Purification and characterization of the heat-stable serine proteinase from *Thermomonospora fusca* YX

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The proteinase secreted from *Thermomonospora fusca* YX grown on cellulose was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and cation-exchange chromatography. The isolated proteinase readily hydrolysed several proteins and demonstrated activity towards casein from 35 to 95 °C (at pH 8.0) with maximum activity at 80 °C. It exhibited broad pH and ionic-strength optima centred at pH 9.0 and 0.2 M-NaCl respectively, and it retained high activity in the presence of 2% (w/v) SDS, 20 mM-dithiothreitol and 1.0 M-NaCl. The proteinase, which was fully inhibited by phenylmethanesulphonyl fluoride, had an M_r of 14500 and an isoelectric point at 9.21. A measurement of proteinase thermal stability demonstrated a $T_{50\%}$ (15 min) of 85 °C at pH 4.5.

INTRODUCTION

A number of bacteria secrete alkaline heat-stable serine proteinases. These include *Thermus* sp. (Matsuzawa *et al.* [1]; Taguchi *et al.* [2]), *Thermoactinomyces vulgaris* (Kleine [3]), *Streptomyces rectus* var. *thermoproteolyticus* (Mizusawa & Yoshida [4]) and *Bacillus* sp. (Aunstrup *et al.* [5]), as well as the thermophilic fungus *Malbranchea pulchella* var. *sulfurea* (Gaucher & Stevenson [6]). Several other species of *Bacillus* produce thermostable neutral metalloproteinases. The most notable among this class is thermolysin from *Bacillus thermoproteolyticus* and neutral proteinase from *Bacillus subtilis* (Sidler & Zuber [7]).

Proteolytic activity has been reported in two strains of the actinomycete *Thermomonospora fusca*. This saprophyte is isolated from thermogenic composts, where it metabolizes primarily plant sugars and polymeric carbohydrates (Crawford [8]). It secretes a set of active cellulases (Crawford & McCoy [9]; Hagerdal *et al.* [10]; Moreira *et al.* [11]; Ferchak & Pye [12,13]; Calza *et al.* [14]) under these conditions, but is unable to metabolize proteinaceous plant materials (Crawford [8]). Weiss *et al.* [15] partially characterized a cysteine peptidase from *Thermomonospora fusca* A.T.C.C. 27730. This enzyme was probably the leucine aminopeptidase observed by Hollick [16], who screened several strains of *Thermomonospora fusca* for proteolytic activity. Desai & Dhala [17] isolated an alkaline heat-stable proteinase from *Thermomonospora fusca* strain A29 that was sensitive to thiol-specific reagents.

Thermomonospora fusca YX secretes a heat-stable proteinase in maximum quantities when grown in culture media with Solka-floc cellulose as the principal carbon source (T. W. Gusek, D. B. Wilson & J. E. Kinsella, unpublished work). The objective of the present research was to isolate the proteinase produced by *Thermomonospora fusca* strain YX and characterize some of its physical and chemical properties.

MATERIALS AND METHODS

Inoculation and incubation

Pure cultures of *Thermomonospora fusca* YX were maintained on Hagerdal *et al.* [13] minimal salts medium

and strips of Whatman cellulose filter paper. The cultures were used to inoculate shake flasks containing 100 ml of media and 0.5 g of Solka-floc, a powdered form of purified cellulose obtained from Brown Co. (Berlin, NH, U.S.A.). The flasks were placed in a closed G24 shaker (New Brunswick Scientific Co.) maintained at 55 °C with a rotational speed of 150 rev./min. After 18 h, 200 ml from these shake cultures was transferred to a sterile 10-litre suspension of Solka-floc in the salt solution and the vessel (14 litres) was attached to a Microferm MF-114 Fermentor (New Brunswick). A constant 55 °C reactor temperature was maintained throughout the incubation period of 43 h. Sterilized air was bubbled in from the bottom at the rate of 2 litres/min, and an impeller shaft rotating at 300 rev./min facilitated good mixing and aeration.

