Caldolase, a chelator-insensitive extracellular serine proteinase from a *Thermus* spp.

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An extracellular alkaline serine proteinase from *Thermus* strain Tok₃ was isolated and purified to homogeneity by (NH₄)₂SO₄ precipitation followed by ion-exchange chromatography on DEAE-cellulose and QAE-Sephadex, affinity chromatography on *N*^α-benzyloxycarbonyl-D-phenylalanyl-triethylenetetraminyl-Sepharose 4B and gel-filtration chromatography on Sephadex G-75. The purified enzyme had a pI of 8.9 and an *M*_r determined by gel-permeation chromatography of 25000. The specific activity was about 37700 proteolytic units/mg with casein as substrate, and the pH optimum was 9.5. Proteolytic activity was inhibited by low concentrations of di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride, but was unaffected by EDTA, EGTA, *o*-phenanthroline, *N*-ethyl-5-phenylisoxazolium-3'-sulphonate, *N*^α-*p*-tosyl-L-phenylalanylchloromethane, *N*^α-*p*-tosyl-L-lysylchloromethane, trypsin inhibitors and pepstatin A. The enzyme contained approx. 10% carbohydrate and four disulphide bonds. No Ca²⁺, Zn²⁺ or free thiol groups were detected. It hydrolysed several native and dye-linked proteins and synthetic chromogenic peptides and esters. The enzyme was very thermostable (half-life values were 840 min at 80 °C, 45 min at 90 °C and 5 min at 100 °C). The enzyme was unstable at low ionic strength: after 60 min at 75 °C in 0.1 M-Tris/acetate buffer, pH 8, only 20% activity remained, compared with no loss in 0.1 M-Tris/acetate buffer, pH 8, containing 0.4 M-NaCl.

INTRODUCTION

Attention is being increasingly focused upon enzymes from extremely thermophilic bacteria. Their thermostability and resistance to denaturing agents and organic solvents make these enzymes increasingly attractive for biotechnological processes (Doig, 1974; Daniel *et al.*, 1981; Hartley & Payton, 1983; Sonnleitner & Fiechter, 1983). Proteinases from extreme thermophiles have an additional advantage in their high specific activity (Cowan *et al.*, 1985, 1987a).

To date, a limited number of proteinases from extremely thermophilic bacteria have been reported and investigated (Heinen & Heinen, 1972; Matsuzawa *et al.*, 1983; Taguchi *et al.*, 1983). Only caldolysin, the extracellular proteinase from *Thermus aquaticus* strain T351 (Cowan & Daniel, 1982a,b; Khoo *et al.*, 1984), and archaelysin, the proteinase from a New Zealand strain of *Desulfurococcus* (Cowan *et al.*, 1987b), have been characterized in detail.

In the present paper the production, purification and characterization of an extracellular proteinase from *Thermus* strain Tok₃ are described. For convenience the proteinase was assigned the trivial name of caldolase. The prefix 'caldo' is derived from the Latin *caldus* (hot) and the suffix 'ase' is a general term for enzymes.

METHODS

Bacterial culture

Thermus strain Tok₃ was isolated from the Tokaanu thermal region, New Zealand. Cells were grown aerobic-

ally at 75 °C on *Thermus* medium (Hickey & Daniel, 1979). Large-scale growth (600 litres) of *Thermus* strain Tok₃ was carried out in an 800-litre fermenter.

Enzyme recovery and purification

The maximum enzyme activity in the culture supernatant occurred after 9 h, during late exponential phase. The culture was harvested by continuous-flow centrifugation in a Sharples model 6 centrifuge at 17000 *g* with a flow rate of 5 litres/min. Protein in the cell-free supernatant was precipitated by addition of crude (NH₄)₂SO₄ to a concentration of 70% saturation (20 °C). The precipitated protein, containing crude extracellular proteinase, was then pelleted in a Sharples model 6 centrifuge at 17000 *g*, and the pellet was homogenized with 1 litre of 0.1 M-Tris/acetate buffer, pH 7, in a Waring blender at high speed for 2–3 min, and left overnight at 4 °C. The suspension was then centrifuged at 10000 *g* in a type GSA rotor in Sorvall RC-2B centrifuge for 30 min. The supernatant, which contained most of the proteinase, was removed and freeze-dried. The enzyme extraction was repeated twice more as above. About 5 g of the freeze-dried crude proteinase was dissolved in 200 ml of 0.1 M-Tris/acetate buffer, pH 8. The enzyme solution was then applied to a DEAE-cellulose column (1.6 cm × 40 cm) equilibrated with 0.1 M-Tris/acetate buffer, pH 8. The non-adsorbed protein fractions, including proteinase, were pooled and applied to QAE-Sephadex in accordance with the above procedure. The non-adsorbed protein, including proteinase, was then applied to a column of Z-D-Phe-TETA-Sepharose 4B

Abbreviations used: Z-, benzyloxycarbonyl-; Bz-, benzoyl-; Tos-, tosyl-; Suc-, 3-carboxypropionyl-; Ac-, acetyl-; -NH-Np, *p*-nitroanilide; -ONp, *p*-nitrophenyl ester; TETA, triethylenetetramine.

