Purification and partial characterization of a thiol proteinase from the thermophilic fungus *Humicola lanuginosa*

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An extracellular thiol proteinase was produced by the growth of a thermophilic fungus, *Humicola lanuginosa*, on a medium containing 2% casein, and was purified to virtual homogeneity by affinity chromatography on organomercurial columns. The essential thiol group for activity was confirmed by the inhibition of the enzyme by *p*-chloromercuribenzoate and mercuric ions. The enzyme, purified 27-fold from the extracellular fluid, exhibited an M_r of 23 700 on gel filtration and sedimentation equilibrium. The *H. lanuginosa* proteinase preferentially cleaves at the C-terminal end of hydrophobic amino acid residues. This proteinase differed from the plant enzyme papain in its interaction with three affinity matrices and its substrate specificity towards synthetic substrates. This enzyme represents a unique example of a thiol proteinase obtained from a fungal source.

Hartley (1960) classified proteinases into three groups: (1) alkaline serine proteinases, (2) neutral metalloproteinases, and (3) thiol proteinases. The first two categories, characterized by their limited specificity for the amino acid esters hydrolysed, include most animal proteinases. The cathepsins, which are less well characterized, do include some thiol proteinases (Mycek, 1970). Most plant proteinases, e.g. papain (Lowe, 1976), ficin (Liener & Friedenson, 1970) and bromelain (Murachi, 1970) are characterized by a free thiol group essential for their activity (Glazer & Smith, 1971), and display broader specificity in the peptide bonds cleaved than the animal proteinases.

Very few thiol microbial proteinases have been identified (Matsubara & Feder, 1971). Only clostripain from *Clostridium histolyticum* (Mitchell & Harrington, 1971), streptococcal proteinase (Liu &

Elliott, 1971) and *Staphylococcus aureus* proteinase II (Arvidson *et al.*, 1973) have been clearly characterized. Ong & Gaucher (1973) first noted the production of an extracellular thiol proteinase by *Humicola lanuginosa* during their study of the production of proteinases by thermophilic fungi. The inactivation of this enzyme by *p*-chloromercuribenzoate under alkaline conditions suggested a thiol proteinase. The extracellular thiol proteinase produced by the fungus *Humicola lanuginosa* (Ong & Gaucher, 1976) appears to be unique as the only thiol proteinase produced by fungi.

This paper describes the purification and partial characterization of the thiol proteinase produced extracellularly by the thermophilic fungus *Humicola lanuginosa*.

Experimental

Materials

Yeast extract (Difco) was obtained from Fisher Scientific Co., Pittsburgh, PA, U.S.A. Casein was obtained from BDH, Poole, Dorset, U.K. Sepharose 4B, Sephadex G-150, and Blue Dextran 2000 were purchased from Pharmacia, Montreal, Canada. Papain was obtained from Worthington Biochemical Corp. (Winley-Morris), Montreal, Canada. Hexanediamine, *p*-aminophenylmercuric acetate, *p*-ClHgBzO⁻ and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate were obtained from Pierce Chemical Co.,

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Abbreviations used: *p*-ClHgBzO⁻, *p*-chloromercuribenzoate; HgPhNH-Sephrose, *p*-mercuriphenylamine-Sephrose; HgPhCONH[CH₂]₆NH-Sephrose, *p*-chloromercuribenzoate immobilized on aminohexyl-Sephrose; HgPhNHCO[CH₂]₃NH-Sephrose, *p*-aminophenylmercuric acetate immobilized on aminohexyl-Sephrose; Cbz-Gly-ONp, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester; PhCH₂SO₂F, phenylmethanesulphonyl fluoride; NbS₂, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent).

Rockford, Illinois, U.S.A. *N*-Benzoyl-L-citrulline ethyl ester was purchased from Nutritional Biochemical Corp., Cleveland, OH, U.S.A. Cbz-Gly-ONp and other benzyloxycarbonyl-amino acid nitrophenyl esters, $\text{PhCH}_2\text{SO}_2\text{F}$ and Nbs_2 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 4-Phenylbutylamine was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Methods

Growth and maintenance. The thermophilic fungus *Humicola lanuginosa* UAMH 1623 was obtained from Dr. R. Emerson, Department of Biology, University of California, Berkeley, CA, U.S.A. (Cooney & Emerson, 1964).

