* spp. are predominantly metalloproteinases, for example: thermolysin from *B. thermoproteolyticus* (Matsubara, 1970), the proteinases from *B. stearothermophilus* NCIB 8924 and *B. stearothermophilus* B-3880 (Sidler and Zuber, 1980) and the proteinase from *B. stearothermophilus* KP 1236 (Takii et al., 1987). These proteinases are destabilized, and at high temperatures inactivated, by Ca^{2+} chelation, but are also inactivated at lower temperatures due to the chelation of the active-site Zn^{2+} ion.

The paper describes the selection, purification, and characterization of a *Thermus* proteinase which is relatively resistant to chelators (and is stable at low ionic strength). Such an enzyme is expected to have high intrinsic stability and may prove useful in biotechnological applications (Cowan et al., 1985; Gusek and Kinsella, 1988).

MATERIALS AND METHODS

Selection of a strain producing a chelator-resistant proteinase

Eleven proteinase-producing *Thermus* and *Bacillus* strains were selected from the Thermophile Research Unit's culture collection and are listed in Table 1. Included as controls in the selection were three isolates with extracellular proteinases of known chelator-sensitivity. These are the EA.1 metalloproteinase which is rapidly deactivated by EDTA (Coolbear et al., 1991), the Rt41A proteinase which is significantly sensitive to EDTA (Peek et al., 1992) and caldolase which is moderately EDTA resistant (Saravani et al., 1989).

Each isolate was grown up on 10 ml of Castenholz medium D (Castenholz, 1969) in 20-ml-capacity universals, incubated at 65 °C (for *Bacillus* strains) or 70 °C (for *Thermus* strains) in an orbital-shaking incubator. Proteinase activity in each culture supernatant was measured at the growth temperature with azocasein as substrate (see below) after preincubation at 70 °C for 30 min in 50 mM Hepes buffers, pH 7.5 (at 70 °C), containing either 5 mM $CaCl_2$ or 10 mM EDTA.

Growth of *Thermus* strain Rt4A2

Thermus strain Rt4A2 was grown on a Castenholz medium modified with 5.0 g/l sodium L-glutamate and 0.3 g/l each of yeast extract and trypticase peptone. A 3.0 l shake flask inoculum was used to inoculate 600 l of prepared medium in an 800 l stainless-steel fermenter. After 13.5 h of growth at 70 °C with

Abbreviations used: Suc, succinyl; NH-Np, nitroanilide; Bz, benzoyl; Z, benzyloxycarbonyl.

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Table 1 Sensitivity of extracellular proteolytic activity of selected strains to EDTA

Culture supernatant from each isolate was preincubated at 70 °C for 30 min in 50 mM Hepes/NaOH buffer, pH 7.5, containing 10 mM EDTA. Proteinase activity was determined at 65 °C or 70 °C (according to the growth temperature of the organism). Results are expressed as a percentage of the activity remaining in a control preincubation of each culture supernatant containing 5 mM $CaCl_2$.

Bacterial strain	Genera	Activity remaining (%)
Rt4A2	*Thermus*	75
TOK3A1*	*Thermus*	32
RT666A1	*Thermus*	16
OK2A1	*Thermus*	6
Rt41A*	*Thermus*	5
Rt460	*Bacillus*	3
Rt648A1	*Bacillus*	0
EA1*	*Bacillus*	0
Ket3A1	*Bacillus*	0
Tp8A1	*Bacillus*	0

* Denotes control strains with proteinases of known EDTA sensitivities.

aeration and pH control, the culture was clarified using a continuous flow centrifuge (Sharples, Model 6) at 100 l/h and 13000 ***g***.

Purification

To the culture supernatant, $(NH_4)_2SO_4$ was dissolved to 70% saturation and left standing overnight. The precipitated protein was harvested by continuous flow centrifugation (Sharples, Model 6). The precipitate (660 g) was stored at −70 °C until required for use.

Frozen precipitate (640 g) was thawed, resuspended in 1900 ml of 50 mM Tes/NaOH buffer, pH 7.5, and insoluble material removed by centrifugation. The precipitate was re-extracted a further two times, and all supernatants were pooled.

To avoid losses due to non-specific binding (Peek et al., 1992) Triton X-100 detergent was added to a final concentration of 0.01 % (v/v) to 5.5 l of pooled extract, which was then diafiltered with an equal volume of 50 mM Tes buffer, pH 7.5/0.01 % (v/v) Triton X-100 using a S1Y100 spiral-wound ultrafiltration cartridge (Amicon).