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affinity-chromatography gel (Pierce Biochemicals). The adsorbed proteinase was eluted with 0.1 M-acetic acid, pH 2.8, and freeze-dried. The freeze-dried enzyme was dissolved in 4 ml of 0.1 M-Tris/acetate buffer, pH 8.0, and then applied to a Sephadex G-75 gel-filtration column (1.6 cm $\times$ 100 cm) equilibrated with 0.1 M-Tris/acetate buffer, pH 8.0, containing 0.1 M-NaCl and 10 mM-CaCl₂.

Proteinase assay

The modified Kunitz (1947) method was used for hydrolysis of casein, albumin, ovalbumin, fibrin and collagen (Cowan & Daniel, 1982a). Hydrolysis of dye-linked proteins was determined by measuring the increase in trichloroacetic acid-soluble chromophores in the supernatant at 440 nm (azo-casein, azo-albumin and Azocoll) or at 495 nm (elastin-Congo Red) (Shotton, 1970; Cowan, 1980). Chromogenic peptides (Bz-Phe-Val-Arg-NH-Np, Bz-Val-Gly-Arg-NH-Np, Tos-Gly-Pro-Arg-NH-Np, Z-Gly-Pro-Arg-NH-Np, Suc-Ala-Ala-Ala-NH-Np, Bz-Pro-Phe-Arg-NH-Np, Bz-Arg-NH-Np, Suc-Phe-NH-Np, Ac-Phe-NH-Np and Z-Phe-NA-Np) were obtained from Sigma Chemical Co. The rate of cleavage was determined at 410 nm (Bergström, 1977). Esterase activity of caldolase was determined with synthetic ester substrates (Z-Ala-ONp, Z-Tyr-ONp, Z-Phe-ONp, Z-Leu-ONp, Z-Trp-ONp, Z-Lys-ONp, Z-Asn-ONp, Z- β -Cys-ONp, Z- β -Asn-ONp, Z-Val-ONp, Z- β -Ala-ONp, Z-D-Nle-ONp, Z-Ile-ONp, and Z-Pro-ONp obtained from Sigma Chemical Co.) by using the method described by Abdelal *et al.* (1977). Hydrolysis of bradykinin and insulin B-chain (substrate/enzyme ratio approx. 10000:1, w/w) was assayed at intervals ranging from 30 s to 30 min at 50 °C.

Protein determination

Protein concentration was determined by using the modified Lowry method (Peterson, 1977), with bovine serum albumin as standard.

M_r determination

The M_r of caldolase was determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) by comparison with the migration of protein markers of known M_r values (bovine serum albumin, ovalbumin, α -chymotrypsinogen and cytochrome *c*). The M_r of the enzyme was also determined by gel-filtration chromatography on a Sephadex G-75 column (1.6 cm $\times$ 100 cm) and gel-permeation chromatography on a 60 cm TSK G3000 SW column (Toyo Soda Co.) with the above-mentioned proteins as standard. Blue Dextran was used for determination of the void volume.

pI determination

Isoelectric focusing was carried out on commercially prepared Servalyt Precotes (pH 3–10; Serva). The voltage was gradually increased from 250 to 1200 V during a 110 min focusing period, while the current declined from 23 mA to below 10 mA. The gel was fixed with 20% (w/v) trichloroacetic acid and then stained with Coomassie Brilliant Blue G-250. A mixture of 11 standard proteins with known pI values (Pharmacia Fine Chemicals) was run in parallel in the same gel.

Carbohydrate determination

The carbohydrate content of the purified enzyme was analysed by the phenol/H₂SO₄ method (Dubois *et al.*, 1956).

Thiol groups and disulphide bonds in caldolase

Free thiol groups were determined colorimetrically with 5,5'-dithiobis-(2-nitrobenzoic acid) by using the method of Robyt *et al.* (1971) at an enzyme concentration of 35 μ g/ml. For the determination of cystine content of caldolase (35 μ g/ml) the method of Anderson & Wetlaufer (1975) was employed.

Presence of metal ions in caldolase

Purified freeze-dried enzyme was dissolved in 10 mM-EDTA and incubated at room temperature for 30 min. The mixture was then applied to a Sephadex G-75 column (1.6 cm $\times$ 65 cm) equilibrated with 0.1 M-Tris/acetate buffer, pH 8.0, containing 0.5 M-NaCl. The fractions of enzyme and EDTA peaks were tested for Ca²⁺ and Zn²⁺ by atomic absorption spectrometry, with Ca²⁺ and Zn²⁺ as the standards. Thermolysin was used as a positive control.

