A pepstatin-insensitive aspartic proteinase from a thermophilic *Bacillus* sp.

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Bacillus sp. strain Wp22.A1 produced a cell-associated aspartic proteinase which was purified to homogeneity using phenyl-Sepharose (hydrophobic and affinity chromatography) and Mono Q. The proteinase has a molecular mass of 45 kDa by SDS/PAGE and a pI of 3.8. It is insensitive to pepstatin, but is sensitive to the other aspartic proteinase-specific inhibitors diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane. Inactivation by DAN was only partial, suggesting that it had non-specifically modified an aspartate residue at a site other than the active site. The enzyme was not inhibited by any of the serine or cysteine proteinase inhibitors tested. Maximum proteolytic activity was observed at pH 3.5. The proteinase had a higher activity with haemoglobin, but was more specific ($V_{max.}/K_m$) for cytochrome *c*. Substrate inhibition was observed with both these substrates. The cleavage of oxidized insulin B chain tended to occur at sites where the P1 amino acid was bulky and non-polar, and the P1′ amino acid was bulky and polar, such as its primary cleavage site of Val^2-Asn^3. The proteinase was stable in the pH range 2.5–5.5. Thermostability was increased in the presence of Ca^{2+}, although to a lesser extent at higher temperatures. The thermostabilities at 60, 70, 80 and 90 °C were 45 h, 102, 21 and 3 min respectively in the presence of Ca^{2+}.

INTRODUCTION

Aspartic proteinases have been isolated and characterized from organisms such as mammals, fungi, plants, retroviruses and more recently archaea and bacteria. They usually contain two aspartate residues at the active site and tend to have a pH optimum in the acidic region. Another characteristic is their inhibition by the transition-state inhibitor pepstatin [1] and the active-site-modifying reagents diazoacetyl-DL-norleucine methyl ester (DAN) [2] and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) [3].

Only a few bacterial aspartic proteinases have been studied to date. Several strains of the genera *Clostridium* (*acetobutylicum* and *butyricum*) [4] and *Lactobacillus* [5] have been shown to produce weak proteolytic activity with acidic pH optima. No inhibitor studies have been conducted on these proteinases, so it is not certain whether they are aspartic proteinases. A microbial rennet from *Bacillus cereus* [6,7] has been partially characterized. This early work focused on the potential use of these enzymes in cheese-making.

More recently, about five bacterial aspartic proteinases have been characterized from mesophilic and thermophilic bacteria. Oda et al. [8,9] isolated mesophilic aspartic proteinases from *Xanthomonas* sp. strain T-22 and *Pseudomonas* sp. strain no. 101. Thermophilic aspartic proteinases have been characterized from *Bacillus* sp. strain MN-32 [10,11] and *Bacillus*. sp. strain Wai21.A1 [12]. The archaebacterium *Sulfolobus acidocaldarius* produces a novel thermophilic aspartic proteinase, thermopsin, that is unique in many of its properties, such as substrate specificity and the lack of sequence similarity to other aspartic proteinases [13].

The present paper describes the purification and characterization of an aspartic proteinase from the thermophilic acidophilic *Bacillus* sp. strain Wp22.A1, isolated from a hot spring at Waiotapu, New Zealand. It is the most thermostable of the eubacterial aspartic proteinases known to date, and has an unusual sensitivity pattern to the inhibitors diagnostic of aspartic proteinases. This paper also describes the use of an amino acid gradient with phenyl-Sepharose for the affinity purification of proteinases.

MATERIALS AND METHODS

Growth and harvesting of *Bacillus* sp. strain Wp22.A1

Bacillus sp. strain Wp22.A1 was grown on a modified acid *Bacillus* medium [14] composed of mineral salts [0.2 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4,7H_2O$, 0.1 g/l $CaCl_2,2H_2O$ and 3.0 g/l KH_2PO_4) with 3.0 g/l yeast extract, 0.75 g/l tryptone and 4.0 g/l cellobiose at pH 3.0. This medium, 45 litres in a series of 2-litre shake flasks, was inoculated with a 16 h culture and incubated, with shaking, at 60 °C for 19 h.

The culture was concentrated on an Amicon Hollow Fibre Cartridge (S1OY10), and the resultant slurry was centrifuged for 15 min at 11170 *g* ($r_{av.}$ 10 cm) in a Sorvall SS-3 centrifuge.

