Purification and characterization of Ak.1 protease, a thermostable subtilisin with a disulphide bond in the substrate-binding cleft

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Ak.1 protease, a thermostable subtilisin isolated originally from *Bacillus* st. Ak.1, was purified to homogeneity from the *Escherichia coli* clone PB5517. It is active against substrates containing neutral or hydrophobic branched-chain amino acids at the P₁ site, such as valine, alanine or phenylalanine. The K_m and k_{cat} of the enzyme decrease with decreasing temperature, though not to the same degree with all substrates, suggesting that specificity changes with temperature. The protease is markedly stabilized by Ca²⁺ ions. At 70 °C, a 10-fold increase in Ca²⁺ concentration increases the half-life by three orders of magnitude. Ak.1 protease is stabilized by Ca²⁺ to a greater extent than is thermitase. This may be due, in part, to the presence of an extra Ca²⁺-binding site in Ak.1 protease. Other metal ions, such as

Sr²⁺, increase the thermostability of the enzyme, but to a significantly lower degree than does Ca²⁺. The structure of the protease showed the presence of a disulphide bond located within the active-site cleft. This bond influences both enzyme activity and thermostability. The disulphide bond appears to have a dual role: maintaining the integrity of the substrate-binding cleft and increasing the thermostability of the protease. The protease was originally investigated to determine its usefulness in the clean-up of DNA at high temperatures. However, it was found that this protease has a limited substrate specificity, so this application was not explored further.

Key words: calcium binding, heat stable, specificity.

INTRODUCTION

One of the most extensively studied families of serine proteases is the subtilisin family. It consists of primarily prokaryotic proteases, such as subtilisin BPN' from *Bacillus amyloliquefaciens* [1], but subtilisins have also been discovered in organisms such as fungi and higher eukaryotes (e.g. [2]). The subtilisin family differ from the chymotrypsin-type serine protease family by significantly different protein scaffolds (β/β folds for trypsin and α/β folds for subtilisin; [3]). However, the two families contain highly similar orientations of the catalytic serine, histidine and aspartate residues.

Ak.1 protease is a thermostable subtilisin (peptidase classification: clan SB, family S8) from *Bacillus* st. Ak.1 which has been partially characterized and crystallized previously [4–6]. It has 68% sequence similarity with thermitase, a thermophilic subtilisin from *Thermoactinomyces vulgaris* [3]. It has a molecular mass of 36 kDa and a pI of 4.0 [6]. Maximum proteolytic activity was observed at pH 7.5 at 75 °C with azocasein as the substrate. It cleaves the substrate Suc-Ala-Ala-Pro-Phe-pNA (where Suc is succinyl and pNA is *p*-nitroanilide) [6]. The half-life of the protease at 90 °C is 19 min in the presence of 5 mM CaCl₂. The pro-protease has been cloned, sequenced and overexpressed in *Escherichia coli*. It contains two cysteine residues [4].

Thermophilic proteases have been shown previously in our laboratories to be useful in the clean-up of DNA at high temperatures [7]. Pretaq is a thermostable Ca²⁺-dependent serine protease isolated from *Thermus* st. Rt41A [7]. It is commercially available as an alternative to proteinase K to clean up DNA. However, due to difficulties in the expression of Pretaq in *E. coli*,

an alternative thermostable Ca²⁺-dependent protease was sought that was more commercially viable to produce. Thus *Bacillus* st. Ak.1 protease was targeted for further characterization to determine its substrate specificity and thermostability, and to assess its potential in DNA clean-up.

MATERIALS AND METHODS

Reagents

Laboratory-grade reagents were used in all cases. The water used for all buffers was Millipore ultrapure water. Media components were obtained from Oxoid. Chromogenic and other substrates were obtained from Sigma Aldrich and Bachem. Chromatography resins and columns were obtained from Pharmacia. All other reagents were obtained from BDH and Sigma Aldrich. Lanthanide chloride and oxovanadium chloride solutions were prepared according to the method of Khoo et al. [8]. All equipment which came into contact with the enzyme prior to thermostability studies with Ca²⁺ was pretreated to remove trace amounts of Ca²⁺ [8]. All buffers for the thermostability work with cations were passed through a metal-ion-chelating column (Chelex 100; Dow Chemical Company) before use to remove traces of Ca²⁺.

Production and purification of Ak.1 protease

Escherichia coli clone PB5517 [4] was grown aerobically in a 10 l LH Series Fermentor on Bulk Growth Medium 2 [4] in a fed-

Abbreviations used: DTT, dithiothreitol; pH₇₅, pH at 75 °C; pNA, *p*-nitroanilide; pNP, *p*-nitrophenol ester; Suc, succinyl.