Amino acid composition of caldolase

Protein hydrolysis was carried out with 6 M-HCl containing 7% (v/v) thioglycolic acid (Böhlen, 1983) in sealed evacuated ampoules for 24 and 72 h at 110 °C. The amino acid composition of the enzyme was determined by using a Waters h.p.l.c. amino acid analyser with post-column reaction with *o*-phthalaldehyde.

pH optimum

The influence of pH on enzyme activity was examined with 0.2% (w/v) casein in the following buffers: Universal buffer (Dawson *et al.*, 1969) (pH 6–11), 0.1 M-Hepes (pH 6.5–10), 0.1 M-Bicine (pH 6.5–9.5) and 0.1 M-Na₂CO₃/NaHCO₃ (pH 8.5–10.3). The pH of buffers was adjusted at 75 °C.

Thermostability

Samples of caldolase (24 μ g/ml in 0.1 M-Tris/acetate buffer, pH 8.0, containing 0.5 M-NaCl and 10 mM-CaCl₂) were incubated in Kimax Hungate tubes or in sealed melting-point capillary tubes at temperatures from 75 to 110 °C. The residual proteinase activity was measured at 75 °C by the standard modified Kunitz method.

Effect of pH on caldolase stability

The enzyme in Universal buffer (Dawson *et al.*, 1969), at pH values ranging from 3 to 12, was incubated for 90 min at room temperature (22 °C) before assay as described above.

Effect of ionic strength on enzyme stability

Caldolase (in 0.1 M-Tris/acetate buffer, pH 8, containing 0.1 M- to 0.7 M-NaCl) was incubated at 22 °C or 75 °C for 60 min. The enzyme was also incubated at 75 °C for 60 min in the presence of various buffers. For the analysis of denaturation and autolysis the freeze-dried salt-free enzyme was dissolved in the following buffers: 0.1 M-Tris/acetate buffer, pH 8, 0.1 M-Tris/acetate buffer, pH 8, containing 10 mM-CaCl₂ and 0.1 M-, 0.3 M- or 0.5 M-NaCl. Each enzyme solution (40 μ g/ml) was then incubated in Kimax Hungate tubes at a chosen

Table 1. Purification of caldolase

For experimental details see the text. One proteolytic unit of activity is defined as 1 μ g of tyrosine released/min measured as absorbance at 280 nm.

Step	Volume (ml)	Total protein (mg)	Total activity (proteolytic units)	Specific activity (proteolytic units/mg)	Purification (fold)	Recovery (%)
600-litre fermenter	600 000	—	8 491 500	—	—	100
Extracted enzyme	3000	—	3 358 400	—	—	40
5 g of freeze-dried extracted enzyme*	200	694	497 300	716	1	40
DEAE-cellulose chromatography	208	321	461 300	1440	2	37
QAE-Sephadex anion-exchange chromatography	208	166	441 500	2690	3.8	36
Affinity chromatography (Z-D-Phe-TETA-Sephadex-4B)	48	17	414 900	24 200	33	33
Sephadex G-75 chromatography	50	7.4	279 200	37 700	53	22

* A 5 g portion of the freeze-dried extracted enzyme was dissolved in 200 ml of 0.1 M-Tris/acetate buffer, pH 8.0. This sample was about 15% of the total enzyme extracted from the $(\text{NH}_4)_2\text{SO}_4$ -precipitated sample.

temperature (in the range 75–95 °C). Samples of enzyme were removed periodically in order to measure the proteinase activity and to determine the M_r distribution of components (by gel-permeation chromatography) on a 60 cm TSK G3000 SW column.

RESULTS AND DISCUSSION

Enzyme purification

About 60% of the original proteinase activity was lost during the $(\text{NH}_4)_2\text{SO}_4$ precipitation and extraction steps (Table 1). A large portion of the non-enzyme protein and coloured material was removed by the subsequent ion-exchange chromatography stages, during which little of the proteinase was lost. Affinity chromatography with Z-D-Phe-TETA-Sephadex 4B was the most effective purification step. The enzyme obtained from the last stage of purification (Sephadex G-75) had a specific activity of about 37 700 proteolytic units/mg. The results of the enzyme purification are presented in Table 1. The purified caldolase was shown to be homogeneous by SDS/polyacrylamide-gel electrophoresis and isoelectric focusing.