Proteinase purification

Extraction of the proteinase from the cell pellet was achieved by resuspending approx. 20 g (wet weight) of cell pellet in 200 ml of 10 mM formate, pH 3.0, containing 1 M NaCl, and incubating it at 4 °C for 2 days with stirring. After the incubation, the slurry was centrifuged as above to pellet the cells and retrieve the supernatant. This process was repeated five times. The extracts were pooled with the original culture supernatant and concentrated to 240 ml using an Amicon YM10 ultrafiltration mem-

Abbreviations used: APMSF, (4-aminophenyl)methanesulphonyl fluoride; Boc, t-butoxycarbonyl; Cbz, benzyloxycarbonyl; IAA, iodoacetic acid; PMSF, phenylmethanesulphonyl fluoride; Suc, succinyl; DAN, diazoacetyl-DL-norleucine methyl ester; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

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brane, and equilibrated to 20 mM formate, pH 3.15, containing 1 M NaCl and 0.5 M $(NH_4)_2SO_4$.

This extract was added to a 300 ml phenyl-Sepharose (Pharmacia) column, pre-equilibrated in the same solution. The column was washed with 10 mM formate, pH 3.15, and the proteinase was eluted with a 2-litre gradient of 0–50% (v/v) ethanediol in 10 mM formate, pH 3.15, at a flow rate of 20 ml/min. The active fractions were pooled and diafiltered to a volume of 26 ml and a final buffer composition of 20 mM Bistris/H_2SO_4, pH 5.8, with 2.5% (w/v) betaine and 0.01% (v/v) Triton X-100.

The non-diffusible material was added to a Mono Q 5/5 (Pharmacia) column, pre-equilibrated in the Bistris buffer. The proteinase was eluted with a 20 ml gradient of 0–0.35 M NaCl in the above buffer, at a flow rate of 1 ml/min. The active fractions were pooled and diafiltered to a volume of 26 ml and a final buffer composition of 20 mM formate, pH 3.15, containing 1 M NaCl and 0.5 M $(NH_4)_2SO_4$.

The non-diffusible material was applied to a 10 ml phenyl-Sepharose (Pharmacia) column, pre-equilibrated in the same solution. The column was washed with 10 mM formate, pH 3.5, and the proteinase eluted with a 60 ml gradient of 0–1.5% (w/v) of each of phenylalanine, histidine and alanine in the above buffer, at a flow rate of 2 ml/min. A gradient of 0–50% (v/v) ethanediol in 10 mM formate, pH 3.15, was then applied to the column to remove any remaining bound protein. Fractions containing proteolytic activity were collected and underwent a buffer exchange and concentration, as before, to a final buffer composition of 10 mM formate, pH 3.15, containing 0.01% (v/v) Triton X-100. The fractions were frozen and stored at −70 °C until required for further use.

Proteinase assay

The assay with protein substrates was based on the method of Peek et al. [15]. The reaction mixture consisted of the substrate and proteinase in 50 mM formate buffer containing 5 mM $CaCl_2$ and 0.0005% (v/v) Triton X-100 (due to its presence in the enzyme buffer), to a final volume of 1 ml. For the purification assays, the substrate was 0.1% (w/v) bovine haemoglobin (Sigma) at pH 3.0. The substrate for the characterization assays was 0.05% (w/v) horse heart cytochrome *c* (Sigma) at pH 3.5.

The reaction was initiated by the addition of the proteinase to a 1.5 ml Eppendorf tube containing the substrate that had been preincubated at 60 °C. After the required incubation time at 60 °C (1 h for haemoglobin, 2 h for cytochrome *c*), the reaction was stopped by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid. After the reaction was stopped, the enzyme was added to the controls. After standing on ice for 15 min, the samples were centrifuged for 5 min at 15000 rev./min in a Beckman Microfuge E. Activity was determined by measuring the absorbance of the trichloroacetic acid-soluble peptides at 280 nm. One unit is defined as the amount of enzyme that produces a change in A_{280} of 1/min.

Protein assay

During the initial stages of purification, the protein concentration was determined by the Bradford method [16]. The final protein concentration was determined by an adaptation of the method of Lowry [17]. In both methods, BSA (Sigma) was used as the protein standard. For enzyme samples containing Triton X-100, the BSA standards contained the same concentration of Triton X-100.

Electrophoresis

The molecular mass and purity were determined by running samples of enzyme on SDS/PAGE 10–15% gradient Phast gels using the Pharmacia Phast System. The samples were prepared and run on the gel as described by Pharmacia (Separation Technique File No. 110). Non-denaturing PAGE at pH 8.8, using 8–25% gradient Phastgels, was carried out by the method of Pharmacia (Separation Technique File No. 120). After electrophoresis, the bands were detected by the silver-staining technique for the Phast system described by Heukeshoven and Dernick [18]. For non-denaturing PAGE, an initial trichloroacetic acid-fixing step was employed before staining.

Purified and non-purified enzyme were focused on Phastgel isoelectric focusing media containing Pharmalytes in the range pH 3–9 by the method of Pharmacia (Separation Technique File No. 100). Proteinase activity was detected by the X-ray-film-digestion method described by Paech et al. [19] at pH 3.0, followed by silver staining. The pI was determined by a comparison with standard proteins (pI 2.5–6.5) from the Pharmacia low-pI calibration kit.

pH profile

The enzyme was preincubated in 50 mM formate at various pH values for 2 h at 60 °C, then assayed for residual activity with 0.05% (w/v) cytochrome *c* at 60 °C to determine stability.