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batch manner at 35 °C for 17 h. The culture was harvested and concentrated using a Hollow Fibre Cartridge (Amicon S10Y10) and centrifuged at 11 100 *g* (Beckman JA-10) for 20 min at 4 °C. The supernatant (3 litres) was retained and incubated with 0.01 g of lysozyme and 0.001 g of DNase at room temperature overnight. The supernatant was heat-treated at 70 °C for 15 min to precipitate the majority of the *E. coli* proteins, followed by centrifugation as before. The supernatant (2.9 litres) was heat-treated for a further 2 h at 70 °C to activate the protease. The active solution was concentrated by ultrafiltration (Amicon YM-10) to 1.6 litres and equilibrated to 10 mM Tris/NaOH, pH 7.0, containing 5 mM CaCl₂ and 1 M NaCl.

The protease was purified to homogeneity in three steps. The extracts were applied to phenyl-Sepharose and Mono Q according to the method of Peek et al. [4]. The final step was a second phenyl-Sepharose step, using the amino acids phenyl-alanine, histidine and alanine in the eluting buffer at pH 7.5 [9].

Protease assays

The protease assay with azocasein was carried out using the method of Peek et al. [4] at 75 °C using a Perkin–Elmer Lambda B spectrophotometer fitted with a thermoelectric cell holder. The assay was linear with respect to enzyme concentration with azocasein over an A_{420} range of only 0.05 unit, so a standard curve with this substrate was constructed and the assay values were adjusted accordingly.

Activity towards a variety of synthetic peptide substrates was determined in the spectrophotometer described above. The assay mixture consisted of 1 ml of substrate in 50 mM Hepes/NaOH, pH 7.5 (at the specified temperature), containing 5 mM CaCl₂. The substrate was preincubated at the required temperature for 10 min, followed by the addition of 10 μ l of the protease solution. The absorbance change was monitored continuously using Perkin–Elmer Computerized Spectroscopy Software at the wavelengths specified. For substrates that were not completely soluble (e.g. *p*-nitrophenol-ester-linked substrates), 50% (v/v) acetonitrile was included in the substrate buffer and assays were conducted at 40 °C. For some experiments the total assay volume was 0.1 ml, composed of 0.09 ml of substrate and 0.01 ml of the protease.

Protein assay

The protein concentration was determined according to the modified Lowry and $A_{280/205}$ methods of Peterson [10], and the Bradford method of Read and Northcote [11].

Determination of disulphide bond content

Ak.1 protease was incubated with 10 mM concentrations of the reducing and modifying reagents for 1 h at room temperature prior to the assays. Samples of the dithiothreitol (DTT)-treated protease were dialysed against water continuously flushed with filtered nitrogen (0.22 μ m filter) at 4 °C for 2 days, changing the water five times, to remove the DTT. A control Ak.1 protease sample (no DTT treatment) was also dialysed, but without nitrogen flushing. Another sample was flushed with O₂ continuously for 1 h, followed by incubation overnight in a high-O₂ environment. A final sample was subjected to pretreatment with DTT and 4 M urea, followed by the addition of 75 mM NaCl. The sample was passed through Sephadex G25, with 10 mM EDTA in the eluting buffer to remove the residual reagents. These samples were subjected to free cysteine analysis and activity determination. The quantification of the disulphide bond

and free thiol content was carried out using the method of Hermanson [12].

Electrophoresis

The purity of the protease was determined by running samples on SDS/PAGE 10–15% (w/v) gradient gels (Pharmacia Phast System method). To prevent autolysis during the pretreatment, the samples were prepared using the trichloroacetic acid/acetone method of Peek et al. [4]. The molecular-mass standards were from the Pharmacia low-molecular-mass calibration kit, and the gels were stained using the silver stain method (Bio-Rad).

Substrate specificity

Activity towards a variety of pNA and *p*-nitrophenol ester (pNP) peptide substrates was determined using a substrate concentration of 1 mM. This was chosen due to the high cost and/or low solubility of some of the substrates. The K_m and V_{max} values were calculated using the direct linear plot method [13]. The range of substrate concentrations used in these experiments varied depending on the affinity of the enzyme for each substrate, but was between 0.05 and 6 mM in all cases. In some of the substrate-specificity experiments, assays were conducted in buffers containing 50% (v/v) acetonitrile. This was due to the poor solubility of some of the substrates. To compare these activity results with those with other more soluble substrates, comparative reactions of all substrates were carried out in 50% acetonitrile, to take into account the effect of 50% acetonitrile on activity and/or stability.