The organism was grown for 14 days on slants of yeast extract (0.05%, w/v)/glucose (1%, w/v)/agar (2%, w/v) at 45°C, and the spores were suspended in sterile aerosol solution. Several drops of the spore suspension were added to filter-paper strips in sterile vials and freeze-dried. The vials were sealed under vacuum and stored at -70°C. Every 6 months a vial was opened and the paper strip containing freeze-dried spores was inoculated. The cells were then transferred to six casein (1%, w/v)/agar (2%, w/v) slants and further incubated for 14 days at 45°C. These casein/agar slants could be stored at 4°C for several months.

Six yeast extract/glucose/agar slants were inoculated with cells from a casein/agar slant and incubated for 14 days at 45°C in 2-litre Erlenmeyer flasks. The spores from two yeast-extract/glucose/agar slants were resuspended in sterile buffered saline (Difco) solution and inoculated into 400 ml of medium. The medium consisted of NaNO_3 (0.8 g), KH_2PO_4 (0.4 g), KCl (0.4 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (4 mg), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (120 µg), casein (4 g), glucose (0.4 g) and yeast extract (1.2 g) in a total volume of 400 ml at pH 7.4. Flasks were shaken in a New Brunswick orbital shaker at 300 rev./min for 30 h at 45°C.

Contents of two flasks (800 ml) were used to inoculate a 14-litre New Brunswick Microferm fermentor containing 8 litres of medium comprised of NaCl (16 g), KH_2PO_4 (8 g), KCl (8 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.08 g), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2.4 mg) casein (80 g), glucose (8 g) and yeast extract (0.8 g). The fermentor growth was carried out at 45°C with an air inflow of 1.5 litres/min for 36 h.

The culture filtrate of the *Humicola lanuginosa* growth was concentrated with an Amicon hollow-fibre concentrator with a PM10 cartridge. The casein, which precipitates out during concentration at 4°C, was filtered, and the concentration was continued to obtain approx. 1/20 of the original volume. The concentrate was dialysed at 4°C overnight against 10 mM-sodium phosphate adjusted

to pH 8.0 with NaOH solution. This solution could be stored for several months at 4°C with negligible loss of enzyme activity.

Preparation of affinity columns. (i) HgPhNH-Sepharese . This was prepared as described by Sluyterman & Wijdenes (1970).

(ii) $\text{HgPhCONH}[\text{CH}_2]_6\text{NH-Sepharese}$. Sepharese (50 ml), in 0.2 M- NaHCO_3 , activated with CNBr (March *et al.*, 1974), was coupled to hexanediamine (3.89 g) in 5 ml of distilled water at 4°C for 20 h. The gel was then washed with 20 vol. each of 0.1 M-sodium acetate adjusted to pH 4.0 with acetic acid, 2 M-urea, and 0.1 M- NaHCO_3 . All washing solutions contained 0.5 M- NaCl .

The gel (25 ml) was finally washed with 0.1 M-sodium acetate buffer, pH 4.0, and incubated with 0.1 M-1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate and *p*- ClHgBzO^- (1 g) in 0.1 M-sodium acetate buffer, pH 4.0, for 20 h at room temperature (Ishikawa & Iwai, 1975). The gel was then washed with 0.1 M- NaHCO_3 , 0.1 M-sodium acetate buffer, pH 4.0, 5 M-urea, and finally 0.1 M-sodium phosphate adjusted to pH 8.0 with NaOH.

The remainder of the aminohexyl-Sepharese (25 ml) was coupled to acetic acid in the presence of 0.1 M-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate at pH 4.75 and room temperature for 16 h to produce *N*-acetylhexylamine-Sepharese.

(iii) $\text{HgPhNHCO}(\text{CH}_2)_3\text{NH-Sepharese}$. CNBr -activated Sepharese (25 ml) in 0.2 M- NaHCO_3 was coupled to ϵ -aminohexanoic acid in 5 ml of distilled water and incubated for 20 h at 4°C with stirring. The gel was sequentially washed with 0.1 M-sodium acetate buffer (pH 4.0)/2 M-urea/0.1 M- NaHCO_3 as described above. The coupling of *p*-aminophenyl acetate (0.325 g) to hexanoylamine-Sepharese was carried out in the presence of 0.1 M-1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate in 0.1 M-sodium acetate buffer, pH 4.0, for 20 h at room temperature with constant stirring.

Proteinase assay. The assay of Ong & Gaucher (1973) was used with casein as substrate in 50 mM-sodium phosphate, adjusted to pH 8.0 with NaOH solution, containing 0.25 M- NaCl .