The activity of the proteinase was determined by assaying the enzyme at various pH values with 0.05% (w/v) cytochrome *c* for 2 h at 60 °C. This experiment was repeated at 45 °C.

Activity towards synthetic substrates

The synthetic peptides Leu-Ser-Phe(NO_2)-NLe-Ala-Leu-OMe and *N*-Boc-Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-4-hydroxymethylpyridine ester (Bachem) were prepared and assayed as described by Martin et al. [20] and Agarwal and Rich [21] respectively, except that the substrate was composed of 0.1 mM peptide (0.037 mM for the Boc-peptide) in 50 mM formate, pH 3.5 (at 60 °C) with 5 mM $CaCl_2$.

Activity against a number of *p*-nitroanilide (-NH-Np) peptides (Sigma, Bachem) was determined by the method of Freeman et al. [22]. The peptides tested were benzoyl-DL-Arg-NH-Np, *N*-Suc-L-Phe-NH-Np, L-Ala-NH-Np, N^{α}-Cbz-L-Arg-NH-Np, *N*-Suc-L-Tyr-L-Leu-L-Val-NH-Np and *N*-Cbz-Gly-Gly-Leu-NH-Np. The enzyme (0.074 units in 15 μl) was added to 1 ml of 0.25 mM peptide-NH-Np in 50 mM formate, pH 3.5, at 60 °C, with 5 mM $CaCl_2$, 0.01% (v/v) Triton X-100 and 10% (v/v) 2-methoxyethanol (20% for the latter two peptides), preincubated at 60 °C in a Perkin–Elmer Lambda 3B spectrophotometer. Hydrolysis was monitored at 410 nm for 3 min.

Cleavage of oxidized insulin B chain

This was carried out by the method of Peek et al. [15]. Oxidized insulin B chain (bovine; Sigma) was purified by reversed-phase HPLC on an MPLC C18 column (Brownlee Laboratories) with the Waters liquid chromatography system. The peptide was eluted from the column using a 60 ml linear gradient of 0–100% (v/v) acetonitrile, containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The peptide was detected by monitoring absorbance at 230 nm, and these fractions were pooled and lyophilized.

The purified peptide (4.5 mg) was dissolved in 10 ml of 50 mM formate, pH 3.5 at 60 °C, containing 5 mM $CaCl_2$, followed by filtration through a 0.22 μm filter to remove any insoluble material. The peptide solution was preincubated at 60 °C for

15 min before the addition of 0.45 μg of protease. Samples were removed at 0, 5, 15 and 30 min and 1, 3, 6 and 24 h. The reaction was stopped by the addition of 5 μl of 6 M HCl to 1 ml of the peptide/protease samples. The samples were cooled, then stored at −70 °C until required. The peptide fragments produced by the proteolysis were separated using reversed-phase HPLC as before, except that the gradient was composed of 0–60 % (v/v) acetonitrile, containing 0.1 % (v/v) trifluoroacetic acid at a flow rate of 1 ml/min.

The eluted peptide fragments were lyophilized, then hydrolysed by heating for 1 h at 150 °C in 6 M HCl containing 1 % (v/v) phenol. The resulting amino acids were derivatized with phenyl isothiocyanate [15] and separated using the Waters Picotag amino acid analysis system. The amino acid compositions of the peptides were matched to the known sequence of oxidized insulin B chain to determine the cleavage sites.

Thermostability of Wp22.A1 proteinase

After preincubation under high- and low-Ca^{2+} conditions in the temperature range 60–90 °C, samples were withdrawn and assayed for residual activity with 0.05 % (w/v) cytochrome *c*.

Effect of metal ions on thermostability

Wp22.A1 proteinase, diluted in 50 mM formate, pH 3.5 at 60 °C, with 10 mM EDTA, was incubated at room temperature for 30 min. The solution was then dialysed overnight at 4 °C against 10 mM formate, pH 3.5 at 60 °C, containing 0.01 % (v/v) Triton X-100. Samples of dialysed enzyme were diluted in the latter buffer containing 5 mM concentrations of bi- and ter-valent cations. These samples were preincubated at 60 °C for 30 min, then assayed for activity with 0.05 % (w/v) cytochrome *c*, containing 5 mM concentrations of the cations in place of the normal 5'mM $CaCl_2$ (control).

Stability towards denaturing and reducing agents

The enzyme was preincubated in the presence of denaturing and reducing agents (see Table 5) at 25 °C for 24 h and 60 °C for 60 min, except for the organic solvents which were tested at 25 °C. Samples were assayed for residual activity with 0.05 % (w/v) cytochrome *c*.