Thermostability determination

Ca²⁺-free Ak.1 protease (apo-Ak.1) was prepared by incubating the protease with 10 mM EDTA in 10 mM Hepes/NaOH, pH_{7.5} (pH at 75 °C) 7.5, containing 0.01% (w/v) Triton X-100 for 1.5 h at room temperature. This was followed by exhaustive dialysis against 10 mM Hepes/NaOH, pH_{7.5} 7.5, containing 0.01% (w/v) Triton X-100 in a 1 ml dialysis chamber with continuous buffer exchange for 2 days at 4 °C.

Apo-Ak.1, in 50 mM Hepes/NaOH, pH 7.5 (at the required temperature), containing 0.01% (v/v) Triton X-100 and the appropriate concentration of metal chloride salt/additive, was incubated at the specified temperature in reaction vials. Samples were withdrawn at various times, fast-frozen in liquid N₂ and then stored on ice until assayed. For high-temperature incubations (> 90 °C) or for very long incubations (> 1 day), samples were sealed in acid-cleaned capillary tubes (including the zero-time control) and heated in a thermostatically controlled oil bath. These tubes were cooled quickly in liquid nitrogen, and stored on ice until assayed. The samples were assayed for remaining activity with 0.2% azocasein at 75 °C.

Effect of the disulphide bond on activity and stability

To determine the effects of the reagents on activity, DTT-treated protease was assayed with 1 mM Suc-Ala-Ala-Pro-Phe-pNA in 50 mM Hepes/NaOH, pH_{7.5} 7.5, containing 5 mM CaCl₂, 0.01% Triton X-100 and 10 mM reductant/modifier at 20 °C. To test the effect of DTT on K_m and k_{cat} , the enzyme was tested with several concentrations of the above substrates under the same conditions. To test the effect of DTT on the k_{cat} of the protease with Suc-Ala-Ala-Pro-Phe-pNA over a wide temperature range, the protease was assayed, as above, over the temperature range 5–75 °C. The duration of the assay was 1 min, to ensure that DTT was present throughout the assay [14].

To determine the half-life of the protease in the presence and absence of a disulphide bond, the protease was preincubated in 10 mM DTT for 1 h at 20 °C, and then incubated at 85 °C. Samples were withdrawn at various times, cooled on ice and assayed for remaining activity with 1 mM Suc-Ala-Ala-Pro-Phe-pNA at 20 °C. To determine the effect of heavy metals on the activity of the protease, the protease was preincubated with a 100 μ M concentration of Hg²⁺, Pb²⁺, Cd²⁺ or Zn²⁺ for 1 h at room temperature. Remaining activity was detected with 1 mM Suc-Ala-Ala-Pro-Phe-pNA at 25 °C.

RESULTS

Extraction and purification of Ak.1 protease

The purified protease had a specific activity of 174 units/mg (1 unit = ΔA_{420} of 1.0 per min at 75 °C with 0.2 % azocasein). The

Table 1 Quantification of the number of cysteine residues in Ak.1 protease

The determination of the free thiol content was performed using the method of Hermanson [12].

Enzyme pretreatment	-SH content (mol/mol of protease)
None	0
Urea and NaCl/Sephadex G25/EDTA	0
DTT/SepPak or dialysis under N ₂	< 1
DTT/SepPak/O ₂ treatment	< 1
DTT/urea and NaCl/Sephadex G25/EDTA	1.69

Table 2 Substrate specificity of Ak.1 protease with pNA substrates

The **bold** residue denotes the P1 amino acid in each case. Abbreviations: Cbz, benzyloxycarbonyl; Ac, acetyl; Bz, benzoyl. Assays at 75 °C were conducted with 1 mM peptide-pNA in 50 mM Hepes/NaOH, pH_{7.5}, containing 5 mM CaCl₂ and 0.01 % Triton X-100. Assays at 40 °C were conducted with 1 mM peptide-pNA in 50 mM Hepes/NaOH, pH_{7.5}, containing 5 mM CaCl₂, 0.01 % Triton X-100 and 50 % acetonitrile. Units represent μ mol of pNA/min. Molar absorption coefficients of 10500 and 9400 were used for 0 and 50 % acetonitrile buffers respectively. In all cases, reactions were carried out in triplicate, and the mean values are recorded. Errors represent $\pm 1\%$ of the mean value, and experiments were repeated if the variation of the replicates exceeded this.