S1Y100 permeate (11.0 l) containing the proteinase was concentrated 9-fold in a 2.5 l stirred ultrafiltration cell at 4 °C using a YM2 membrane (Amicon) and dialysed at 4 °C against 20 mM Tes/NaOH buffer, pH 7.5/5 mM $CaCl_2$/0.01 % (v/v) Triton X-100 (Tes buffer).

The dialysed extract was then applied in two runs to a Hiload 26/10 S Sepharose cation-exchange f.p.l.c. column (Pharmacia) equilibrated with Tes buffer (without Triton X-100) at a flow rate of 7 ml/min. The unbound protein was discarded and the proteinase eluted with a 420 ml linear gradient of 0–1 M NaCl in Tes buffer (without Triton X-100). The active fractions were pooled, desalted and concentrated to 50 ml by diafiltration on a YM5 membrane (Amicon), using Tes buffer.

The two 50 ml desalted concentrates were pooled and further concentrated (by ultrafiltration) to 10 ml. This was then applied to the Hiload 26/60 Superdex 200 (preparative-grade) gel-filtration f.p.l.c. column (Pharmacia) equilibrated with Tes buffer at a flow rate of 3 ml/min and 3 ml fractions were collected. The active fractions were pooled (150 ml) and stored in aliquots at −70 °C.

A portion (27 ml) of the Superdex-processed material was diafiltered and concentrated to 5 ml using 25 mM Caps [3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid]/NaOH buffer, pH 10.75, containing 5 mM $CaCl_2$ and 0.01 % (v/v) Triton X-100, and applied to a Mono P HR 5/20 anion-exchange column (Pharmacia), equilibrated in the same buffer. The unbound fraction containing pure proteinase (10 ml) was collected, diafiltered and diluted to 25 ml using Tes buffer, and frozen in aliquots at −70 °C until required for use.

Proteinase assay

Proteinase activity was determined by the release of trichloroacetic-acid-soluble peptides from azocasein at 75 °C (or 65 °C and 70 °C in the initial screening work). The pH of all buffers was adjusted at the temperature at which they were used.

The assay reaction was started by the addition of a proteinase-containing sample to a preincubated Eppendorf containing 0.2 % (w/v) azocasein substrate (final concentration) in 50 mM Hepes/NaOH, pH 7.5/5 mM $CaCl_2$ in a final volume of 1 ml. Where required, samples were diluted in 50 mM Hepes buffer/0.01 % (v/v) Triton X-100.

The reactions were stopped after 10 min with the addition of 500 μl of 15 % (w/v) trichloroacetic acid. The mixture was left for 10 min at room temperature and centrifuged at 15000 ***g*** for 5 min. The absorbance of the supernatant was determined at 420 nm. The assay was linear up to a change in absorbance of 0.2. One unit is defined as that amount of enzyme activity producing a change in absorbance at 420 nm of 1.0 in 1 min.

Esterase assays (1.5 ml) containing 0.67 unit of proteinase were incubated at 50 °C in the presence of each ester at a concentration of 1 mM in 50 mM Hepes/NaOH, pH 7.0/50 % acetonitrite/5 mM $CaCl_2$/0.01 % (v/v) Triton X-100. Initial rates of reaction for ester hydrolysis were determined by monitoring the increase in absorbance at 410 nm.

For determination of the effect of metal ions on activity, the proteinase was preincubated at 75° for 5 min in 50 mM Hepes/NaOH buffer, pH 7.5, in the presence of 2 mM metal ion. Assays were carried out at a 1 mM concentration of metal ion, carried over from the preincubation.

For the determination of inhibitor sensitivity, proteinase was preincubated at room temperature for 10, 30 and 60 min in 50 mM Hepes/NaOH buffer, pH 8.3/5 mM $CaCl_2$/0.01 % (w/v) Triton X-100 and one of the following inhibitors: 10 mM phenylmethanesulphonyl fluoride, 1 mM E64, 10 μg/ml pepstatin and 10 mM *o*-phenanthroline (Dunn, 1989). Residual activity was determined using the standard proteinase assay.

Protein assay

Protein was determined by a modified method of Bradford et al. (Saravani, 1985) using BSA as a standard. Interfering substances (Peterson, 1983) were removed by dialysis or dilution. Samples containing Triton X-100 were assayed by comparison to BSA standards containing Triton X-100.

Electrophoresis

Proteins were subjected to SDS/PAGE by using the Pharmacia PhastSystem with 10–15 % gradient gels. To prevent autolysis proteinase-containing samples (4 μg) were treated with 10 mM phenylmethanesulphonyl fluoride for 4 h then denatured by adjusting to 0.1 M HCl and incubating for 30 min at room

temperature. Samples were lyophilized ready for the addition of sample buffer.