Assay for proteolytic activity

During incubation, samples of filtered medium were assayed for proteolytic activity by using a modified procedure of the Reimerdes & Klostermeyer [19] caseinolytic assay. A 0.27% solution of sodium caseinate (Sigma Chemical Co.) dissolved in 0.13 M-Tris/HCl buffer, pH 8.0, plus the enzyme sample were incubated at 65 °C for 20 min. An equivalent volume of 5% (w/v) trichloroacetic acid was then added to quench the reaction, and the clarified supernatant was analysed spectrophotometrically at 280 nm to quantify acid-soluble casein-hydrolysis products. The units for specific activity were expressed as mg of casein hydrolysed/min per mg of proteinase (mg of proteinase based on the Lowry *et al.* method for protein determination [20] with bovine serum albumin as the standard protein).

Purification of proteinase from the culture supernatant

The proteolytic activity approached a maximum value after 43 h and the contents of the fermenter were then separated with a Sharples continuous-flow centrifuge, and the clear proteinase-containing supernatant (9 litres) was cooled to 2 °C and adjusted to 45% saturation with analytical-grade $(\text{NH}_4)_2\text{SO}_4$. The precipitated proteinase along with other proteins were collected as a pellet by centrifugation at 6100 *g* for 15 min.

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The redissolved proteinase pellet was desalted on Sephadex G-25 (fine grade) (Pharmacia) equilibrated in 0.05 M-Tris/HCl buffer, pH 8.0, and the salt-free fractions were next applied to a 2.5 cm × 60 cm column packed with CM-Sephadex C-50 resin (Pharmacia) equilibrated in the same buffer. The proteins retained were eluted at a flow rate of 33 ml/h with a buffered 0–0.5 M-NaCl continuous gradient. The major peak containing proteolytic activity was pooled and freeze-dried. The material was redissolved in water and desalted on Sephadex G-15 (Pharmacia). The purified proteinase solution was frozen dropwise in liquid N₂ and stored at –70 °C. All of the chromatographic operations were conducted at 6 °C.

Determination of temperature and pH optima

The caseinolytic assay was used to analyse enzyme activity over the temperature range 25–95 °C at pH 8.0. Measurements of pH optimum were made with a Radiometer pH-stat (London Corp., Cleveland, OH, U.S.A.). The operation of this instrument was described previously by Rothenbuhler & Kinsella [21]. All reactions were conducted at 55 °C in unbuffered 10.0 ml solutions at 0.3% bovine serum albumin (Sigma). The desired pH was maintained by alkali titration.

Effect of ionic strength, dithiothreitol or SDS

The caseinolytic assay was used to determine proteolytic activity in NaCl solutions (0–1.1 M) or in solutions of 0, 1.0 mM- or 20 mM-dithiothreitol at 65 °C and pH 8.0.

The pH-stat was employed to measure proteolytic activity in 0.3% bovine serum albumin solutions containing 0%, 0.2% or 2.0% (w/v) SDS. The reactions were conducted at 55 °C, pH 9.0 and 0.2 M-NaCl.

Thermostability

Pure proteinase preparations were heated in 0.2 M-NaCl (either 0.05 M-acetate-buffered, pH 4.5, or 0.05 M-Tris/HCl-buffered, pH 8.0) for 15 min at temperatures from 25 to 95 °C. Residual activity was determined by using the caseinolytic assay at 65 °C and pH 8.0.

Determination of M_r and isoelectric point

Gel filtration. Pure preparations of the proteinase were chromatographed by h.p.l.c. on a Protein Pak 125 gel-filtration column (Waters Associates, Milford, MA, U.S.A.). The column was calibrated with purified bovine serum albumin (M_r 66000), egg albumin (M_r 43000) and chymotrypsinogen A (M_r 25000) (Pharmacia) and cytochrome *c* (M_r 12400) and aprotinin (M_r 6500) (Sigma Chemical Co.). The eluting buffer was 0.1 M-KCl/0.1 M-potassium phosphate buffer, pH 6.8. A Hewlett-Packard 3390A integrator monitored peak area and retention time.