Substrate	Temp. (°C)	Specific activity (units/mg)
(a) Buffer		
Suc-Ala-Ala-Pro- Phe -pNA	75	313 $\pm$ 3
Suc-Ala-Ala-Pro- Leu -pNA	75	103 $\pm$ 1
Suc-Ala-Ala-Pro- Ala -pNA	75	25 $\pm$ 0.3
Suc-Ala-Ala- Ala -pNA	75	16 $\pm$ 0.2
Suc-Ala- Ala -pNA	75	0.56 $\pm$ 0.06
Ala- Ala -pNA	75	0.75 $\pm$ 0.08
Glu- Phe -pNA; Cbz- Phe -pNA; Cbz- Phe -pNA; Suc- Phe -pNA; Ac- Phe -pNA; Phe -pNA; Gly-pNA; Ala -pNA	75	All < 0.10 $\pm$ 0.10
(b) 50 % (v/v) acetonitrile in buffer		
Suc-Ala-Ala-Ala- Ala -pNA	40	0.59 $\pm$ 0.06
Suc-Phe-Ala-Ala- Phe -pNA	40	2.82 $\pm$ 0.03
Suc-Ala-Ala- Ala -pNA	40	0.22 $\pm$ 0.02
Ala-Ala- Phe -pNA	40	0.18 $\pm$ 0.02
Val-Leu- Lys -pNA; Bz- Arg -pNA; Suc-Ala- Ala -pNA; Bz- Tyr -pNA; Cbz-Lys- Arg -pNA; Cbz- Arg -pNA	40	All < 0.10 $\pm$ 0.10

Figure 1 Structures of the active sites of Ak.1 protease and thermitase

Shown is a comparison of Ak.1 protease and thermitase in the active-site region. The substrate Suc-Ala-Ala-Pro-Phe-pNA is modelled into the active site. This structure was generated using the program Molscript and rendered using the Raster3D program suite [6].

purification factor achieved was 404-fold, with 46 % of the protease recovered. The protease has an apparent molecular mass of approx. 36 kDa by SDS/PAGE [4].

The sequence of the protease revealed the presence of two cysteine residues [5]. An analysis of the free thiol content revealed that the two cysteine residues exist in the protease as a disulphide bond (Table 1). This was confirmed by the three-dimensional structure of the protease, which showed the presence of a disulphide bond [6]. The presence of EDTA in the samples was found to be required after treatment with DTT (and removal of the excess) to prevent oxidation of the free thiols.

Substrate specificity

Table 2 shows the specific activity of Ak.1 protease towards a variety of pNA substrates. The substrates are ordered approximately according to decreasing size. These results show that there was no significant activity of Ak.1 protease with pNA substrates which occupy only the P₁-P₁' sites (e.g. Phe-pNA). Activity was detected with only a few substrates which occupy the P₂-P₁' sites, with the best being Ala-Ala-pNA. The specific activity is significantly higher when the P₃-P₁' sites are filled.

The protease was most specific for the substrate Suc-Ala-Ala-Pro-Phe-pNA (phenylalanine at the P₁ site) compared with the other substrates tested. However, no activity was detected with a variety of smaller substrates containing Phe in this position (see Table 2), such as Phe-pNA and acetyl-Phe-pNA.

Figure 1 shows the structure of the active site of Ak.1 protease, with the substrate Suc-Ala-Ala-Pro-Phe-pNA modelled into this site. This shows that, at the P₂ position of the protease, the protein chain juts into the substrate-binding cleft. Proline at

Table 3 K_m , k_{cat} and specificity of Ak.1 protease with several substrates at three temperatures

Reactions were carried out with 1 ml of substrate in 50 mM Hepes/NaOH, pH 7.5 (at the specified temperatures), containing 5 mM CaCl_2 and 0.01% Triton X-100. In all cases, reactions were done in triplicate, and the mean values are recorded in the Table. Errors represent $\pm 1\%$ of the mean value, and experiments were repeated if the variation of the replicates exceeded this.