The isoelectric point was estimated by focusing on a 1% (w/v) agarose (Pharmacia) gel containing 6.3% (v/v) Pharmalyte 8.0–10.5 (Pharmacia) and 12% (w/v) sorbitol, as described by Pharmacia (in Isoelectric Focusing Principles and Methods). Gels were run at 600 V for 3 h at 15 °C on a Pharmacia flat-bed electrophoresis unit, flushed continuously with nitrogen. High-isoelectric-point markers (Pharmacia) were run alongside proteinase samples (5 μg). After electrophoresis, gels were fixed immediately with a solution of 10% (w/v) trichloroacetic acid/5% (w/v) sulphosalicylic acid and stained with Coomassie Blue R-250, as described by Pharmacia (in Isoelectric Focusing Principles and Methods).

Determination of thermal stability

Experiments were performed with proteinase samples (20 μg/ml) in 10 mM Hepes/NaOH buffer, pH 7.5 (adjusted to the working temperature), containing 0.01% (v/v) Triton X-100 and $CaCl_2$. Proteinase depleted of metal cations was prepared by dialysis against Hepes buffer containing 1 mM EDTA, followed by exhaustive dialysis against Hepes buffer. Thermal stability experiments below 90 °C were performed in sealed Eppendorfs which were completely immersed in a water bath at the required temperature. At higher temperatures, samples (50 μl) were sealed into glass capillaries to ensure rapid temperature equilibration. After incubation samples were chilled on ice until required for assay of residual proteinase activity.

Gel-filtration chromatography

Gel filtration was performed on a TSK G3000SW column, 7.5 mm × 60 cm (Toyo Soda Co., Japan). The column was equilibrated with 20 mM Tes/NaOH buffer, pH 7.5, containing 500 mM NaCl, 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100 at a flow rate of 1 ml/min, and protein was detected by measuring the absorbance at 280 nm. Native molecular mass markers (18–300 kDa) were used as standards (Pierce Chemical Co.)

Amino acid composition and N-terminal sequence

These were determined essentially as described previously (Peek et al., 1992), except that proteinase samples were prepared by acid precipitation at 4 °C with an equal volume of 20% (w/v) trichloroacetic acid. The precipitated enzyme was pelleted by centrifugation at 13000 *g*, lyophilized and resuspended in 6 M HCl containing 0.5% (w/v) phenol. Oxidized samples for the determination of total cysteine levels were prepared by incubation in the presence of performic acid at −5 °C for 2 h and lyophilized before the addition of 6 M HCl.

RESULTS AND DISCUSSION

Selection of a strain producing a chelator-resistant proteinase

The results of the initial screening of ten proteinase-producing isolates for EDTA-resistant activity are shown in Table 1. It is noticeable that the *Bacillus* isolates have shown the greatest chelator sensitivity and this may be due to a greater frequency of metalloproteinases among these isolates.

On the basis of these results *Thermus* strain Rt4A2 was chosen for further study. Isolated from a natural hot pool in the Rotorua area, it is non-sporulating, Gram-negative aerobe with rod-shaped morphology and yellow–orange pigmentation with optimal growth at 70 °C (Hudson, 1985).

Purification

The Rt4A2 proteinase was purified to homogeneity by the sequence shown in Table 2. A loss of 19% of the cell-free proteinase activity occurred during the $(NH_4)_2SO_4$ precipitation of the culture supernatant, most of which was due to the incomplete pelleting of the fine protein precipitate. Because of this large initial loss, overall recovery of proteinase from the culture supernatant was 26%. The unexpected increase in total activity following the Superdex stage may be due to the removal of a soluble inhibitory factor during this procedure.

Purity of the proteinase was demonstrated by the presence of a single band on Coomassie Blue stained SDS/PAGE Phast gels and on isoelectric focusing gels. The proteinase focused beyond the last pI marker (pI 10.25) and at the alkaline extreme of the Pharmalyte pH range. The isoelectric point of the Rt4A2 proteinase is strongly alkaline as are those of two other serine proteinases produced by *Thermus* species, namely aqualysin I,

Table 2 Purification of the Rt4A2 proteinase

Stage	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Buffer extraction of $(NH_4)_2SO_4$ precipitate	50300	4830	10	1	100
Ultrafiltration and dialysis	39700	1467	27	3	79
S Sepharose	25200	199	127	12	50
Superdex	30200	143	211	20	60
Mono P	24700	79	313	30	49

Table 3 Amino acid composition of the Rt4A2 proteinase and comparison with *Thermus* spp. Rt41A proteinase and aqualysin I

Compositions of Rt41A and aqualysin were obtained from Peek et al. (1992) and Matsuzawa et al. (1988) respectively.