M_r

The M_r of caldolase was estimated by SDS/polyacrylamide-gel electrophoresis to be 32 000, whereas both the Sephadex G-75 gel-filtration chromatography and TSK G3000 SW h.p.l.c. showed it to be 25 000 on the basis of comparison with standards of known M_r values. Substantial differences in values for M_r determined by different methods have also been described by other authors (Voordouw *et al.*, 1974; Leach *et al.*, 1980; Roitsch & Hageman, 1983; Zlotnik *et al.*, 1984). Anomalous behaviour of glycoproteins on SDS/polyacrylamide-gel electrophoresis has been discussed pre-

viously (Segrest & Jackson, 1972). This is mainly due to the lower SDS binding of glycoproteins compared with standard proteins (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970; Clarke, 1975), resulting in a decreased mobility during SDS/polyacrylamide-gel electrophoresis, and thus a higher apparent M_r (lower SDS binding could also be caused by incomplete unfolding of the highly stable caldolase). In view of the high carbohydrate content of caldolase the M_r derived by gel permeation is probably the more reliable of the two values.

pI

Isoelectric focusing of the purified proteinase revealed a single band with a pI of 8.9 when compared with standards of known pI values (in the range 3.50–9.50).

Carbohydrate content

The carbohydrate content of caldolase (70 μ g/ml), with glucose as a standard, was determined to be approx. 10%, equivalent to 16 mol of hexose/mol of protein on the basis of an M_r of 25 000. The presence of carbohydrate has been also reported in other proteinases (Cowan & Daniel, 1982a; Ogrydziak & Scharf, 1982; Roitsch & Hageman, 1983), but is unusual in a prokaryotic enzyme. Although this carbohydrate may help to stabilize caldolase, its function is perhaps more likely to be connected with secretion. In caldolyisin (Cowan & Daniel, 1982a), another extracellular *Thermus* proteinase, which also contains about 10% carbohydrate, the high stability seems to be due to Ca^{2+} binding.

Free thiol groups and disulphide bonds

It was calculated that the ratio of 3-carboxylato-4-nitrothiophenolate produced per mol of enzyme (Robyt

Fig. 1. Effect of pH on proteolytic activity of caldolase

The enzyme reaction was carried out in the following buffers at the indicated pH values with 0.2% (w/v) casein as outlined in the Methods section: Hepes buffer (●), Bicine buffer (□), $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (▲) and Universal buffer (○).

et al., 1971) corresponded to less than 0.1 thiol group per molecule. This result, suggesting the absence of free thiol groups, is in agreement with the conclusion based on the response of the enzyme to cysteine-proteinase inhibitors (see below).

On the basis of the molar absorption coefficient of 3-carboxy-4-nitrothiophenolate (ϵ $11400 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Robyt *et al.*, 1971) and the Anderson & Wetlaufer (1975) stoichiometric reaction of 1.2 ± 0.3 mol of 3-carboxy-4-nitrothiophenolate/mol of disulphide groups, the presence of four disulphide bonds per molecule of caldolase was inferred. However, neither 2-mercaptoethanol nor dithiothreitol had a significant effect on caldolase stability. It may be that the disulphide bonds are well protected from the reagents. Bridgen *et al.* (1973) have reported that thiol groups in alcohol dehydrogenase from *Bacillus stearothermophilus* were unreactive towards iodoacetate, and attributed this to the localization of these groups within the protein molecule.

Metal ions in caldolase

Neither Ca^{2+} nor Zn^{2+} was present in caldolase ($64 \mu\text{g/ml}$) in significant amounts (< 0.2 mol of Ca^{2+} /mol and < 0.03 mol of Zn^{2+} /mol of enzyme). Caldolase retained full activity and thermostability after EDTA treatment (the enzyme was incubated at 75°C for 30 min and the proteinase activity was measured), indicating that no EDTA-susceptible essential metal ion was present in the enzyme. In the thermolysin sample used as a positive control (Feder *et al.*, 1971; Dalquist *et al.*, 1976) both Ca^{2+} and Zn^{2+} were present in the EDTA fractions but not in the enzyme fractions ($83 \mu\text{g/ml}$) (see the Methods section). Treatment by EDTA entirely inactivated thermolysin.

Amino acid composition of caldolase

The partial amino acid composition of caldolase based on an M_r of 25000 was $\text{Lys}_5\text{His}_6\text{Arg}_7\text{Asx}_{21}\text{Thr}_{19}\text{Ser}_{17}\text{Glx}_{10}\text{Gly}_{29}\text{Ala}_{31}\text{Val}_{19}\text{Met}_2\text{Ile}_8\text{Leu}_{16}\text{Tyr}_9\text{Phe}_4\text{Trp}_3$. Cysteine, proline and hydroxyproline residues were not determined.

Fig. 2. Arrhenius plot for the hydrolysis of casein by caldolase

The temperature-activity relationship of the enzyme was determined at the indicated temperatures with 0.2% (w/v) casein in 0.1 M-Tris/acetate buffer, pH 8.0.

Fig. 3. Arrhenius plot for the hydrolysis of Suc-Ala-Ala-Ala-NH-Np by caldolase

The temperature-activity relationship of the enzyme was determined at the indicated temperature with Suc-Ala-Ala-Ala-NH-Np.