Substrate	K_m (mM)			k_{cat} (s^{-1})			k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)		
	5 °C	40 °C	75 °C	5 °C	40 °C	75 °C	5 °C	40 °C	75 °C
Suc-Ala-Ala-Pro-Phe-pNA	0.07 $\pm$ 0.01	0.30 $\pm$ 0.01	1.00 $\pm$ 0.01	1.48 $\times 10^5 \pm 2$	1.3 $\times 10^5 \pm 2 \times 10^3$	1.1 $\times 10^7 \pm 2 \times 10^5$	2.1 $\times 10^3 \pm 2 \times 10^1$	4.3 $\times 10^5 \pm 5 \times 10^3$	1.1 $\times 10^7 \pm 2 \times 10^5$
Suc-Ala-Ala-Pro-Leu-pNA	0.68 $\pm$ 0.01	1.01 $\pm$ 0.01	3.37 $\pm$ 0.03	3.89 $\times 10^5 \pm 4$	8.8 $\times 10^4 \pm 9 \times 10^2$	8.2 $\times 10^6 \pm 9 \times 10^4$	5.7 $\times 10^4 \pm 9$	8.7 $\times 10^4 \pm 9 \times 10^2$	2.4 $\times 10^6 \pm 3 \times 10^4$
Suc-Ala-Ala-Pro-Ala-pNA	0.33 $\pm$ 0.01	0.33 $\pm$ 0.01	0.92 $\pm$ 0.01	2.22 $\times 10^5 \pm 3$	8.8 $\times 10^3 \pm 9 \times 10^1$	1.3 $\times 10^5 \pm 2 \times 10^3$	6.7 $\times 10^2 \pm 7$	2.7 $\times 10^4 \pm 3 \times 10^2$	1.4 $\times 10^5 \pm 2 \times 10^3$
Suc-Ala-Ala-pNA	0.28 $\pm$ 0.01	0.63 $\pm$ 0.01	2.25 $\pm$ 0.02	2.22 $\times 10^5 \pm 3$	2.2 $\times 10^4 \pm 3 \times 10^2$	9.7 $\times 10^4 \pm 1 \times 10^3$	7.9 $\times 10^2 \pm 8$	3.5 $\times 10^4 \pm 4 \times 10^2$	4.3 $\times 10^4 \pm 5 \times 10^2$
Suc-Ala-Ala-pNA	—	—	17.0 $\pm$ 1.0	—	—	3.7 $\times 10^3 \pm 4 \times 10^1$	—	—	2.2 $\times 10^2 \pm 3$

Figure 2 Plot of half-life of Ak.1 protease against calcium concentration at 70 °C

Ak.1 protease was incubated at 70 °C for various time periods in the presence of 0–5 mM Ca^{2+} . The half-life was determined by assaying for remaining activity with 0.2% azocasein as the substrate. In all cases, reactions were carried out in triplicate, and the mean values were plotted. Experiments were repeated if the variation of the replicates exceeded $\pm 1\%$ of the mean value.

the P_2 position of the substrate causes the substrate to bend at this point, allowing a better fit into the binding cleft.

Ak.1 protease was unable to cleave a number of trypsin- and chymotrypsin-type substrates which contained arginine or tyrosine at the P_1 site (Table 2). However, these substrates were small (e.g. benzoyl-Arg-pNA), and the protease prefers longer-chained substrates, so cleavage of substrates with these amino acids at the P_1 site cannot be excluded.

Table 3 shows the K_m , k_{cat} and specificity data of Ak.1 protease with five substrates in the temperature range 5–75 °C. The K_m of the protease with Suc-Ala-Ala-pNA at 75 °C was considerably higher than with larger substrates.

The protease was most specific for Suc-Ala-Ala-Pro-Phe-pNA at all temperatures tested. The K_m values for Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Ala-pNA at 40 °C and 75 °C were similar, while the k_{cat} values differed considerably. In almost all cases, both the K_m and k_{cat} for each substrate decreased with decreasing temperature.

The decrease in K_m with temperature was not the same with all substrates. For example, a comparison of the decrease in K_m for the substrate Suc-Ala-Ala-Pro-Xaa-pNA, where Xaa is Phe, Leu and Ala respectively, shows that, as the size and hydrophobicity of the R-group of the P_1 amino acid decreases, the percentage change in K_m decreases.

The percentage decrease in k_{cat} with temperature was similar with all substrates tested. The greatest changes in both K_m and k_{cat} occurred within the temperature range 40–75 °C.

Thermostability

Figure 2 shows the relationship between the half-life of Ak.1 protease activity and the Ca^{2+} concentration at 70 °C. Low Ca^{2+} concentrations ($< 10 \mu\text{M}$) had no significant effect on thermostability. As the calcium concentration was increased from 30 to 200 μM , the half-life increased from 4 min to 1500 min. At 5 mM Ca^{2+} , the half-life of the protease was increased by four orders of magnitude at 70 °C over that of the EDTA-treated enzyme.

Replotting the thermostability data as $\log(\% \text{ activity})$ against time and $1/(\% \text{ activity})$ against time allowed the determination

Figure 3 First- and second-order thermostability plots of Ak.1 protease in the presence of 5 μM or 5 mM Ca^{2+}

Ak.1 protease, pre-equilibrated in 5 μM (upper panel) or 5 mM (lower panel) CaCl_2 , was incubated at 70 °C for several minutes to 10 days and then assayed for remaining activity with 0.2% azocasein in 50 mM HEPES/NaOH, pH_{7.5} 7.5, containing 5 mM CaCl_2 at 75 °C. In all cases, reactions were carried out in triplicate, and the mean values were plotted. Experiments were repeated if the variation of the replicates exceeded $\pm 1\%$ of the mean value.

of whether the loss of activity was predominantly due to a first-order (thermal unfolding) or second-order (autolytic) process. An analysis of some of these plots (Figure 3) shows that, at low Ca^{2+} concentrations ($\leq 30 \mu\text{M}$), where Ca^{2+} has little stabilizing effect, the first-order plots were approximately linear, suggesting that the most significant cause of loss of activity is thermal denaturation. At higher Ca^{2+} concentrations the second-order plots became more linear, suggesting that autolysis is becoming more significant, and at $> 200 \mu\text{M}$ Ca^{2+} autolysis was the predominant cause of loss of activity.