The convenient spectrophotometric assay utilizing the production of *p*-nitrophenol by the hydrolysis of Cbz-Gly-ONp substrate was also used. A portion (2.8 ml) of the enzyme diluted in 50 mM-sodium phosphate buffer, pH 8.0, and a buffer blank (2.8 ml) were placed in the sample and reference cuvettes of a thermostatically controlled cell compartment of a Beckman DB-G recording spectrophotometer at 30°C. The enzyme reaction was started by the addition of 0.2 ml portions of 1.5 mM-Cbz-Gly-ONp in acetonitrile to both

cuvettes with rapid stirring. A standard unit, defined as the enzyme required to yield $1\text{ }\mu\text{mol}$ of *p*-nitrophenol/min, was calculated based on an ϵ_{400} for the *p*-nitrophenol anion of $18\,300\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 25°C (Kirsch & Ingelström, 1966).

Protein determination. The protein concentration was determined by A_{280} . The concentration of the thiol proteinase was calculated using an $A_{280}^{1\%}$ value for the purified mercuri-enzyme (dialysed and freeze-dried) of 27.0 ± 3 .

Determination of molecular weights. (a) Gel filtration. The molecular weight was determined as described by Andrews (1964) by gel chromatography on Sephadex G-150. The void volume of the column ($2.5\text{ cm} \times 40\text{ cm}$) was determined with Blue Dextran 2000. Standard proteins [ribonuclease, α -chymotrypsinogen, aldolase, and γ -globulin (Pharmacia calibration kit)] were used to calibrate the column.

(b) Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The molecular weight of the proteinase was estimated by electrophoresis on 10% (w/v) polyacrylamide gels (Weber & Osborn, 1969) in the presence of sodium dodecyl sulphate. The proteins ribonuclease, α -chymotrypsinogen, pepsin, aldolase, and bovine serum albumin were used as markers. All proteins were boiled for 10 min in 1% (w/v) sodium dodecyl sulphate in 10 mM-sodium phosphate adjusted to pH 7.0 with NaOH solution, then dialysed overnight against 0.1% (w/v) sodium dodecyl sulphate and 0.1% (w/v) β -mercaptoethanol in 10 mM-sodium phosphate buffer, pH 7.0, before electrophoresis. The gels were stained with 0.05% (w/v) Coomassie Blue in 7.5% (v/v) acetic acid containing 5% (v/v) propanol.

(c) Sedimentation equilibrium. A sample of mercuri-proteinase ($A_{280} = 0.6$), dialysed against 0.1 M-sodium phosphate buffer, pH 7.0, was centrifuged overnight at $20\,000\text{ rev./min}$ ($r_{\text{av.}} 7.32\text{ cm}$) with the diffusate in the counter cell in a Beckman model E analytical ultracentrifuge. The interference pattern produced was photographed on Kodak type II-6 plates and viewed with a Nikon profile projector (model 6C). The molecular weight was calculated as described by Yphantis (1964), assuming a partial specific volume of 0.73 g/ml .

Results

Purification

The stored concentrate of the culture filtrate was chromatographed on HgPhNH-Sephrose equilibrated and eluted with 50 mM-sodium acetate adjusted to pH 5.0 with acetic acid. Although a considerable amount of pigment bound to the column, the proteinase was selectively eluted with

Fig. 1. Selective isolation of the thiol proteinase from the concentrated filtrate of *Humicola lanuginosa* by affinity chromatography on HgPhNH-Sephrose

The sample (15 ml) of the concentrated culture filtrate was fractionated on a HgPhNH-Sephrose column ($0.9\text{ cm} \times 10\text{ cm}$) and eluted with 50 mM-sodium acetate buffer, pH 5.0, at 25°C . The flow rate was 30 ml/h , with 3.25 ml fractions being collected. The arrow (↓) indicates the stage at which the elution with 50 mM-sodium acetate buffer containing 2 mM-HgCl_2 was commenced.

50 mM-sodium acetate buffer, pH 5.0, containing 2 mM-HgCl_2 (Fig. 1). A 27-fold purification was obtained by affinity chromatography on HgPhNH-Sephrose (Table 1), which was preferred to the other two Sephrose-mercurials tested.

The introduction of spacer arms (hexanediamine and ϵ -aminohexanoic acid) appeared to reduce the capacity of the mercurial-Sephrose columns to bind the *Humicola* proteinase or papain. The Sephrose-mercurial ligands had greater capacity to bind the fungal proteinase than papain (Table 2).

The three organomercurial-Sephrose columns had different capacities towards the *Humicola* thiol proteinase, but the resulting enzyme preparations had essentially the same specific activity (Table 3). In order to check the selectivity of the binding of the *Humicola* proteinase, the binding of the enzyme to Sephrose-4B, 2-aminoethanol-Sephrose (Stewart & Stevenson, 1973), *N*-acetylhexyl-1-amine-Sephrose, and 4-phenylbutylamine-Sephrose (Stevenson & Landman, 1971) under similar conditions to those used for the mercurial columns, was investigated. The proteinase was unretarded by all these columns, indicating an affinity of the enzyme for organomercurial-Sephrose.