Table 2. Inhibition of caldolase

Standard error values in all determinations were less than 5%. The following inhibitors had negligible (< 5%) effect: soya-bean trypsin inhibitor, lima-bean trypsin inhibitor, chicken egg-white trypsin inhibitor, antipain, α -antitrypsin, leupeptin, gramicidin S (each at 0.1 mg/ml), Tos-Lys-CH₂Cl at 5 mM, *p*-chloromercuribenzoate at 10 mM, 4-hydroxymercuribenzoate at 10 mM and pepstatin A at 2 mM concentration.

Inhibitor (class)	Inhibitor	Preincubation time (at 20 °C)	Concn. of inhibitor	Enzyme inhibition (%)
Serine-proteinase inhibitor	Phenylmethane-sulphonyl fluoride	60 min	0.1 mM	84
			1 mM	92
			5 mM	100
	Di-isopropyl phosphorofluoridate	105 min	0.1 mM	30
			1 mM	90
			5 mM	100
Chymotrypsin inhibitors	Chymostatin	60 min	0.1 mg/ml	66
		60 min	0.5 mg/ml	78
Metal-ion chelators	EDTA (disodium salt)	120 min	10 mM	3
	EGTA	120 min	10 mM	0
	<i>O</i> -Phenanthroline	120 min	5 mM	2
	<i>N</i> -Ethyl-5-phenylisoxazolium-3'-sulphonate	120 min	5 mM	0
Cysteine-proteinase inhibitors	Iodoacetic acid	120 min	1 mM	3
			10 mM	58
		60 min (75 °C)	1 mM	3
			10 mM	97
Heavy-metal ions	CuCl ₂	60 min	1 mM	0
			10 mM	39
	HgCl ₂	60 min	1 mM	70
			10 mM	100

pH optimum

Caldolase exhibited a broad pH-activity profile with an apparent pH optimum of about 9.5 (Fig. 1). However, in view of the sensitivity of caldolase activity to changes in ionic strength (see below), this datum must be viewed with caution. The ionic strengths of Universal buffer (calculated by using the Henderson-Hasselbach equation) at pH 6, 8, and 10 are 0.114, 0.243 and 0.368 respectively. Increasing ionic strength might account for the increased pH-dependence of activity below pH 7 in Universal buffer, but probably has little effect on enzyme activity above pH 9.

Temperature-activity relationship in caldolase

The Arrhenius plots for casein and peptide substrate (Suc-Ala-Ala-Ala-NH-Np) exhibit a sharp discontinuity at about 92 °C, due to denaturation (Figs. 2 and 3). Neither of the plots is a straight line, but the evidence for a discontinuity at any particular temperature or temperatures below 90 °C seems poor (e.g. see Wolfe & Bagnall, 1979). For the casein plot it is difficult to separate the effect of temperature on the enzyme from that on the substrate. However, the E_a at 80 °C is 58 kJ/mol. For the peptide substrate there is a declining apparent E_a with increasing temperature (E_a at 40 °C 22 kJ/mol and at 80 °C 6 kJ/mol): the Q_{10} at 80 °C, approx. 1.1, is surprisingly low.

Effect of inhibitors on caldolase activity

Caldolase was strongly inhibited by low concentrations of di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride and inhibited by relatively high concentrations of diphenylcarbamoyl fluoride, iodoacetic acid, HgCl₂, CuCl₂ and chymostatin (Table 2). It was not inhibited by metal-ion chelators, trypsin inhibitors Tos-Phe-CH₂Cl, Tos-Lys-CH₂Cl and pepstatin A. The above results indicate that caldolase is a serine proteinase. The chelating agents had no effect on caldolase, confirming that metal ions, in particular Ca²⁺ and Zn²⁺, are not required for enzyme activity or stability. Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl, which inhibit chymotrypsin and trypsin respectively (Walsh, 1970), had no effect on caldolase. Iodoacetic acid was only able to inhibit caldolase at high concentration and high temperature. At a high concentration iodoacetic acid can react with the side groups of a number of amino acids (Means & Feeney, 1971).

Substrate specificity

(a) **Native proteins.** Caldolase hydrolysed several protein substrates (Table 3). There was significant hydrolysis of collagen even at 35 °C, but only a very weak activity towards elastin.

Table 3. Relative rates of protein hydrolysis by caldolase

Actual rates of casein hydrolysis at 75 °C and 35 °C were $\Delta A_{280}/\text{min} = 0.069$ and $\Delta A_{280}/\text{min} = 0.0027$ respectively. Actual rates of azo-casein hydrolysis at 75 °C and 35 °C were $\Delta A_{440}/\text{min} = 0.061$ and $\Delta A_{440}/\text{min} = 0.0024$ respectively.