Electrophoresis. SDS/polyacrylamide gel electrophoresis was performed as described by Weber & Osborn [22] in a vertical slab gel plate (12%) with the Protean II (Bio-Rad Laboratories, Richmond, CA, U.S.A.) electrophoresis system. Purified proteinase samples were first inactivated with 1.0 mM-phenylmethanesulphonyl fluoride to prevent autolysis (Weber & Osborn [23]). Low- M_r (2300–43000) protein standards (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) were used to approximate the size of the proteinase. Silver

Fig. 1. Elution of the proteinase from *Thermomonospora fusca* YX on CM-Sephadex with an NaCl gradient

Elution buffer was 0.05 M-Tris/HCl buffer, pH 8.0, containing a 0–0.5 M-NaCl continuous gradient. The fraction volume was 6.0 ml. —, Protein detection (A_{280}); ---, conductivity of eluate.

staining was performed with a kit from Bio-Rad Laboratories.

Isoelectric focusing. This was performed with the electrofocusing gel-electrophoresis system from Separation Sciences (Attleboro, MA, U.S.A.). The precast gels encompassed the pH range 3.5–9.5. A mixture of ten standard proteins of pI 3.6–10.2 (FMC Corp., Rockland, ME, U.S.A.) and the pure proteinase preparations were electrofocused and then stained with Coomassie Brilliant Blue R.

RESULTS

Proteinase purification

Most of the proteins present in the desalted crude protein pool were anionic at pH 8.0 and did not bind to the cation-exchanger resin. Two proteins did bind, and each was eluted as a distinct peak when the NaCl gradient was applied (Fig. 1). Both peaks contained high levels of proteolytic activity. The specific activity and M_r of the proteinase constituting the minor peak were very similar to those of the proteinase in the major peak. The difference in their relative affinity for the cationic resin may have resulted from deamidation of one or more basic amino acids to form a second population of closely related molecules. A 39-fold purification was achieved for the proteinase with a final yield of 17% (19% if the minor proteinase was included) (Table 1).

The proteinase preparation exhibited high purity by h.p.l.c., isoelectric focusing and SDS/polyacrylamide-gel electrophoresis (Figs. 2 and 3). Two faint bands corresponding to lower- M_r species in the silver-stained SDS/polyacrylamide gel probably resulted from proteinase breakdown during sample preparation for electrophoresis. These additional protein bands were

Table 1. Summary of purification of proteinase from *Thermomonospora fusca* YX

For experimental details see the text. Overall purification of the proteinase was 39-fold. Specific activity is expressed as mg of casein hydrolysed/min per mg of protein.

Purification step	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
1. Cell-free supernatant	8860	11 550	1.70	100
2. Redissolved 0–45% -satn.-(NH ₄) ₂ SO ₄ pellet	122	6040	2.20	52
3. Sephadex G-25 (fine grade)	600	2250	1.12	19.5
4. CM-Sephadex C-50	102	1980	66.0	17.1
5. Sephadex G-15	30	1980	66.0	17.1

absent from the h.p.l.c. chromatogram and the electrofocusing gel.

Temperature optimum

Although little proteolytic activity was observed at or below 25 °C, the level of activity doubled for each 10 °C temperature increment from 35 to 75 °C and reached a maximum at 80 °C (Fig. 4). Activity fell off sharply above 85 °C, with 37% of the maximum value at 90 °C and 10% at 95 °C. The enzyme was completely inactivated in less than 5 min at 100 °C.