	Number of residues per molecule		
Amino acid	Rt4A2	Rt41A	Aqualysin
Asx	29	31	28
Glx	18	11	9
Ser	30*	26	28
Gly	40	35	38
His	7	6	5
Arg	12	11	16
Thr	25*	38	24
Ala	32	39	41
Pro	16	16	12
Tyr	16	17	17
Val	28†	26	27
Met	4	4	2
Cys	3‡	4	4
Ile	13†	12	10
Leu	24	24	19
Phe	9	6	3
Trp	5§	5	5
Lys	6	5	2

* Value extrapolated to $t = 0$ hydrolysis.
† Determined after 2 h hydrolysis at 150 °C.
‡ Determined after oxidation of sample with performic acid (see text).
§ Determined spectrophotometrically.

pI > 10 (Matsuzawa et al., 1988), and proteinase Rt41A, pI > 10.25 (Peek et al., 1992).

Molecular-mass determination

The molecular mass of the Rt4A2 proteinase was 31.6 kDa as determined by SDS/PAGE. Elution from a TSK G3000SW gel-filtration column gave an anomalous molecular mass estimate of 10.0 kDa. This indicates interaction with the column matrix leading to retardation. Anomalous molecular mass estimations using gel-filtration chromatography have been observed for a number of extracellular microbial proteinases (Voordouw et al., 1974). There has been speculation that the basicity of a proteinase might contribute to retardation (Ensign and Wolfe, 1966; Arvidson et al., 1973), but hydrophobic interaction and hydrogen bonding with the matrix, as well as the effect of molecular shape, may also be important.

Amino acid composition and N-terminal sequencing

Table 3 shows the amino acid composition of Rt4A2 proteinase and a comparison with Rt41A (Peek et al., 1992) and aqualysin I (Matsuzawa et al., 1988). As expected the proteinases are similar in terms of composition, however, a number of interesting differences do occur. Of this group Rt41A proteinase is the least stable (particularly in the absence of Ca^{2+}) but its only significant difference in amino acid composition from Rt4A2 and from aqualysin I is its high threonine content. The notable difference between Rt4A2 and the other two proteinases is the higher content of glutamate plus glutamine and phenylalanine, and the lower content of alanine. Also Rt4A2 appears to have only three cysteines in contrast with Rt41A and aqualysin I which form two disulphide bonds. The results suggest Rt4A2 may have at least one free thiol group per enzyme molecule. In addition the arginine/lysine ratio is higher for aqualysin I than either Rt4A2 or Rt41A proteinases.

The Rt4A2 proteinase (Figure 1) shows homology to four other *Thermus* proteinases (proteinase K being of fungal origin), but differs at residues six and possibly 13.

pH optimum

Maximum activity of the Rt4A2 proteinase was observed at pH 9.0 and 75% of this activity was retained over the pH range 6.5–10.5, using universal buffer (Dawson et al., 1969) (results not shown). The pH optimum of the proteinase falls within the range established for serine proteinases of *Thermus* origin by proteinase Rt41A, pH optimum 8.0 (Peek et al., 1992), and aqualysin I, pH optimum 10.4 (Matsuzawa et al., 1988), and is close to that of caldolase, pH optimum 9.5 (Saravani et al., 1989).

Substrate inhibition

The Michaelis–Menten relationship showed significant substrate inhibition above an azocasein concentration of 0.1% (w/v) and the Lineweaver–Burk plot shows deviations from linearity at high substrate concentrations as a result of the substrate inhibition (results not shown). Theoretical values for K_m were 0.029% (w/v), and for $V_{max.}$ 446 units/mg. The inhibition constant was estimated to be 0.78% (w/v) from a plot of $1/V$ against S (Dixon and Webb, 1979).

Substrate inhibition has also been observed with the proteinases caldolysin, caldolase, proteinase Rt41A, subtilisin BPN and proteinase K, with high-molecular-mass substrates (Cowan, 1980; Saravani, 1985; Bajorath et al., 1988; Peek et al., 1992). The mechanism of inhibition is unknown but a number of explanations are possible. Studies on the specificity of enzymes, in particular proteinases, suggest many enzymes have several groups which combine with particular parts of the substrate molecule before catalysis, and it is possible to imagine an ineffective enzyme–substrate complex in which some of the binding groups are combined with one substrate molecule while other groups are combined with a second substrate molecule. At high substrate concentrations the chances of formation of ineffective complexes would increase (Dixon and Webb, 1979). However, other mechanisms such as steric hinderance from binding close to the active site, or the presence of an inhibitory contaminant in the substrate must be considered (Cowan et al., 1987a).