Substrate	Relative rate of hydrolysis	
	At 75 °C	At 35 °C
Casein	100	100
Bovine serum albumin	62	34
Ovalbumin	31	8
Haemoglobin	99	68
Collagen type I	61	31
Fibrin	52	39
Elastin	< 1	< 1
Azo-casein*	100	100
Azo-albumin*	133	127
Azocoll*	124	115
Elastin-Congo Red†	1.8	1.2

* Measured at 440 nm.
† Measured at 495 nm.

(b) **Dye-linked proteins.** The susceptibility of azo-albumin and Azocoll was greater than that of azo-casein, in contrast with the results for the unmodified substrates. There was little hydrolysis of elastin-Congo Red (Table 3).

(c) **Chromogenic peptide substrates.** Caldolase was capable of hydrolysing several synthetic *N*-terminally blocked tripeptide *p*-nitroanilide substrates. Reaction rates followed the order: Bz-Phe-Val-Arg-NH-Np > Bz-Val-Gly-Arg-NH-Np > Tos-Gly-Pro-Arg-NH-Np > Suc-Ala-Ala-Ala-NH-Np > Bz-Pro-Phe-Arg-H-Np; the enzyme failed to hydrolyse any of the single amino acid *p*-nitroanilide substrates.

(d) **Chromogenic ester substrates.** Caldolase hydrolysed all synthetic ester substrates tested (see the Methods section). Reaction rates followed the order: Z-Ala-ONp > Z-Tyr-ONp $\approx$ Z-Phe-ONp $\approx$ Z-Leu-ONp > Z-Trp-ONp > Z-lys-ONp > Z-Asn-ONp > Z- β -Cys-ONp $\approx$ Z- β -Asn-ONp > Z-Val-ONp > Z- β -Ala-ONp $\approx$ Z-D-Nle-ONp > Z-Ile-ONp $\approx$ Z-Pro-ONp. Esterase activity was not the result of a contaminant, since the ratio of proteinase to esterase activity was the same for all the later purification stages, and these ratios remained unchanged after partial thermal denaturation and after treatment with EDTA, *p*-chloromercuribenzoate and phenylmethanesulphonyl fluoride (results not shown).

(e) **Peptides.** Caldolase was not able to hydrolyse bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) during the reaction periods tested, suggesting that the enzyme lacked specificity towards non-polar amino acids. In contrast, cleavage of insulin B-chain even for short periods resulted in a very complex pattern (results not shown), indicating a low degree of enzyme specificity.

Fig. 4. Stability of caldolase at temperatures between 75 °C and 100 °C

Purified enzyme (24 $\mu\text{g}/\text{ml}$ in 0.1 M-Tris/acetate buffer, pH 8.0, containing 0.5 M-NaCl and 10 mM- CaCl_2) was incubated at 75 °C ($\square$), at 80 °C ($\blacksquare$), at 85 °C ($\triangle$), at 90 °C ($\blacktriangle$), at 95 °C ($\circ$) and at 100 °C ($\bullet$). Samples of enzyme solution were removed at intervals, and the residual proteinase activity was determined at 75 °C by the modified Kunitz method.

Effect of denaturing and reducing agents

Caldolase activity was affected little by incubation in 8 M-urea and 6 M-guanidinium chloride at low temperature, but was decreased significantly after incubation at high temperatures (69 % loss and 80 % loss respectively after 60 min incubations at 75 °C).

2-Mercaptoethanol and dithiothreitol had no significant effect on enzyme stability at either low or high temperatures. This suggests either that the disulphide bonds are protected from the solvent, or that they are not involved in the stability of caldolase.

Stability

Stability profiles at temperatures between 75 °C and 110 °C are shown in Figs. 4 and 5. The half-life of the enzyme was about 840 min at 80 °C and 45 min at 90 °C. Loss of proteinase activity above 100 °C was very rapid, and at 106 °C the half-life of the enzyme was less than 1 min.

Caldolase was unstable at low salt concentration (Fig. 6), and was stabilized by various salts (Table 4). An analysis of denaturation and autolysis of caldolase in the presence of various salt concentrations (0.1 M-Tris/acetate buffer, pH 8, and 0.1 M-Tris/acetate buffer, pH 8, containing 0.1 M-, 0.3 M- or 0.5 M-NaCl plus 10 mM- CaCl_2) and at temperatures ranging from 75 to 95 °C revealed that at high ionic strength and temperatures up to 85 °C second-order kinetics were predominant (autolysis was dominant), whereas at 90 °C and 95 °C first-order kinetics were significant (denaturation was important) (Voordouw & Roche, 1975). At low ionic strength second-order kinetics were predominant up to 95 °C. TSK gel-filtration chromatography demonstrated that, by increasing the incubation time, by raising the temperature or by a combination of both, autolysis products were increased. This was shown by the decrease in height

Fig. 5. Stability of caldolase above 100 °C

Samples (30 μ l) of enzyme (24 μ g/ml in 0.1 M-Tris/acetate buffer, pH 8.0, containing 0.5 M-NaCl and 10 mM- CaCl_2) were sealed in melting-point capillary tubes and heated in a poly(ethylene glycol) 400 bath at 100 °C ($\square$), at 102 °C ($\blacksquare$), at 104 °C ($\triangle$), at 106 °C ($\blacktriangle$), at 108 °C ($\circ$) and at 110 °C ($\bullet$) for the periods indicated. The residual proteinase activity of each sample was measured at 75 °C by the modified Kunitz method.