pH optimum

Proteolytic activity occurred throughout the alkaline pH range 8.0–11.0 (the highest pH tested), with the maximum rate around pH 9.0 (Fig. 5). An assay involving bovine serum albumin hydrolysis in buffered solutions at 65 °C (and measurement of the acid-soluble proteolysis fragments) indicated that the minimum pH for activity was between 6.0 and 7.0. The pH-stat method did not detect any activity at pH 7.0, conceivably because peptide-bond hydrolysis produced both protons and free terminal amino groups, which participated in acid–base equilibria since the reaction pH was near the

pK of the α -amino group. The average pK of the α -amino group of di-, tri- and oligo-peptides produced by protein hydrolysis is estimated to be around 7.6 (Mihalyi [24]) or 7.8 at 25 °C (Steinhardt & Beychok [25]). This value is temperature-dependent and was calculated to be 7.13 at 55 °C by using the van't Hoff equation:

$$\ln(k_2/k_1) = (\Delta H^\circ/R)(T_2 - T_1)/T_1 T_2$$

where $k_1 = 7.8$, $\Delta H^\circ = 41.84$ kJ/mol (10 kcal/mol), $T_1 = 298.15$ K, $T_2 = 328.15$ K and $R = 8.309$ J·mol⁻¹·K⁻¹ (1.986 cal·mol⁻¹·K⁻¹). At an unbuffered reaction pH of 7.0 (55 °C) more than 50% of the protons released during peptide-bond hydrolysis are associated with a terminal amino group, which decreases the extent of pH fall (and thus alkali consumption) in the pH-stat system. Hence the actual rate of proteolysis is underestimated. This may account for the non-detection of proteolytic activity at pH 7.0 in the pH-stat system. At higher pH values the final corrected rates of activity were obtained by dividing

Fig. 2. H.p.l.c. gel-filtration chromatogram of purified proteinase from *Thermomonospora fusca* YX

H.p.l.c. was on a Protein Pak 125 gel-filtration column with a protein fractionation range of M_r 2000–80000. Elution buffer was 0.1 M-KCl/0.1 M-potassium phosphate buffer, pH 6.8. The flow rate was 0.4 ml/min.

Fig. 3. SDS/polyacrylamide-gel electrophoresis (a) and isoelectric focusing (b) of the purified proteinase from *Thermomonospora fusca* YX

The SDS/polyacrylamide gel was stained with silver and the isoelectric-focusing gel with Coomassie Brilliant Blue R.

Fig. 4. Activity of the proteinase from *Thermomonospora fusca* YX as a function of temperature

Specific activity is expressed as mg of casein hydrolysed/min per mg of enzyme at the specific assay temperature at pH 8.0.

the observed rate by the fraction of unprotonated α -amino groups. (The fraction approaches unity with increasing pH.)

Effect of ionic strength, dithiothreitol, SDS or phenylmethanesulphonyl fluoride

The proteinase retained high activity in concentrated solutions of NaCl, dithiothreitol or SDS (Figs. 6 and 7). In the presence of 2% (w/v) SDS or 1.1 M-NaCl enzymic activity was decreased by 25 or 40% respectively. The activity of the proteinase was unaltered in 20 mM-dithiothreitol.

Complete inhibition of the proteinase was achieved with the serine-specific reagent phenylmethanesulphonyl fluoride at concentrations of 0.1–1.0 mM. This reagent irreversibly inhibits serine proteinases by forming a sulphonylserine group at the active site (Gold [26]; James [27]), which is analogous to the reaction of organophosphate esters, such as di-isopropyl phosphorofluoridate, with the serine proteinases trypsin and chymotrypsin.

M_r and isoelectric point

The major proteinase that was isolated by using CM-Sephadex ion-exchange chromatography had a retention time on h.p.l.c. that corresponded to an M_r of 9460, based upon the regression equation for the retention times of five standard proteins ($r^2 = 92\%$). The same enzyme exhibited an M_r of 14500 by SDS/polyacrylamide-gel electrophoresis and linear-regression analysis of the five standard proteins ($r^2 = 94\%$).