Hydrolysis of proteins

At 75 °C collagen and casein appear to be the most susceptible to proteolytic attack (Table 4); however, at this temperature heat effects on the substrate are likely to be significant, especially on collagen (which rapidly forms gelatin) and haemoglobin (which denatures and coagulates). Susceptibility of the native protein to

	Position														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Proteinase															
Rt4A2	A	V	Q	S	P	V	T	W	G	L	D	?	V/A	D	Q
Rt41A	A	V	Q	S	P	A	T	W	G	L	D	N/A	I	D	Q
Caldolase	A	V	Q	S	P	A	T	?	G	L	D	?	V/I	D/I	
Aqualysin I	A	T	Q	S	P	A	P	W	G	L	D	R	I	D	Q
Caldolysin	A	V	Q	S	E	A	T	W	G	L	D	R	Y	D	Q
Proteinase K	A	A	Q	T	N	A	P	W	G	L	A	R	I	S	S

Figure 1 N-terminal sequence similarities of the Rt4A2 proteinase

Fifteen N-terminal amino acids of the Rt4A2 proteinase compared with those of four other *Thermus* proteinases (Peek et al., 1992), aqualysin 1 (Matsuzawa et al., 1988), caldolysin (K. Peek, unpublished work), caldolase (M. Prescott, unpublished work) and the proteinase K (Jany et al., 1986).

Table 4 Activity of the Rt4A2 proteinase against natural proteins

Proteins were dissolved or suspended at a concentration of 0.2% (w/v) in 50 mM Hepes/NaOH buffer, pH 7.5 (at 75 °C), containing 5 mM $CaCl_2$. Assays (1 ml vol.) were performed with agitation where necessary, at 75 °C and 35 °C. The release of trichloroacetic-acid-soluble peptides was determined by measuring the absorbance at 280 nm: for assay procedure see Cowan and Daniel (1982). Units are ΔA_{280}/min.

Natural protein (0.2%, w/v)	Activity (units) Temp.... 75 °C	35 °C
Fibrin	2.07	0.24
Casein	4.39	0.22
Haemoglobin	2.18	0.19
Collagen	6.06	0.11
Albumin	1.68	0.07
Elastin	0.13	0.02
Keratin	0.00	0.00

proteolysis is best seen at 35 °C; at this temperature, fibrin, casein and haemoglobin were the most susceptible to proteolytic attack by the Rt4A2 proteinase. The Rt4A2 proteinase shows similar relative activity toward casein, haemoglobin and collagen as do caldolysin, caldolase and proteinase Rt41A (Cowan et al., 1987b). It shows greatest similarity to caldolase, which is most active against casein and haemoglobin, and has moderate activity against collagen, albumin and fibrin with trace activity against elastin (Sarvani et al., 1989).

The Rt4A2 proteinase was also active against dye-bound derivatives of proteins. However, it was noted that while the rate of hydrolysis of casein is three times that of albumin, the reverse is found for the azo-dye derivatives (rate of hydrolysis of azoalbumin being twice that of azocasein). A similar observation has been made with caldolysin (Cowan, 1980) and two explanations are possible; first, structural changes in the protein during azo-derivatization may have resulted in increased accessibility of susceptible albumin peptide bonds, or the steric interference of peptide bonds in casein (Cowan, 1980). Secondly, there may be a difference in the average distribution (or substitution number) of azo-groups between the two protein derivatives, with the net effect of over-estimation of azoalbumin hydrolysis relative to that of azocasein.

Hydrolysis of synthetic substrates

Of seven peptides tested, Rt4A2 proteinase showed greatest activity against Suc-Ala-Ala-Pro-Phe-NH-Np. Although this peptide was originally synthesized as a substrate for chymotrypsin (Del Mar et al., 1979) it is readily cleaved by the subtilisins and other serine proteinases. Lesser activity was seen against *N*-Bz-Phe-Arg-NH-Np, Suc-Ala-Ala-Pro-Leu-NH-Np, and Suc-Phe-Leu-Phe-NH-Np (no activity was detected against D-Val-Gly-Arg-NH-Np, D-Val-Leu-Lys-NH-Np or Z-Lys-Arg-NH-Np). Due to the presence of terminal blocking groups on these peptides, activity of the Rt4A2 proteinase against them indicates an endopeptidase mode of action. The results are similar to those obtained with Rt41A proteinases and show a preference for aromatic or hydrophobic amino acids at the P_1 position. Activity against *N*-Bz-Phe-Arg-NH-Np also shows that positively charged amino acid side chains can also be accommodated.

The Rt4A2 proteinase was able to hydrolyse (in order of activity) the following benzyloxycarbonyl amino acid *p*-nitrophenol esters: alanine > glycine > tryptophan = tyrosine = phenylalanine > leucine > benzylcysteine. Valine, proline, isoleucine and benzylaspartate esters were not hydrolysed. Caldolase (Saravani et al., 1989) and aqualysin I (Matsuzawa et al., 1988) also show most activity toward the alanine ester, and generally cleave a similar type of amino acid (aromatic and aliphatic).