Figure 4 shows the half-lives of Ak.1 protease in the presence of 5 mM concentrations of several lanthanides and other metal ions. These results show that lanthanum stabilizes the protease, but to a much smaller degree than Ca^{2+} . The first- and second-order plots (results not shown) show that, in all cases, stabilization was due to the prevention of thermal denaturation, as for Ca^{2+} . Sr^{2+} increased the thermostability of the protease by about 500-fold at 70 °C. This is quite significant, but still only about 4% of the stabilization observed with Ca^{2+} ions.

Figure 4 Effects of various metal ions on the half-life of Ak.1 protease at 70 °C

The protease was incubated at 70 °C in the presence of 0.5 mM metal ion for various time periods, then assayed for remaining activity with 0.2% azocasein. In all cases, reactions were carried out in triplicate, and the mean values were plotted. Experiments were repeated if the variation of the replicates exceeded $\pm 1\%$ of the mean value.

The effects of a variety of additives on the thermostability of Ak.1 protease in the presence of 5 mM CaCl_2 were determined at 90–115 °C (results not shown). Only 90% solutions of sorbitol, trehalose and glycerol significantly stabilized the enzyme. At 105 °C, complete loss of activity was virtually instantaneous in the absence of sorbitol. In the presence of 90% sorbitol, the half-life was 104 min. The major cause of loss of activity in the presence of sorbitol is thermal denaturation (results not shown). Stabilization by these solutions has been noted with other enzymes [15].

Effect of reducing the disulphide bond on substrate specificity

Substrate specificity was determined in the presence and absence of DTT, a thiol reagent; i.e. with the disulphide bond open and intact respectively (Table 4). The results show that, as the size of the substrate decreased, the effect on the K_m of opening the disulphide bond decreased. In fact, the smallest substrate, benzyloxycarbonyl-Gly-pNP, showed no change in K_m in the presence (free -SH) or absence (-S-S-) of DTT. The K_m increased in the presence of DTT, suggesting that the substrate was bound more weakly to the enzyme. The k_{cat} also decreased in the presence of DTT.

It was found that some metals at micromolar concentrations decreased the activity of the protease. For example, 100 μM Hg^{2+} inhibited the activity of the protease by 60%. At 100 μM , the metals inhibiting the protease were, in the order of decreasing inhibition: $\text{Hg}^{2+} > \text{Pb}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+}$.

Effect of reducing the disulphide bond on thermostability

The half-lives of Ak.1 protease at 85 °C in the presence and absence of DTT were 34 and 317 min respectively in the presence of 5 mM Ca^{2+} (results not shown). This shows that the disulphide bond increases the thermostability of the protease.

Table 4 Substrate specificity of Ak.1 protease with peptide substrates at 20 °C in the presence and absence of DTT

Abbreviation: Cbz, benzyloxycarbonyl. Units: $^1\mu\text{mol}$ of pNA/min per mg; $^2\mu\text{mol}$ of pNP/min per mg. In all cases, reactions were done in triplicate, and the mean values are recorded in the Table. Errors represent $\pm 1\%$ of the mean value, and experiments were repeated if the variation of the replicates exceeded this.

Substrate	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	
	Control	+ DTT	Control	+ DTT	Control	+ DTT
Suc-Ala-Ala-Pro-Phe-pNA ¹	0.15 ± 0.01	0.44 ± 0.01	$1.0 \times 10^4 \pm 1 \times 10^2$	$2.9 \times 10^3 \pm 30$	$6.7 \times 10^4 \pm 7 \times 10^2$	$6.6 \times 10^3 \pm 70$
Suc-Ala-Ala-Ala-pNA ¹	0.32 ± 0.01	0.58 ± 0.01	$1.2 \times 10^3 \pm 20$	$7.5 \times 10^2 \pm 8$	$3.8 \times 10^3 \pm 40$	$1.3 \times 10^3 \pm 20$
Suc-Ala-Ala-pNA ¹	4.00 ± 0.1	6.67 ± 0.1	93 ± 1	93 ± 1	24 ± 0.3	14 ± 0.2
Cbz-Gly-pNP ²	8.30 ± 0.1	8.30 ± 0.1	$1.9 \times 10^3 \pm 20$	$2.5 \times 10^3 \pm 30$	$2.3 \times 10^2 \pm 3$	$3.0 \times 10^2 \pm 3$