Table 4. Effect of various salts on caldolase stability

Buffer is 0.1 M-Tris/acetate buffer, pH 8.0. The salt-free freeze-dried enzyme was dissolved in 0.1 M-Tris/acetate buffer, pH 8.0, and diluted 10-fold in various salt solutions (10 mM of each salt was dissolved in 0.1 M-Tris/acetate buffer, pH 8.0, unless otherwise stated). The mixtures were then incubated at either 22 °C or 75 °C for 60 min or at 85 °C for 30 min. Proteinase activity of each sample was determined at 75 °C as described in the Methods section.

Addition to buffer	Substrate	Enzyme activity remaining (%) after incubation at:		
		22 °C	75 °C	85 °C
0.5 M-NaCl + 10 mM- CaCl_2	0.2% casein	100	100	100*
None	0.2% casein	63	14	7
CaCl_2	0.2% casein	75	32	33
MgCl_2	0.2% casein	71	26	24
SnCl_2	0.2% casein	77	24	21
KCl	0.2% casein	61	14	9
Sodium acetate	0.2% casein	63	8	7
0.5 M-NaCl + 10 mM- CaCl_2	0.1% azo-casein	100	100	100
VCl_3	0.1% azo-casein	62	14	14
$\text{Co}(\text{NO}_3)_2$	0.1% azo-casein	98	70	44

* Activity remaining after incubation at 85 °C was 84% of that remaining after incubation at 22 °C and 75 °C.

Fig. 6. Effect of ionic strength on caldolase activity

The salt-free caldolase was dissolved in various salt concentrations (0.1 M-Tris/acetate buffer, pH 8.0, containing 0.1–0.7 M-NaCl) and incubated at 22 °C ($\bullet$) or at 75 °C ($\blacktriangle$) for 60 min. The proteinase activity of each sample was determined at 75 °C by the modified Kunitz method. The ionic strength shown is that calculated for each concentration according to the formula $I = \frac{1}{2} \sum m_i Z_i^2$ where m_i is the molarity of the ion and Z_i is the net charge of the ion. The conductivity of Tris was measured with a conductivity meter and converted into ionic strength by using NaCl as a standard curve.

of the main (enzyme) peak and the increase in the low- M_r peaks. It was concluded that ionic strength plays a major role in structural stabilization of caldolase. The activation and stabilization of several other enzymes by high salt concentration has been reported (Griffiths & Sundaram, 1973; Crabb *et al.*, 1977; Shannon *et al.*, 1982). Assay of autolytic fragments separated by the TSK G3000 SW gel-permeation column showed that some of these were catalytically active, so the integrity of the whole enzyme is apparently not necessary for activity.

Reversible denaturation of caldolase

Incubation at low ionic strengths caused loss of activity both at room temperature and at 85 °C. At 85 °C it was not possible to recover the activity, whereas the recovery of activity at 22 °C was considerable (Table 5). This

Table 5. Determination of the reversibility of caldolase inactivation at low salt concentrations

Salt-free enzyme samples were incubated at 85 °C for 15 min, at 75 °C for 30 min and at 22 °C for 60 min. Proteinase activity of each sample was determined by the modified Kunitz method before and after the addition of salt (0.4 M-NaCl + 10 mM- CaCl_2) at 75 °C.

Temperature (°C)	Incubation time (min)	Enzyme activity (%)	
		Before NaCl	After addition of NaCl
0–2	0	96	100
0–2	60	95	100
22	60	14	81
75	30	12	42
85	15	2	8

suggests that low ionic strength induces reversible unfolding of the enzyme, leading to loss of activity. At 0 °C the unfolding apparently does not occur, and at 22 °C the unfolding is reversible. At temperatures where the enzyme is substantially active, say 75 °C, this unfolding will render the enzyme particularly susceptible to autolytic attack by the few remaining active enzyme molecules. At higher temperatures (e.g. 85 °C) it may be that further thermally induced unfolding occurs that is irreversible, yielding a very low recovery of activity.

G. A. S. thanks the Meat Industries Research Institute of New Zealand for a post-graduate scholarship. We thank the Development Finance Corporation of New Zealand for its financial support.