Table 1. *Purification of Humicola thiol proteinase*
Specific activity was expressed as units of proteinase activity/ml divided by A_{280}

	Total activity (units)	Activity (units/ml)	A_{280}	Specific activity (units/ A_{280})	Yield (%)	Purification (fold)
Culture filtrate	63 365	4.37	1.3	3.36	100	1
Concentrate (PM10 membrane)	55 653	48.69	8.2	5.94	87.8	1.8
HgPhNH-Sephadex	49 944	28.57	0.31	92.16	78.3	27.4

Table 2. *Capacities of the organomercurial-Sephadex synthesized*

The capacity for Nbs₂ was estimated by the method of Sluyterman & Wijdenes (1974). Similarly, the capacity for papain was calculated by using $A_{280}^{1\%} = 25.0$ and an M_r of 23 500. The capacity for the *Humicola* thiol proteinase was calculated by using $A_{280}^{1\%} = 27.0 \pm 3.0$ and an M_r of 23 700.

	Capacity (mm)		
	Nbs ₂	Papain	Thiol proteinase
HgPhNH-Sephadex	1.54	0.52	1.97
HgPhNHCO(CH ₂) ₅ NH-Sephadex	0.71	0.077	1.09
HgPhCONH(CH ₂) ₆ NH-Sephadex	0.68	0.016	0.97

Gel filtration

The enzyme was fractionated from the concentrated culture filtrate by re-chromatography on a column (2.5 cm × 40 cm) of Sephadex G-150 to obtain the specific activities achieved by affinity chromatography. Affinity chromatography retains the advantages of ease and rapidity of separation and the concentration of the thiol proteinase in the inhibited form.

Enzyme purity

Disc-gel electrophoresis (Ornstein, 1964) with 7.5% (w/v) polyacrylamide gels at pH 9.5 indicated several minor protein contaminants, which varied during each sample preparation. The mercuri-enzyme, re-activated with 5 mM-cysteine and 1 mM-EDTA, autolysed rapidly under the conditions of the sample preparation and electrophoresis in the Tris/glycine system of Davis (1964). The mercuri-proteinase (100 g) from the elution of the enzyme from HgPhNH-Sephadex gave a faint single band on 10% (w/v) polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Thus re-activation and subsequent auto-

lysis of the enzyme remained a problem during the sample preparation.

Better criteria of enzyme purity were provided by gel filtration of the mercuri-proteinase on Sephadex G-150. A single uniform peak of constant specific activity, comparable with that of the applied enzyme, was obtained. The observation of a minor peak of low-molecular-weight material was eliminated by elution with 50 mM-sodium acetate buffer, pH 5.0, containing 2 mM-HgCl₂.

Molecular weight

The mercuri-proteinase was observed as a major protein band (R_F 0.57 with respect to Bromophenol Blue of M_r 23 700 by 10%-polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The relative molecular mass of mercuri-proteinase (K_{av} 0.56) as determined by Sephadex G-150 chromatography was 23 400. The sedimentation analysis of the enzyme at pH 7.0 showed linear plots of the log (fringe displacement) versus r^2 . An upward curvature at the bottom of the cell was observed and perhaps indicates the aggregation of the proteinase. The relative molecular size of the observed aggregates varied according to the speed of centrifugation. At 20 000 rev./min (r_{av} 7.32 cm) an M_r of 96 200 was found, indicating the possible formation of a tetramer of 24 050- M_r subunits.

Inhibitors

The enzyme was inhibited by p -ClHgBzO⁻ and by Hg²⁺, indicating the importance of a thiol group for activity. p -ClHgBzO⁻ at a molar ratio of 40:1 at 37°C inhibited the proteinase in less than 10 min. The extent of inhibition by p -ClHgBzO⁻ under these conditions was slightly dependent on the pH. The percentage of inhibition was greater at pH 5.0 (97%) than at pH 7.0 (87%) or at pH 8.0 (83%) with 0.2 M- p -ClHgBzO⁻ at 37°C in 10 min. In contrast, Hg²⁺ at a molar ratio of 200:1 completely inhibited the proteinase at pH 5.0 or at pH 8.0 in less than 10 min.