DISCUSSION

Substrate specificity

The activity profile of Ak.1 protease with the substrates Suc-Ala-Ala-Pro-Xaa (where Xaa is Phe, Ala or Leu) shows that the specificity with these substrates increases as the hydrophobicity and size of the P₁ amino acid increases. This is not surprising, as the X-ray structure shows that the active-site cleft is very hydrophobic [6]. The decrease in activity with smaller substrates suggests that the protease has a requirement for the P₂ site to be occupied before significant hydrolysis of the substrate can be achieved. Kinetic analysis with several differently sized substrates suggests that the lower activity with smaller substrates could be due, in part, to a lower binding affinity for the enzyme (higher K_m).

Substrate size, as well as substrate composition, appears to be important in determining the specificity of the protease. This was supported by the increased specificity for alanine-based substrates as the chain length increased (Table 2). An analysis of the specific activities of the protease in relation to the number of alanine residues in the substrate (Suc-Ala_{*n*}-pNA; *n* = 2, 3 or 5) showed a linear correlation between increased specific activity and increased chain length (results not shown).

The decrease in K_m of the enzyme as the temperature decreases is not surprising, since, when the temperature decreases, the flexibility of a protein also decreases, so a substrate is likely to be more tightly bound. However, the degree of change in K_m (and k_{cat}) with temperature was not the same with all substrates. This suggests that the specificity of Ak.1 protease changes with temperature.

The K_m of the protease with the substrate Suc-Ala-Ala-Pro-Ala-pNA was unchanged in the temperature range 5–40 °C, but there was a significant decrease in the K_m of the protease with Suc-Ala-Ala-Ala-pNA over this temperature range. This suggests that the change in K_m with temperature is not solely dependent on the nature of the P₁ amino acid.

The protease exhibited high specificity towards only a few of the substrates tested. This makes it less useful as a general DNA clean-up reagent than Pretaq, which has a very broad substrate specificity [7].

The structure of Ak.1 protease [6] shows that the disulphide bond is located in the substrate-binding cleft, at the S₄ site. This is unusual for a protease. Even more surprising is the fact that the two cysteine residues are separated from each other by only one amino acid. Disulphide-bond formation in these circumstances is rare. Mise and Bahl [16] reported the presence of a disulphide bond in gonadotrophin that is formed between two cysteine residues separated only by a histidine residue. Some proteases, such as trypsin, are known to contain disulphide

bonds in the active-site/binding cleft [17]. Some subtilisins are known to contain a single cysteine residue in the active site which influences catalysis, such as thermitase from *Thermoactinomyces vulgaris* [18].

To determine the effect of the disulphide bond on the activity of the enzyme, the k_{cat} and K_m of the enzyme in the presence and absence of the disulphide bond (i.e. $\pm$ DTT) were determined with several different-sized substrates. It was found that, the smaller the substrate, the less the effect on K_m and/or k_{cat} . This could be due to the lower likelihood of smaller substrates interacting at the P₄ site, where the disulphide bond is located. The difference in the k_{cat}/K_m values may be dependent on whether the P₄ site is occupied by amino acids or not. The absence of the disulphide bond could cause a change in the shape of the binding cleft, opening it up and potentially making substrate binding more difficult. Thus the disulphide bond affects Ak.1 protease by influencing both K_m and k_{cat} , differing from trypsin, in which only the acylation rate is affected [17].

Thermostability

A common feature among proteases is the binding of one or more Ca²⁺ ions, resulting in an increase in thermostability [3]. This binding is at specific sites, often in external loops, which reduces the flexibility of the molecule and therefore its denaturation and/or autolysis rate. A comparison of the structures and sequences of a large number of subtilisins [3] has identified three distinct Ca²⁺-binding sites. Thermitase contains all three binding sites, while subtilisin BPN' and proteinase K contain two and one of the distinct sites respectively [3].

The three-dimensional structure of Ak.1 protease [6] shows that it contains four Ca²⁺-binding sites. Three of these sites correspond to the Ca²⁺-binding sites of thermitase [19], while the fourth site is unique among subtilisins. The thermostability of thermitase only doubles on binding of Ca²⁺ to the third, and weakest, Ca²⁺-binding site [20]. Ca²⁺ binding to the other two sites does not significantly influence thermostability. The dramatic change in the stabilization of Ak.1 protease by Ca²⁺ over the concentration range 20–200 μM compared with the effect on thermitase suggests that the fourth Ca²⁺-binding site in Ak.1 protease plays a significant role in increasing the thermostability of the protease.