Inhibitor studies

Pepstatin, EDTA, EGTA, bestatin and phenylmethanesulphonyl fluoride (PMSF)

The enzyme was preincubated with the inhibitors in a buffer consisting of 50 mM formate, pH 3.5 at 60 °C, with 5 mM $CaCl_2$ and 0.01 % (v/v) Triton X-100 for 30 min at room temperature. Samples of enzyme were then assayed for residual activity with 0.05 % (v/v) cytochrome *c* at pH 3.5, with the inhibitors present at the same final concentration as in the preincubations (except for PMSF).

Tos-Lys-CH_2Cl, Tos-Phe-CH_2Cl, (4-aminophenyl)methanesulphonyl fluoride (APMSF) and iodoacetic acid (IAA)

The enzyme was preincubated with the inhibitors in a buffer consisting of 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.0 (pH 8.0 for IAA), with 0.01 % (v/v) Triton X-100 for 30 min at room temperature. The enzyme was then assayed for residual activity with 0.05 % (w/v) cytochrome *c* at pH 3.5.

DAN

The method used was essentially that described by Rajagopalan et al. [2]. DAN additions (4 μl of a stock solution in methanol) were made to a solution containing 50 μl of 0.1 M $CuCl_2$, 76 μl of proteinase (38.2 μg/ml) and 100 μl of 0.2 M formate, pH 5.5, buffer at room temperature (final DAN concentration 12.7 mM). Aliquots (5 μl) were withdrawn at various times over a 6 h period and assayed for residual activity with 0.05 % (w/v) cytochrome *c* at pH 3.5.

EPNP

This is essentially the method described by Tang [3]. Wp22.A1 proteinase (2.3 μg), in a final volume of 200 μl containing 0.1 M citrate, pH 3.5, was shaken vigorously with EPNP at a final concentration of 0.01 mM for 20 h at room temperature. Samples (5 μl) were withdrawn every few hours and assayed for residual activity with 0.05 % (w/v) cytochrome *c* at pH 3.5.

Determination of the presence of disulphide bonds

The enzyme was preincubated for 24 h in the buffer described by Pharmacia Separation Technique File no. 110. For the non-reduced sample, no 2-mercaptoethanol was present. Qualitative detection of the presence of disulphide bonds was made by comparing the distance the proteinase band migrated on SDS/PAGE (Phast system) under reducing and non-reducing conditions.

RESULTS AND DISCUSSION

Extraction and purification of Wp22.A1 proteinase

Table 1 shows the purification of Wp22.A1 proteinase from the culture extracts using phenyl-Sepharose and Mono Q. In spite of six extractions of the cell pellet, less than 30 % of the activity detected in the whole cell suspension was found in the combined extracts. The activity in the supernatant was negligible compared with the cell-associated activity. In each purification step, more than 100 % of the proteinase was apparently recovered. Presumably, substances in the crude extract that may have been inhibiting the proteinase, such as proteins and peptides (substrate inhibition), were removed by subsequent purification steps. Another possibility is a slow activation of the proteinase by autolysis during purification, which was carried out at room temperature. So the actual losses in each step, including the recovery from the cell pellet, are unknown.

Phenyl-Sepharose was used first as a hydrophobic matrix, then as an affinity matrix. In both steps, the pH of the buffers had to

Table 1 Purification of the *Bacillus* sp. strain Wp22.A1 aspartic proteinase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Pooled extracts	5.95	94.2	15.8	1	100
Phenyl-Sepharose	0.73	104.6	143.8	9.1	111
Mono Q FPLC	0.58	112.0	194.5	12.3	119
Phenyl-Sepharose	0.47	112.1	236.7	15.0	119

Figure 1 SDS/PAGE of Wp22.A1 proteinase

The purified protease was pretreated by the method of Pharmacia (Separation Technique File No. 110) and run on a 10–15% T Phastgel (Pharmacia) with SDS buffer strips.

Table 2 Aspartic proteinase activity with protein substrates

Wp22.A1 proteinase was assayed with the substrates in 50 mM formate, pH 3.5 at 60 °C, containing 5 mM $CaCl_2$. The concentrations of haemoglobin, cytochrome *c* and BSA used were those optimal for activity. The other five substrates were insoluble at pH 3.5, so activity may have been limited by this.

Substrate	Specific activity (units/mg)
Haemoglobin (0.1%, w/v)	129.0
Cytochrome *c* (0.05%, w/v)	67.87
BSA (0.1%, w/v)	7.6
Keratin (0.2%, w/v)	14.46
Collagen (0.2%, w/v)	8.96
Elastin (0.2%, w/v)	7.74
Casein (0.2%, w/v)	7.47
Fibrin (0.2%, w/v)	3.53

be close to the pH optimum of the proteinase to obtain good binding capacity on phenyl-Sepharose. This requirement, as well as the use of an amino acid gradient as the eluting conditions, supports the claim that phenyl-Sepharose acts as an affinity matrix for proteinases [23]. The amino acids in the gradient were ones frequently found at the P1 site of substrates cleaved by aspartic proteinases.