Inhibitors

Of the inhibitors tested, phenylmethanesulphonyl fluoride was the only one to significantly reduce the proteolytic activity of the Rt4A2 proteinase (reacting to give complete inhibition within 10 min). This places the proteinase in the serine class. Rt4A2 is not a cysteine proteinase, as E64 was not inhibitory. Pepstatin, the aspartic (acid) proteinase inhibitor, had no effect. The metalloproteinase inhibitor 1,10-phenanthroline (a zinc chelator) also had no effect.

Observed effects of metal ions (1 mM; see the Materials and methods section) on activity ranged from no effect (Li^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{3+}) to a strongly inhibitory effect. Moderate inhibitory effects were seen in the presence of a number of metal ions, which reduced the activity to between 45 and 84% of the full activity (V^{3+}, Co^{3+}, Zn^{2+}, La^{3+}, Cu^{3+}, Ni^{3+}, Cd^{3+} and Ag^{3+}). Only Hg^{2+} showed extreme inhibitory effects and reduced the activity remaining to less than 10%. The poisoning of enzymes by heavy metal ions is well documented (Valle and Ulmer, 1972). The ions of mercury, cadmium, and lead are known to react with protein thiol groups (converting them into mercaptides), as well as with histidine and tryptophan residues. In addition, disulphide bonds can be hydrolytically degraded by the action of silver or mercury (Torchinsky, 1981).

None of the metal chelators tested (EDTA, EGTA and 1,10-phenanthroline) had a significant effect on the activity of the Rt4A2 proteinase during the standard 10 min assay, and the zinc chelator 1,10-phenanthroline had no effect on enzyme activity or stability. However, the general metal chelators, EGTA and EDTA, did cause a moderate reduction in the stability of the Rt4A2 proteinase (23% and 29% respectively). The observation that full activity could not be regained by the subsequent inclusion of 5 mM $CaCl_2$ in the assay mix confirms that the chelation of the metal ions present decreases stability (causing irreversible denaturation or more probably, reversible denaturation followed by autolysis) and not activity (which would be reversible upon addition of activating ion).

Effect of temperature and $CaCl_2$

The effect of temperature on activity, when represented as an Arrhenius plot, shows a linear relationship over the range 0–90 °C with an apparent activation energy of 65 kJ/mol. A downwards deviation of the plot is seen above 90 °C due to the effects of increased thermal denaturation.

It is evident from Figure 2 that the thermostability of Rt4A2 is markedly affected by the presence of $CaCl_2$. At 90 °C, maximum stability ($t_{\frac{1}{2}}$ = 132 min) was observed in the presence of 50 mM $CaCl_2$ (I = 0.15 M) and is greatly decreased at lower concentrations. A similar observation has been reported for the *Thermus* proteinase caldolysin (Cowan and Daniel, 1982; Khoo et al., 1984). Even at 5 mM the Ca^{2+} ions are far in excess of potential calcium-binding sites (enzyme concentration is about 0.6 μM). It has been suggested for caldolysin that a change in the concentration of free ions influences the period during which the calcium sites are occupied and hence stability. NaCl at equivalent ionic strengths could not substitute for $CaCl_2$, thus indicating that increased thermostability is not conferred, in part, by increased ionic strength as has been reported for caldolase (Saravani et al., 1989).

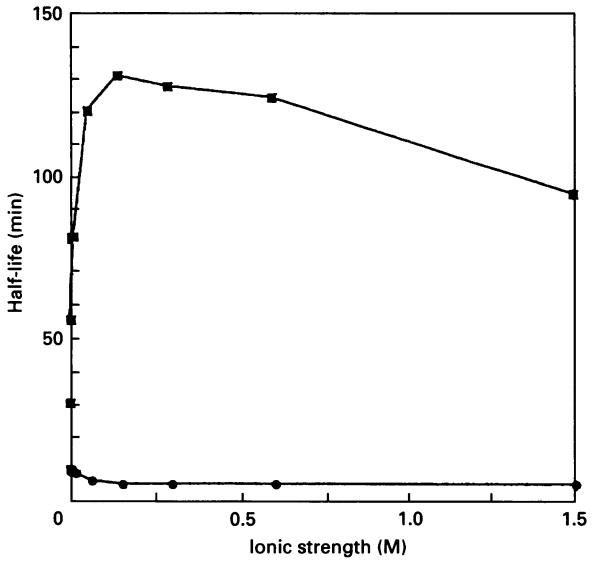

Figure 2 Effect of Ca^{2+} on stability of the Rt4A2 proteinase

Half-life values were determined as described in Table 6. Either $CaCl_2$ (■) or NaCl (●) were added to the incubations for the final ionic strengths indicated.