The enzyme inhibited by p -ClHgBzO⁻ could be re-activated by the addition of free thiol. L-Cysteine was a more effective re-activator (100% with

Table 3. *Purification of the Humicola thiol proteinase on three organomercurial-Sepharoses*

The stored concentrate of the culture filtrate of *Humicola lanuginosa* (20 ml) was chromatographed on affinity columns (0.9 cm × 10 cm) equilibrated and eluted with 50 mM-sodium acetate buffer, pH 5.0. The thiol proteinase was selectively eluted with 50 mM-sodium acetate buffer containing 2 mM-HgCl₂. The specific activity was expressed as units/min divided by A₂₈₀.

	Volume (ml)	A ₂₈₀	Activity (units/ml)	Total activity (units)	Specific activity (units/A ₂₈₀)
HgPhNH-Sepharese	26.0	0.31	28.57	743	92.16
HgPhNHCO(CH ₂) ₃ NH-Sepharese	18.5	0.24	22.62	418	94.25
HgPhCONH(CH ₂) ₆ NH-Sepharese	2.0	0.18	17.46	384	97.0

Table 4. *Comparison of the hydrolysis of synthetic substrates by Humicola proteinase and papain*

Purified proteinase was used to hydrolyse the synthetic substrate (0.15 mM) in 6.7% (v/v) acetonitrile in 50 mM-sodium phosphate buffer, pH 8.0, at 30°C. Relative rates of hydrolysis are compared by using Cbz-Gly-ONp as 100% in the spectrophotometric assay.

Substrate	Relative rate (%)	
	<i>Humicola</i> proteinase	Papain
Cbz-Gly-ONp	100	100
Cbz-Ala-ONp	142	140
Cbz-Cys-ONp	0	23
Cbz-Leu-ONp	18	7
Cbz-Lys-ONp	0	127
Cbz-Phe-ONp	33	0
Cbz-Tyr-ONp	76	—
Cbz-Trp-ONp	42	0

5 mM-cysteine in the presence of 1 mM-EDTA at pH 5.0) than β-mercaptoethanol (58% re-activation with 12 mM-β-mercaptoethanol). The re-activation of mercuri-papain by thiols in the presence of EDTA has been well studied (Kimmel & Smith, 1954). In this respect the *Humicola* proteinase shows all the characteristics of a thiol enzyme.

PhCH₂SO₂F and *N*-*p*-phenylazophenyl-*N*-phenyl-carbamyl chloride, potent inhibitors of serine proteinases, also inhibit this enzyme. PhCH₂SO₂F is far more commonly associated with the inhibition of serine proteinases (Lindsay & Stevenson, 1976), but has also been observed to inhibit the thiol proteinase papain (Whitaker & Perez-Villasenor, 1968).

Enzyme specificity towards synthetic substrates

By using a variety of benzyloxycarbonyl-amino acid *p*-nitrophenyl esters the *Humicola* proteinase (purified by chromatography on Sephadex G-150) demonstrated some interesting differences in substrate specificity as compared with papain (Table 4).

Two commonly used substrates for papain, benzoyl-L-citrulline ethyl ester and benzoyl-L-arginine ethyl ester were not hydrolysed by *Humicola* thiol proteinase. Thus this enzyme appears to resemble much more closely the streptococcal proteinase (Liu & Elliott, 1971), with a specificity for hydrolysis at the C-terminal side of hydrophobic-amino-acid residues.

Discussion

Humicola lanuginosa produces an extracellular thiol proteinase when grown on a high-protein medium. The enzyme is noteworthy because it is the only known thiol proteinase to be identified from a fungal source.

The enzyme was purified by an essentially one-step procedure employing affinity chromatography on organomercurial-Sepharese. Three different organomercurial-Sepharese were equally effective in the purification of the thiol proteinase; each matrix resulted in an enzyme preparation having a specific activity of 250 units/mg of protein. However, these affinity matrices differed considerably in their capacities. The problems of autolysis, and thus the criteria for enzyme purity, are shared with other proteinases [papain (Sluyterman, 1964); bromelain (Ota *et al.*, 1964); ficin (Kramer & Whitaker, 1964); α-chymotrypsin (Lindsay & Stevenson, 1976)].

The comparison of this proteinase with papain showed some differences in the binding capacity to the organomercurial columns tested, suggesting a difference in the availability of the thiol groups in these enzymes to mercurial binding. The fungal proteinase (*M_r* 23 700) demonstrated some structural similarity to thiol proteinases from plant sources [papain, *M_r* 23 000 (Drenth *et al.*, 1968); ficin, *M_r* 23 800 (Jones & Glazer, 1970)], but its substrate specificity may be more like those of the bacterial thiol proteinases. The characterization of the *Humicola* enzyme may provide a greater understanding at the molecular level of the evolutionary position of fungi relative to plants and bacteria.

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