An interesting point to note is that phenyl-Sepharose has been used as an affinity ligand for the purification of a UDP-glucose:flavone O^7-glucosyltransferase from *Silene latifolia* [24]. The eluting buffer contained isovitexin (6-*C*-glucosyl-apigenin) which is the enzyme's substrate. This suggests that phenyl-Sepharose may be suitable for the affinity purification of a variety of enzymes that have substrates containing a phenyl group.

Overall, the enzyme was apparently purified by a factor of 15 and had a specific activity of 237 units/mg with 0.1% (w/v) haemoglobin (Table 1). After being stored at −70 °C, the specific activity with haemoglobin was about 129 units/mg. The specific activity with 0.05% (w/v) cytochrome *c* after storage at −70 °C was 67.9 units/mg.

On SDS/PAGE, 80 ng of the purified proteinase appeared as a single band after silver staining (Figure 1). Using isoelectric focusing/PAGE, 80 ng of proteinase appeared as a single band after silver staining, coincident with the single activity band detected on the same gel by the X-ray film method [19]. The molecular mass and pI were estimated to be 45 kDa and 3.8 respectively. Both the reduced and non-reduced samples migrated the same distance on SDS/PAGE, indicating the absence of disulphide bonds in the native enzyme. Minor additional bands were present on non-denaturing PAGE, including some of much lower molecular mass. Autolysis of pure proteinase preparations before, or during, electrophoresis is not uncommon [e.g. 15,25].

Substrate specificity

Protein substrates

The specific activity with haemoglobin was higher than with any other substrate tested (Table 2). The proteinase had a K_m and $V_{max.}$ of 0.036% and 97 units/mg respectively with haemoglobin. For cytochrome *c*, the K_m and $V_{max.}$ were 0.022% and 84 units/mg respectively, giving a higher specificity ($V_{max.}/K_m$) than for haemoglobin. These results, combined with the lack of linearity of the haemoglobin assay with respect to enzyme concentration (results not shown), made cytochrome *c* the assay substrate of choice for Wp22.A1 proteinase. The activities of the proteinase with the other protein substrates were quite low (Table 2), possibly because of their insolubility at pH 3.5 (except for BSA).

The aspartic proteinase from *Bacillus* sp. strain Wai21.A1 [26] has a specific activity of 177.7 units/mg with 0.2% haemoglobin, as compared with 74 units/mg for Wp22.A1 proteinase at this substrate concentration. An aspartic proteinase from the fungus *Hebeloma crustuliniforme* [27] has a specific activity of 56 units/mg with cytochrome *c* (comparable units) which is similar to the specific activity of Wp22.A1 proteinase (Table 2).

Wp22.A1 proteinase showed substrate inhibition with haemoglobin above 0.2% and cytochrome *c* above 0.05% (results not shown). The apparent k_i values were 0.9% and 0.5% respectively. Substrate inhibition is a common feature among proteinases [15,28].

Synthetic peptide substrates

No activity was detected with the two aspartic proteinase substrates Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe [20] and *N*-Boc-Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-4-hydroxymethylpyridine ester [21]. These peptides are used to assay pepsin/chymosin and cathepsin D respectively. The same results were obtained with the proteinase from *Bacillus* sp. strain Wai21.A1 [26]. The proteinase did not liberate *p*-nitroanilide from any of the *p*-nitroanilide-linked peptides tested (see the Materials and methods section).

Hydrolysis of oxidized insulin B chain

The hydrolysis of oxidized insulin B chain by Wp22.A1 proteinase is shown in Figure 2. The primary cleavage site was at Val^2-Asn^3, with 50% of the bonds cleaved in approx. 20 min. At 1 h, cleavage of the sites His^5-Leu^6, Leu^{15}-Tyr^{16} and Phe^{25}-Tyr^{26} had occurred, with about 50% of each of these bonds being cleaved at 3 h. In contrast, the other bacterial and archaeal aspartic proteinases tend to have a strong specificity for cleavage at Leu^{15}-Tyr^{16}, and are not cleaved at Val^2-Asn^3 [11,26,29]. At 3 h, there was a minor cleavage of the decapeptide Leu^6 to Leu^{15} to yield a nonapeptide with one less leucine than the original decapeptide. This led to some uncertainty as to whether the leucine had been cleaved from the N- or C-terminus of the decapeptide as the peptides were only analysed by amino acid composition.

These results suggest that Wp22.A1 proteinase has a preference for sites where the P1 amino acid is bulky and non-polar, and the P1′ amino acid is bulky and polar. These results correlate with the general observations that microbial aspartic proteinases prefer to hydrolyse between bulky or hydrophobic residues [30].