Table 5 Stability of Rt4a2 proteinase at temperatures between 70 °C and 105 °C

Proteinase samples (20 μg/ml) contained 10 mM Hepes/NaOH pH 7.5, 0.01% (v/v) Triton X-100 and $CaCl_2$ as indicated. Incubation was in Eppendorfs or in sealed glass capillaries at temperatures above 90 °C. Samples were withdrawn at appropriate time intervals for assay of residual activity. Half-lives were determined from first-order plots of the data. ND denotes experiment not done.

Temperature (°C)	Half-life		
	+5 mM $CaCl_2$	+50 mM $CaCl_2$	EDTA-treated
70	> 170 h	ND	10 h
80	43 h	ND	1.4 h
90	1.5 h	ND	9.8 min
100	ND	19.4 min	ND
105	ND	4.4 min	ND

Table 5 indicates that preparations of Rt4A2 treated with EDTA retained a significant degree of thermostability at 80 °C ($t_{\frac{1}{2}}$ = 1.4 h) and 90 °C ($t_{\frac{1}{2}}$ = 9.8 min) and is therefore somewhat more stable under the same conditions than aqualysin ($t_{\frac{1}{2}}$ = 1 h at 80 °C) (Matsuzawa et al., 1988) and considerably more stable than both caldolysin ($t_{\frac{1}{2}}$ = 4.8 min at 75 °C) (Cowan and Daniel, 1982) and Rt41A proteinase ($t_{\frac{1}{2}}$ = 2.8 min at 70 °C) (Peek et al., 1992) which is particularly sensitive to temperature after treatment with EDTA. Even low calcium concentrations confer increased stability, however, and in 50 μM $CaCl_2$ the half-life of the Rt4A2 proteinase was 18 min at 90 °C. The data in Table 5 were obtained at 10 μM buffer concentration, demonstrating the much greater stability of the Rt4A2 proteinase at relatively low ionic strength compared with that of caldolase. The latter is rapidly denatured and then autolysed even in 100 mM Tris/acetate buffer at 85 °C (98% loss in 15 min) (Saravani et al., 1989).

Table 6 Stability of the Rt4A2 proteinase towards denaturing and reducing agents

Proteinase was incubated for 24 h at 4 °C or 1 h at 75 °C in the presence of reducing or denaturing agent, 20 mM Tes/NaOH, pH 7.5, 5 mM $CaCl_2$ and 0.01% (v/v) Triton X-100. Residual activity was determined and expressed as a percentage of a control with no additions.

Denaturing/ reducing agent	Incubation conditions...	Activity remaining (%)	
		24 h at 4 °C	1 h at 75 °C
1% (w/v) SDS		78	79
6 M Urea		95	73
6 M Guanidium chloride		93	7
1% (w/v) Mercaptoethanol		78	81
10 mM Dithiothreitol		96	99

Effect of denaturing and reducing agents

At 4 °C the Rt4A2 proteinase showed significant resistance to the denaturing agents, losing less than 7% activity after 24 h in 6 M urea and 6 M guanidinium chloride, and only 22% in 1% (w/v) SDS (Table 6). During a 1 h incubation at 75 °C, guanidinium chloride became much more effective as a denaturant, causing a 93% loss in activity. Loss of activity in 1% (w/v) SDS remained essentially the same (21%) during the 1 h incubation at 75 °C.

Although the denaturation powers of such agents result from a combination of effects, two general features include: a reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure, and direct interactions with the protein molecule (Volkin and Klibanov, 1989).

Under both incubation conditions tested the proteinase showed similar sensitivity to the reducing agents. An insignificant 1% and 4% loss of activity was seen in 10 mM dithiothreitol after 24 h at 4 °C and 1 h at 75 °C respectively. A greater loss in activity was seen with 1% (v/v) mercaptoethanol, which caused losses of 21% and 22% after incubations at 4 °C and 75 °C respectively.

These thiol-containing reagents inactivate enzymes by reducing disulphide bonds, and their action is usually reversible (Volkin and Klibanov, 1989). The 20% loss of activity seen with mercaptoethanol may be due to a decrease in the stability of the proteinase caused by the reduction of a disulphide bond.

Effect of organic solvents

The Rt4A2 proteinase showed good stability at 4 °C in 90% (v/v) isopropanol, acetone, ethanol and methanol (< 10% loss). A 25% and 59% loss in activity was observed in 90% (v/v) acetonitrile and 90% (v/v) butanol respectively. The Rt41A proteinase shows similar sensitivity to acetonitrile and butanol (Peek et al., 1992). Butanol was the only immiscible solvent tested and the deleterious effect on enzyme activity seen in its presence may be due to denaturation of the proteinase at the solvent interface.