The isoelectric points of the proteinases comprising the major and minor peaks were 9.21 and 8.44 respectively (based upon the regression equation for eight proteins of known pI with $r^2 = 99\%$). These values verified the

Fig. 5. Activity of the proteinase from *Thermomonospora fusca* YX as a function of pH

Specific activity is expressed as μmol of OH^- consumed/min per mg of enzyme at 55°C in the pH-stat system with bovine serum albumin as substrate.

Fig. 6. Activity of the proteinase from *Thermomonospora fusca* YX as a function of ionic strength

Specific activity is expressed as mg of casein hydrolysed/min per mg of enzyme at 65°C at pH 8.0.

cationic behaviour of each enzyme on the cation-exchanger resin at pH 8.0, and the difference in affinities that the two enzymes demonstrated for the resin. The difference of 0.77 pH unit between the pI of each proteinase also accounted for the narrow salt gradient that was sufficient to elute both enzymes.

Fig. 7. Effect of SDS or dithiothreitol on activity of the proteinase from *Thermomonospora T. fusca* YX

Specific activity is expressed as (a) μmol of OH^- consumed/min per mg of enzyme at 55°C at pH 9.0 in the pH-stat system with bovine serum albumin as substrate or (b) mg of casein hydrolysed/min per mg of enzyme at 65°C at pH 8.0.

Assessment of proteinase thermostability

The thermal-stability profile of the proteinase is shown in Fig. 8. The heat treatments were conducted at a lower pH (4.5) to prevent autolysis. (The use of a reversible inactivating agent was avoided to exclude the possibility that it might confer additional stability to the proteinase.) At pH 4.5 the proteinase was completely stable up to 75°C and exhibited only 12% inactivation at 80°C . A 46% decrease in activity was recorded at 85°C , and 100% loss at 90°C . The same experiment was also conducted at pH 8.0 (results not shown) and produced a similar curve for heat treatments up to 70°C . Above 70°C the magnitude of enzyme inactivation was 2-fold greater at each temperature tested than at pH 4.5. This difference was possibly caused by autolysis at the higher pH.

DISCUSSION

The proteinase from *Thermomonospora fusca* YX demonstrated several properties that distinguish it from previously isolated heat-stable bacterial proteinases (Table 2). These include its temperature optimum (80°C), pH optimum (9.0) and low M_r (14500). The strain YX enzyme described here is a serine proteinase, whereas the proteinase isolated from *Thermomonospora fusca* strain A29 by Desai & Dhala [17] apparently

Fig. 8. Thermostability profile of the proteinase from *Thermomonospora fusca* YX at pH 4.5

Samples were heated for 15 min at the appropriate temperature and then assayed for residual activity. Specific activity is expressed as mg of casein hydrolysed/min per mg of enzyme at 65°C at pH 8.0.

belonged to the cysteine proteinase class because it was sensitive to oxidizing agents and *p*-chloromercuribenzoate. The isoelectric point of the *Thermomonospora fusca* YX proteinase (9.21) was close to that of thermitase from *Thermoactinomyces vulgaris* (9.1) and subtilisin carlsberg from *Bacillus licheniformis* (9.4). The proteinase from *Thermomonospora fusca* YX was much more resistant to heat denaturation than thermitase, which was rapidly inactivated at 55°C at both pH 4.5 and 8.0 (Klein [3]). The strain YX proteinase demonstrated heat-stability up to 80°C at both pH values.

The existence of two closely related *Thermomonospora fusca* YX proteinases differing only in their isoelectric points may result from limited deamidation of the native enzyme, which can occur during protein purification (Robinson *et al.* [32]). Two highly homologous proteinases from *Streptomyces* sp. with similar specific activities, M_r values and amino acid composition were purified by Mizusawa & Yoshida [4]. The only difference was that proteinase B contained one additional amide group per molecule, suggesting deamidation of an asparagine or glutamine residue of proteinase B (which had a higher isoelectric point of 9.50) to give rise to proteinase A (which had a pI of 9.35). Ahern & Klivanov [33] observed a progressive decrease in the isoelectric point of lysozyme depending upon the number of amide groups that had been deamidated.