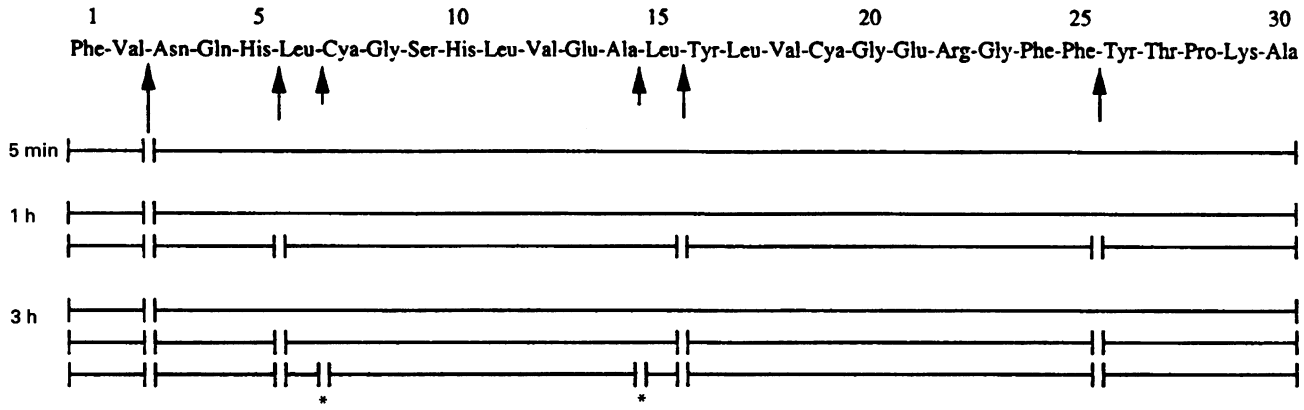

Figure 2 Hydrolysis of the oxidized insulin B chain (bovine) by Wp22.A1 proteinase

The fragments produced by proteolytic cleavage of insulin B chain by Wp22.A1 proteinase after 5 min, 1 and 3 h incubations at 60 °C are shown. The identification of the fragments, and therefore the sites of cleavage of insulin B chain, were made by determining the amino acid composition of the fragments, and mapping them to the known sequence of insulin B chain. As the fragments Cya^7 to Leu^{15} and Leu^6 to Ala^{14} are indistinguishable on the basis of composition analysis, it is not clear which of the two * sites have been cleaved. A mixture of the two, i.e. some cleavage at both sites, is also possible.

Table 3 Effect of proteinase inhibitors on Wp22.A1 proteinase

The proteinase was preincubated with the inhibitors (see the Materials and methods section), then assayed for remaining activity with 0.05% cytochrome *c* in 50 mM formate, pH 3.5 at 60 °C, containing 5 mM $CaCl_2$ for 2 h at 60 °C.

Inhibitor	Proteinase class	Concentration (mM)	Activity remaining (%)
Pepstatin	Aspartic	0.005	93
DAN (three additions)	Aspartic	12.7	3.6
EPNP	Aspartic	0.01	0
Bestatin	Aminopeptidase	0.13	77.2
EDTA	Metallo	10	49.5
EGTA	Metallo	10	74.1
Tos-Phe-CH_2Cl	Cysteine	2	95.8
IAA	Cysteine	0.01	110.7
Tos-Phe-CH_2Cl	Serine	10	105.1
PMSF	Serine	10	103.5
APMSF	Serine	10	103.6

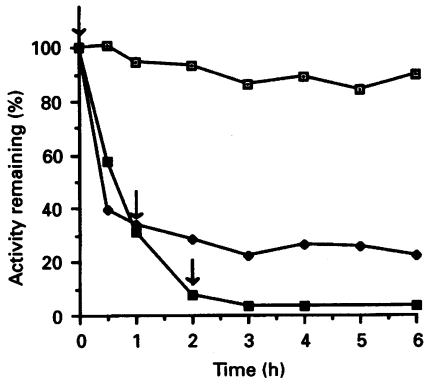

Figure 3 Inactivation of Wp22.A1 proteinase by DAN

The method used was essentially that of Rajagopalan et al. [2]. The arrows indicate when the additions of DAN, each of 4.23 mM, were made. ⊡, No DAN; ◆, one addition of DAN; ■, three additions of DAN.

However, the specificity for Val^2-Asn^3 is unusual, as very few other microbial proteinases cleave at this site [30].

Effect of proteinase inhibitors

Almost no inhibition was seen with a concentration of pepstatin that completely inhibits pepsin (Table 3). This suggests that it belongs to the pepstatin-insensitive *Scytalidium*-type subclass of aspartic proteinases [31]. However, members of this subclass are also DAN- and EPNP-insensitive, whereas Wp22.A1 proteinase is sensitive to both these inhibitors. Tang [3] suggested that DAN and EPNP may each modify different aspartate residues involved in the active site (Asp^{215} and Asp^{32} respectively; pepsin numbering).