The first- and second-order plots suggest that Ca²⁺ ions stabilize the protease by the prevention of thermal denaturation, rather than by having a dramatic effect on the autolysis rate. However, as unfolded or partially folded proteins tend to be more susceptible to proteolysis, a decrease in thermal denaturation could also cause a decrease in autolysis. Thus an

analysis of first- and second-order plots serves only as a guide to determine the major cause of loss of activity.

The lower degree of stabilization by Mg^{2+} , Co^{2+} and Mn^{2+} could be attributed, in part, to the fact that each of these ions has an effective ionic radius considerably less than that of Ca^{2+} . Cations such as Mn^{2+} and Cd^{2+} possess a high degree of directional specificity, as opposed to Ca^{2+} and the lanthanides [8]. Another problem with Mg^{2+} and Cd^{2+} is their preference to bind to nitrogen, rather than oxygen ligands like Ca^{2+} and the lanthanides. Cd^{2+} binds strongly to sulphur ligands. It exhibits considerable covalency in its bonds, in contrast with the essentially ionic nature of the Ca^{2+} bonds [21].

Disulphide bonds have been commonly implicated in increasing the stability of many proteins, including subtilisins such as proteinase K [19] and aqualysin [22]. Therefore it is not surprising that cleavage of this bond caused a decrease in the thermostability of Ak.1 protease.

Comparison of Ak.1 protease with other subtilisins

In general, subtilisins exhibit a broad specificity, preferring to cleave substrates containing neutral residues at the P_1 site [23]. This is consistent with the action of Ak.1 protease and thermitase, which cleave substrates containing Leu and Ala at the P_1 site. These proteases also have a high specificity for Phe at the P_1 site [24]. Subtilisins also cleaves substrates containing hydrophobic residues, such as Phe, at the P_1 site and small neutral residues, such as Val or Pro, at the P_2 site [25]. This is consistent with Ak.1 protease, which has a high specificity for a substrate containing Pro-Phe at the P_2 - P_1 sites.

Thermitase and subtilisin BPN' also cleave several alanine-based peptide substrates [25]. As with Ak.1 protease, increasing the chain length of Ala-based substrates with thermitase causes an increase in the binding affinity for that substrate. Thermitase, subtilisin BPN' and proteinase K are known to prefer substrates which occupy subsites P_3 - P_2 ' [26]. Unlike Ak.1 protease, thermitase cleaves a variety of chymotrypsin-like substrates (e.g. acetyl-Tyr-ethyl ester), though not trypsin ones (e.g. tosyl-Arg-methyl ester) [27].

Ak.1 protease and thermitase have half-lives at 80 °C of 15 h and 19 min respectively in the presence of Ca^{2+} ions, but only < 1 min and 9 min in the absence of Ca^{2+} [20]. Thus, while thermitase is intrinsically more thermostable than Ak.1 protease, the latter is stabilized to a significantly greater extent by Ca^{2+} ions. Other subtilisins have fewer Ca^{2+} -binding sites, and have significantly lower thermostability.

Many subtilisins contain disulphide bonds, such as proteinase K [3]. However, the presence of a disulphide bond in the substrate-binding cleft is unique to Ak.1 protease, based on sequence alignments of 126 known subtilisins (results not shown). Thermitase contains one cysteine residue, Cys-75, located near the active-site residues [18]. It has been shown that this residue plays a role in catalysis. Ak.1 protease does not contain a cysteine residue in this position.

Conclusions

Ak.1 protease is a thermostable subtilisin capable of cleaving protein and some peptide substrates at 75 °C in the presence of Ca^{2+} ions. It is highly active against larger substrates containing neutral or hydrophobic branched-chain amino acids at the P_1 site (e.g. Phe and Ala) and neutral amino acids at the P_2 site (e.g. Pro). Cleavage of the other substrates tested was significantly

slower. This makes the enzyme less useful in DNA clean-up, as a broad substrate specificity is required for efficient protein removal, such as with Pretaq [7].

Ak.1 protease is dramatically stabilized by Ca^{2+} ions, and to a lesser extent by other metal ions, such as Sr^{2+} and Cd^{2+} . This appears to be due, at least in part, to the presence of an extra (fourth) Ca^{2+} -binding site, compared with three Ca^{2+} -binding sites in thermitase.

The presence of a disulphide bond in the substrate-binding site of the protease appears to have a dual role. The opening of this bond causes a decrease in thermostability, and a decrease in the binding affinity of larger substrates. Thus it appears that the disulphide bond is important in maintaining the optimal structure and size of the substrate-binding site, as well as contributing to the thermostability of the enzyme.

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