REFERENCES

- Abdelal, A. T. H., Kennedy, E. H. & Ahearn, D. G. (1977) *J. Bacteriol.* **130**, 1125–1129
- Anderson, W. L. & Wetlaufer, D. B. (1975) *Anal. Biochem.* **67**, 493–502
- Bergström, K. (1977) *Sci. Tools* **24**, 8–9
- Böhlen, P. (1983) *Methods Enzymol.* **91**, 17–26
- Bridgen, J., Kolb, E. & Harris, J. I. (1973) *FEBS Lett.* **33**, 1–3
- Clarke, S. (1975) *J. Biol. Chem.* **250**, 4549–4569
- Cowan, D. A. (1980) D.Phil. Thesis, University of Waikato
- Cowan, D. A. & Daniel, R. M. (1982a) *Biochim. Biophys. Acta* **705**, 293–305
- Cowan, D. A. & Daniel, R. M. (1982b) *Biotechnol. Bioeng.* **24**, 2053–2061
- Cowan, D. A., Daniel, R. M. & Morgan, H. W. (1985) *Trends Biotechnol.* **3**, 68–72
- Cowan, D. A., Daniel, R. M. & Morgan, H. W. (1987a) *Int. J. Biochem.* **19**, 741–743
- Cowan, D. A., Smolenski, K. A., Daniel, R. M. & Morgan, H. W. (1987b) *Biochem. J.* **247**, 121–133
- Crabb, J. W., Murdock, A. L. & Amelunxen, R. E. (1977) *Biochemistry* **16**, 4840–4847
- Dalquist, F. W., Long, J. W. & Bigbee, W. L. (1976) *Biochemistry* **5**, 1103–1111
- Daniel, R. M., Cowan, D. A. & Morgan, H. W. (1981) *Chem. N.Z.* **45**, 94–97
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., p. 485, Oxford University Press, London
- Doig, A. R. (1974) in *Enzyme Engineering* (Engineering Foundation Conference on Enzyme Engineering), (Pye, E. K. & Wingard, L. B., eds.), vol. 2, pp. 17–24, Plenum Press, London
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Biochem.* **28**, 350–356
- Feder, J., Garrett, L. R. & Wildi, B. S. (1971) *Biochemistry* **10**, 4552–4561
- Griffiths, M. W. & Sundaram, T. K. (1973) *J. Bacteriol.* **116**, 1160–1169
- Hartley, B. S. & Payton, M. A. (1983) *Biochem. Soc. Symp.* **48**, 133–146
- Heinen, U. J. & Heinen, W. (1972) *Arch. Microbiol.* **82**, 1–23
- Hickey, C. W. & Daniel, R. M. (1979) *J. Gen. Microbiol.* **114**, 195–200
- Khoo, T. C., Cowan, D. A., Daniel, R. M. & Morgan, H. W. (1984) *Biochem. J.* **221**, 407–413
- Kunitz, M. (1947) *J. Gen. Physiol.* **30**, 291–310
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leach, B. S., Collawn, J. F., Jr. & Fish, W. W. (1980) *Biochemistry* **19**, 5734–5741
- Matsuzawa, H., Hamaoki, M. & Ohta, T. (1983) *Agric. Biol. Chem.* **47**, 25–28
- Means, G. F. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 105–138, Holden-Day, San Francisco
- Ogrydziak, D. M. & Scharf, S. J. (1982) *J. Gen. Microbiol.* **128**, 1225–1234
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Pitt-Rivers, S. & Impiombato, F. S. A. (1968) *Biochem. J.* **109**, 825–830
- Reynolds, J. A. & Tanford, C. (1970) *J. Biol. Chem.* **245**, 5161–5165
- Robyt, J. F., Ackerman, R. J. & Chittenden, C. G. (1971) *Arch. Biochem. Biophys.* **147**, 262–269
- Roitsch, C. A. & Hageman, J. H. (1983) *J. Bacteriol.* **155**, 145–152
- Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63
- Shannon, J. D., Bond, J. S. & Bradley, S. G. (1982) *Arch. Biochem. Biophys.* **219**, 80–88
- Shotton, D. M. (1970) *Methods Enzymol.* **19**, 113–140
- Sonnleitner, B. & Fiechter, A. (1983) *Trends Biotechnol.* **1**, 74–84
- Taguchi, H., Hamaoki, M., Matsuzata, H. & Ohta, T. (1983) *J. Biochem. (Tokyo)* **93**, 7–13
- Voordouw, G. & Roche, R. S. (1975) *Biochemistry* **14**, 4667–4673
- Voordouw, G., Gaucher, G. M. & Roche, R. S. (1974) *Biochem. Biophys. Res. Commun.* **58**, 8–12
- Walsh, K. A. (1970) *Methods Enzymol.* **19**, 41–63
- Wolfe, J. & Bagnall, D. (1979) in *Low Temperature Stress in Crop Plants* (Lyons, J. M., Graham, D. & Raisin, J. K., ed.), pp. 527–533, Academic Press, New York
- Zlotnik, H., Schramm, V. L. & Buckley, H. R. (1984) *J. Bacteriol.* **157**, 627–631