The inactivation pattern of Wp22.A1 proteinase with EPNP is similar to that seen with pepsin [3], suggesting that this proteinase may contain one of the two active-site aspartate residues.

Figure 3 shows the time course of inactivation of Wp22.A1 proteinase with DAN. Complete inhibition was seen only after three DAN additions and 6 h of incubation. This is in contrast with pepsin, which requires only one DAN addition and less than 30 min of incubation to achieve total inhibition. Other aspartic proteinases from *Bacillus* sp. strain Wai21.A1 and *S. acidocaldarius* showed a similar inhibition profile with DAN to that found for Wp22.A1 (see Table 6). It has been suggested that in this situation, DAN may be non-specifically modifying an aspartic acid residue somewhere other than at the active site [13], such as in the binding cleft. Such a modification could affect the binding affinity or specificity of the enzyme and hence lower its activity.

Even though the proteinase was sensitive to the metal chelators EDTA and EGTA, it is not a member of the metalloproteinase

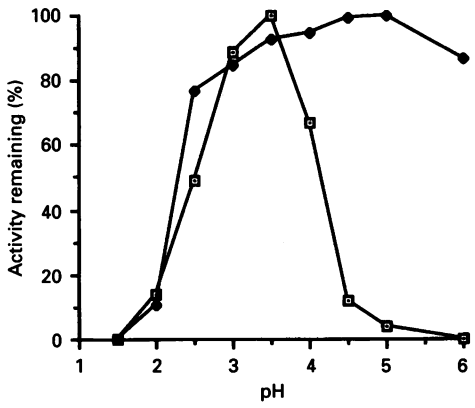

Figure 4 Effect of pH on activity (□) and stability (◆) of Wp22.A1 proteinase at 60 °C

The enzyme was preincubated in 50 mM formate for 2 h at 60 °C, then assayed for residual activity with 0.05% (w/v) cytochrome *c* at 60 °C to determine stability. The activity of the proteinase was determined by assaying the enzyme with 0.05% (w/v) cytochrome *c* for 2 h at 60 °C.

class, as the inhibition was due to the chelation of stabilizing Ca^{2+} ions rather than the loss of a catalytic active-site metal (see below). This was demonstrated by the lack of inhibition of the proteinase by the chelators at 40 °C where the loss of activity as a result of thermal instability is not significant (results not shown).

Wp22.A1 proteinase was not inhibited by any of the serine or cysteine proteinase inhibitors tested. However, it was partially inhibited by bestatin, an inhibitor specific for aminopeptidases [32]. This inhibition was probably not significant as the k_i of aminopeptidase B with bestatin is 0.06 μM and Wp22.A1 was tested with 130 μM bestatin.

Effect of pH on activity and stability of Wp22.A1 proteinase

Maximum activity with cytochrome *c* was obtained at pH 3.5 at 60 °C (Figure 4) and pH 3.0 at 45 °C (results not shown). Maximal stability was in the range pH 4–5, where less than maximal activity was observed. The increased stability at higher pH could be due to a decrease in autolysis.

Effect of temperature on activity

An Arrhenius plot with the substrate cytochrome *c* (results not shown) shows what appears to be a change in slope at about 40–45 °C. The E_a and Q_{10} values in the range 20–40 °C are 79.1 kJ/mol and 3.3 respectively, whereas those for the 45–60 °C range are 127.4 kJ/mol and 4.5 respectively.

These high Q_{10} values are quite surprising. However, as the substrate was a mesophilic protein, it was expected to be thermally unstable. It is also possible that cyochrome *c* is more sensitive to temperature at pH 3.5 than at neutral pH. A combination of these factors could result in an increased tendency of the substrate to unfold, and therefore expose more potential proteolytic cleavage sites. This could explain the high Q_{10} values.

Thermostability of Wp22.A1 proteinase

Wp22.A1 proteinase is the most thermostable of the eubacterial aspartic proteinases characterized to date (see Table 6). At 60 °C, the $t_{1/2}$ in the presence and absence of Ca^{2+} was 45 and 12 h respectively (Table 4). Half-lives measured in the presence of 50 μM Ca^{2+}, no added Ca^{2+} and the presence of EDTA were all similar (results not shown). In the presence of 5 mM Ca^{2+}, at 60 °C, both the first- and second-order plots approached linearity, suggesting that both thermal denaturation and autolysis contributed significantly to the loss of activity (results not shown). With lower levels of Ca^{2+}, only the first-order plots were linear, suggesting that thermal denaturation was dominant under these conditions. This suggests that high Ca^{2+} levels stabilize the enzyme by the prevention of thermal denaturation, making autolysis more significant.