The inactivation observed when a water-miscible organic solvent (such as acetonitrile) is added to an aqueous enzyme solution is a consequence of the solvent binding directly to the protein via hydrophobic interactions and/or altering the dielectric constant of the solution. The latter affects the balance of non-covalent forces that maintain the protein in its native conformation. Therefore organic solvents may tend to 'turn proteins inside out' by increasing the solubility of the hydro-

phobic core while decreasing the solubility of the charged surface (Volkin and Klibanov, 1989).

REFERENCES

Arvidson, S., Holme, T. and Lindholm, B. (1973) Biochim. Biophys. Acta **302**, 135–148

Bajorath, J., Scvenger, W. and Pal, G. P. (1988) Biochim. Biophys. Acta **954**, 176–182

Castenholz, R. W. (1969) Bacteriol. Rev. **33**, 476–504

Coolbear, T., Eames, C. W., Casey, Y. and Daniel, R. M. (1991) J. Appl. Bacteriol. **71**, 252–264

Cowan, D. A. (1980) PhD Thesis, University of Waikato, Hamilton

Cowan, D. A. and Daniel, R. M. (1982) Biochim. Biophys. Acta **705**, 293–305

Cowan, D. A., Daniel, R. M. and Morgan, H. W. (1985) Trends Biotechnol. **3**, 68–72

Cowan, D. A., Daniel, R. M. and Morgan, H. W. (1987a) Int. J. Biochem. **19**, 741–743

Cowan, D. A., Daniel, R. M. and Morgan, H. W. (1987b) FEMS Microbiol. Lett. **43**, 155–159

Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1969) Data for Biochemical Research (2nd ed.), p. 485, Oxford University Press, Oxford

Del Mar, E. G., Largman, C., Broderick, J. W. and Geokas, M. C. (1979) Anal. Biochem. **99**, 316–320

Dixon, M. and Webb, E. C. (1979) Enzymes (3rd ed.), pp. 126–137, The Chaucer Press, Suffolk

Dunn, B. M. (1989) in Proteolytic Enzymes – A Practical Approach (Beynon, R. J. and Bond, J. S., eds.), pp. 57–81, IRL Press, Oxford

Ensign, J. C. and Wolfe, R. S. (1966) J. Bacteriol. **91**, 524–534

Gusek, T. W. and Kinsella, J. E. (1988) Food Technol. **42**, 102–107

Hudson, J. A. (1985) PhD Thesis, University of Waikato, Hamilton

Jany, K. D., Lederer, G. and Meyer, B. (1986) FEBS Lett. **199**, 139–144

Khoo, T. C., Cowan, D. A., Daniel, R. M. and Morgan, H. W. (1984) Biochem. J. **221**, 407–413

Matsubara, H. (1970) Methods Enzymol. **19**, 642–650

Matsuzawa, H., Hamaoki, M. and Ohta, T. (1983) Agric. Biol. Chem. **47**, 25–28

Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwon, S. and Takahisa, O. (1988) Eur. J. Biochem. **171**, 441–447

Peek, K., Daniel, R. M., Coolbear, T., Monk, C. and Parker, L. (1992) Eur. J. Biochem. **207**, 1035–1044

Peterson, G. L. (1983) Methods Enzymol. **91**, 95–119

Saravani, A. G. (1985) PhD Thesis, University of Waikato, Hamilton

Saravani, G. A., Cowan, D. A., Daniel, R. M. and Morgan, H. W. (1989) Biochem. J. **262**, 409–416

Sidler, W. and Zuber, H. (1980) Eur. J. Appl. Microbiol. **10**, 197–209

Taguchi, H., Hamaoki, M., Matsuzawa, H. and Ohta, T. (1983) J. Biochem. **93**, 7–13

Takii, Y., Tagushi, H., Shimoto, H. and Suzuki, Y. (1987) Appl. Microbiol. Biotechnol. **27**, 186–191

Torchinsky, Y. M. (1981) Sulfur in Proteins, Pergamon Press, Oxford

Valle, B. L. and Ulmer, D. D. (1972) Annu. Rev. Biochem. **41**, 91–128

Volkin, D. B. and Klibanov, A. M. (1989) in Protein Function: A Practical Approach (Creighton, T. E., ed.), pp. 1–24, IRL Press, Oxford

Voordouw, G., Gaucher, G. M. and Roche, S. (1974) Biochem. Biophys. Res. Commun. **58**, 8–12

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