The M_r value of the *Thermomonospora fusca* YX proteinase is more accurate for the denatured rather than for the native form. In SDS/polyacrylamide-gel electrophoresis, where proteins are reduced and unfolded, the relative rate of migration is determined by the size of the random-coil form and not by differences in native tertiary configuration, whereas h.p.l.c. gel filtration

Table 2. Comparison of the properties of the proteinase from *Thermomonospora fusca* YX with those of other thermostable bacterial proteinases

Enzyme (source)	Class	M_r	Optimum pH	Optimum temp. (°C)	Reference
Aqualysin I (<i>Thermus aquaticus</i> YT-1)	Serine	28 500	Alkaline	70–80	[1]
Aqualysin II (<i>Thermus aquaticus</i> YT-1)	Serine	?	Neutral	95	[1]
Proteinase (<i>Thermus caldophilus</i> GK24)	Serine	31 000	7.8	90	[2]
Proteinase B (<i>Streptomyces rectus</i> var. <i>thermoproteolyticus</i>)	Serine	21 500	10.7	80	[4,28]
Subtilisin carlsberg (<i>Bacillus licheniformis</i>)	Serine	27 300	9.0	60	[5,29]
Thermitase (<i>Thermoactinomyces vulgaris</i>)	Serine	28 400	8.5	85	[3,30]
Thermolysin (<i>Bacillus thermoproteolyticus</i>)	Metal	34 500	7.2	80	[7,31]
Proteinase (<i>Thermomonospora fusca</i> A29)	Cysteine?	21 500	8.5	65	[17]
Proteinase (<i>Thermomonospora fusca</i> YX)	Serine	14 500	9.0	80	Present work

separates proteins on the basis of size and configuration. The thermostable proteinase from *Thermomonospora fusca* YX may possess a much tighter globular structure than the mesophilic proteins that were used to calibrate the h.p.l.c. column. This could explain why the proteinase was eluted as a smaller protein (M_r 9460) than cytochrome *c* (12400) in h.p.l.c. gel filtration, but migrated as a larger protein (14500) than cytochrome *c* in electrophoresis. [A preliminary M_r value of 13400 (minus the contribution from tryptophan) was calculated for the proteinase from the amino acid analysis.] A compact globular conformation for the proteinase is a likely structural arrangement, since it would enable dense packing of hydrophobic residues at the core of the molecule, as well as exclude water from the interior. Both of these factors have been implicated in protein thermostabilization (Wright *et al.* [34]; Walker *et al.* [35].

The molecular basis of the thermostability demonstrated by the proteinase has not yet been determined. Surface ion-pairing does not appear to constitute a major stabilizing force, since the enzyme tolerates high concentrations of NaCl without significant decreases in activity at high temperatures, and it exhibits similar inactivation-temperature profiles at both acidic and alkaline pH. Either of these factors could affect the charged residues comprising a salt bridge and hence diminish thermostability (Rügg *et al.* [36]). It also does not appear that disulphide bridges have an important role in maintaining the structure of the proteinase at elevated temperatures, since high concentrations of dithiothreitol did not adversely affect enzymic activity.

The fact that hydrophobic association increases with increasing temperature suggests that these interactions constitute a major force in protein thermostability (Ben-Naim [37]; Ikai [38]; Merkler *et al.* [39]). The thermal-stability profile for thermolysin is very similar to that for the enzyme from *Thermomonospora fusca* YX, as both proteinases undergo 50% inactivation during heat treatment at 84–85 °C for 15 min (Pangburn *et al.* [40]). These investigators suggested that, in addition to Ca^{2+}

binding, the hydrophobic nature of thermolysin promotes a more thermostable protein. Such a mechanism may contribute to the heat-stability of the *Thermomonospora fusca* YX proteinase.

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