At higher temperatures, Ca^{2+} had less of an effect on the thermostability of the proteinase than at 60 °C (Table 4). At 70 °C, 5 mM Ca^{2+} had little significant stabilizing effect. The first-order plots at higher temperatures also showed that thermal denaturation was the cause of loss of activity (results not shown).

Table 4 Thermostability of Wp22.A1 proteinase

Proteinase samples in 50 mM formate, pH 3.5 at 60 °C, containing 0.01% Triton X-100 and $CaCl_2$ (as indicated) were preincubated at the temperatures indicated. Samples were withdrawn at appropriate time intervals and assayed for remaining activity with 0.05% cytochrome *c* in the above buffer. The preincubation buffer was composed of 50 mM formate, pH 3.5, containing 0.01% (v/v) Triton X-100, either with or without 5 mM $CaCl_2$.

	Half-life ($t_{1/2}$)	
Temperature (°C)	5 mM Ca^{2+}	No added Ca^{2+}
60	45 h	12 h
70	102 min	70 min
80	21 min	16 min
90	3 min	2.4 min

Stability towards denaturing and reducing agents

Table 5 shows the stability of Wp22.A1 proteinase to a variety of denaturing and reducing agents. These data need to be interpreted with caution as there is about a 5% (v/v) carry-over of the denaturants into the assay. As the substrate is a protein, it could also be susceptible to unfolding by the denaturants, causing the exposure of more potential proteolytic cleavage sites. This could explain the greater than 100% retention of proteinase activity in the presence of some denaturants. The results indicate that the

Table 5 Effect of various denaturing agents on Wp22.A1 proteinase

Samples of the proteinase were diluted in 50 mM formate, pH 3.5 at 60 °C, containing the denaturing agent. These samples were incubated for 24 h at 4 °C or 1 h at 60 °C, as indicated, then assayed for residual activity with 0.05% cytochrome *c* in 50 mM formate, pH 3.5 at 60 °C, containing 5 mM $CaCl_2$. The activity was expressed as a percentage of a control which was preincubated without the agent. ND, Not determined.

	Activity remaining (%)	
Condition	25 °C, 24 h	60 °C, 1 h
Control (just 50 mM formate, pH 3.5)	100	100
1% (w/v) SDS	0	0
6 M urea	102.5	137.6
10 mM dithiothreitol	123.7	123.0
1% (v/v) 2-mercaptoethanol	136.0	141.6
90% (v/v) ethanol	44.9	ND
90% (v/v) acetonitrile	35.3	ND

Table 6 Properties of some prokaryotic aspartic proteinases

+, Sensitive to the inhibitor; −, insensitive to the inhibitor. +*, non-specific inhibition; n.a.l., no activity loss.

Source	Temperature (°C)	Molecular mass (kDa)	pI	$pH_{opt.}$	Thermostability ($t_{1/2}$)	Inhibition			Reference
						Pepstatin	DAN	EPNP	
S. acidocaldarius	75	46–51	4.0	2.7	n.a.l. 48 h, 80 °C, pH 4.5	+	+*	+	[13]
Xanthomonas sp. T-22	55	40–41	4.0	2.7	< 15 min, 60 °C, pH 2.7	−	−	+	[8]
Pseudomonas sp. st. 101	50	42–43	3.2	3.0	< 15 min, 65 °C, pH 4.0	−	−	+	[9]
Bacillus sp. st. MN-32	70	40–41	3.5	3.0	~ 10 min, 80 °C, pH 4.0	−	−	−	[10,11]
Bacillus sp. st. Wai21.A1	60	45	3.8	3.0	2 min, 80 °C, pH 3.0	−	+*	+	[12,26]
Bacillus sp. st. Wp22.A1	60	45	3.8	3.5	21 min, 80 °C, pH 3.5	−	+	+	This work

proteinase is probably stable under these conditions. This is not surprising as it has been observed previously that thermophilic proteins tend to be more resistant to denaturing agents than mesophilic proteins [33]. The proteinase was also resistant to 2 M NaCl, showing no loss of activity after 1 h at 60 °C.

Effect of metal ions

In most cases, the effects of 5 mM concentrations of the cations on the stability of the proteinase were small. Mg^{2+}, Zn^{2+} and Ni^{3+} appeared to stabilize the enzyme to the same degree as Ca^{2+}. Co^{2+} stabilized the enzyme partially, while V^{2+} and Cu^{2+} appeared to destabilize the enzyme compared with the control in which no cation had been added.

Comparison of bacterial aspartic proteinases

Table 6 compares the properties of Wp22.A1 with the other characterized prokaryotic aspartic proteinases. This shows that these proteinases tend to have a molecular mass in the range 40–46 kDa, and an isoelectric point between 3 and 4. Most are insensitive to pepstatin and some are insensitive to the other group-specific inhibitors. Wp22.A1 can be distinguished from the other bacterial and archaeal aspartic proteinases by its pH optimum, isoelectric point and thermostability.

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