Purification and characterization of major extracellular proteinases from *Trichophyton rubrum*

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Two extracellular proteinases that probably play a central role in the metabolism and pathogenesis of the most common dermatophyte of man, *Trichophyton rubrum*, were purified to homogeneity. Size-exclusion chromatography and Chromatofocusing were used to purify the major proteinases 42-fold from crude fungal culture filtrate. The major enzyme has pI 7.8 and subunit M_r 44000, but forms a dimer of M_r approx. 90000 in the absence of reducing agents. A second enzyme with pI 6.5 and subunit M_r 36000, was also purified. It is very similar in substrate specificity to the major enzyme but has lower specific activity, and may be an autoproteolysis product. The major proteinase has pH optimum 8, a Ca^{2+} -dependence maximum of 1 mM, and was inhibited by serine-proteinase inhibitors, especially tetrapeptidyl chloromethane derivatives with hydrophobic residues at the P-1 site. Kinetic studies also showed that tetrapeptides containing aromatic or hydrophobic residues at P-1 were the best substrates. A $k_{\text{cat.}}/K_m$ of $27000 \text{ M}^{-1} \cdot \text{s}^{-1}$ was calculated for the peptide 3-carboxypropionyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The enzyme has significant activity against keratin, elastin and denatured type I collagen (Azocoll).

INTRODUCTION

Trichophyton rubrum is an anthropophilic fungus that is the most common and widely distributed dermatophyte of man (Rippon, 1982). This organism is responsible for superficial skin infections such as tinea pedis (athlete's foot). It is never cultured from soil and must use human skin protein as a nutrient source. It is not surprising, therefore, that a number of proteinases or 'keratinases' have been identified in crude culture filtrates of *T. rubrum* or related *Trichophyton* species (Chattaway *et al.*, 1963; Rippon & Varadi, 1968; Day *et al.*, 1968; Yu *et al.*, 1969; Meevootisom & Niederpruem, 1979).

We now report on the purification of two proteinases released from *T. rubrum* into culture medium. Characterization of their substrate specificity allows clarification of their possible role in fungal metabolism and disease.

MATERIALS AND METHODS

Materials

Sabouraud broth was from Difco Laboratories. Ox ligamentum-nuchae elastin (E60 mesh) was from Elastin Products Co., St. Louis, MO, U.S.A. [^{14}C]Acetic anhydride, NaB^3H_4 and [^3H]di-isopropyl phosphorofluoridate were from Amersham. Electrophoresis M_r standards were from Bio-Rad. Suc-Ala-Ala-Pro-Leu- CH_2Cl and all peptide-AMC substrates were from Enzyme Systems Products, Livermore, CA, U.S.A. All other peptidyl chloromethane inhibitors were a gift from Dr. James Powers, Georgia Institute of Technology, Atlanta, GA, U.S.A. Gelatin, Azocoll, chymotrypsin, 4,4'-dithiopyridine, EDTA, Trasylol, 1,10-phenanthro-

line, *N*-ethylmaleimide, soya-bean trypsin inhibitor, α_1 -proteinase inhibitor and pancreatic elastase were all from Sigma Chemical Co., St. Louis, MO, U.S.A. Suc-Ala-Ala-Pro-Phe-pNA was from Vega Biomedicals, Tucson, AZ, U.S.A. All other peptide-pNA substrates were a gift from Dr. Corey Largman, Veterans' Administration Hospital, Martinez, CA, U.S.A.

Culture of *T. rubrum*

T. rubrum (I.F.O. 9185) from Kyushu University, Fukuoka, Japan, was inoculated into 100 ml of Sabouraud broth (Difco Laboratories) in plastic tissue-culture flasks (Falcon) and incubated at 22 °C. Medium was replaced every 2 weeks. Filtered (Millipore, 0.45 μm pore size) conditioned medium from four flasks of 12-week-old cultures was pooled for each purification.

Purification of *T. rubrum* proteinase

All procedures were carried out at 4 °C. Protein from the culture filtrate was precipitated with 70% -saturated $(\text{NH}_4)_2\text{SO}_4$, collected by centrifugation at 20000 *g* for 20 min, dialysed against water and freeze-dried. Freeze-dried samples (50–70 mg) were dissolved in 3 ml of 150 mM-NaCl/20 mM-Tris/HCl buffer, pH 8, and applied to a Sephadex G-100 column (1.9 cm $\times$ 110 cm). Samples were eluted at a flow rate of 15 ml/h, and 2 ml fractions were collected.

Fractions representing the main peak of proteolytic activity from Sephadex chromatography were pooled and dialysed against 20 mM-Tris/acetate buffer, pH 8.3. They were further resolved on a Mono P Chromatofocusing column (Pharmacia) fitted with a fast-protein-liquid-chromatography gradient programmer (Pharmacia). A

Abbreviations used: Suc, 3-carboxypropionyl; Bzl, benzyl; AMC, 7-amino-4-methylcoumarin; pNA, 4-nitroaniline; SDS, sodium dodecyl sulphate.

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gradient of pH 8–5 was generated with 20 mM-Tris/acetate buffer, pH 8.3, and Polybuffer, pH 5 (Pharmacia). Samples were eluted at a flow rate of 0.5 ml/min, and 1 ml fractions were collected.

Purification was monitored by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). A 4% stacking gel and a 10% running gel were used. Sample buffer included 5% (v/v) 2-mercaptoethanol, and samples were boiled for 5 min before electrophoresis. Protein bands were identified by silver stain (Wray *et al.*, 1981).

Purification was also monitored by using SDS/polyacrylamide gels co-polymerized with gelatin. Heussen & Dowdle (1980) introduced the use of gelatin co-polymerized in SDS/polyacrylamide-gel electrophoresis to detect proteinases and determine M_r values simultaneously. Polyacrylamide gels (10%) were polymerized in the presence of 0.1% pig skin type I gelatin. Boiling and reducing agents were not used, but otherwise the procedure was that of Laemmli (1970) with a non-gelatin 4% stacking gel. Gels were prepared on a Hoefer small-slab-gel apparatus (0.75 mm thickness). A pH 8.3 Tris/glycine running buffer was used. Fungal enzyme samples (approx. 5 μ g of protein in 2.5 μ l of water) were diluted 1:1 in sample buffer [5% (w/v) SDS, 2% (w/v) sucrose and 0.005% Bromophenol Blue] in stacking gel buffer (0.5 M-Tris/HCl, pH 6.8, containing 0.4% SDS). Electrophoresis was run at 20 mA/gel. After electrophoresis, the gel was washed once in 100 ml of 2.5% (w/v) Triton X-100 for 2 h to remove SDS. The gel was incubated in 1 mM- CaCl_2 /100 mM-Tris/HCl buffer, pH 7.8, for 12–24 h at 37 °C. Reaction was stopped by washing the gel for 1 h in 50% (v/v) trichloroacetic acid at room temperature. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained in 45% (v/v) methanol/10% (v/v) acetic acid until proteolytic bands that cleared the dye from the gel were optimal for photography (approx. 30 min).

For calculation of M_r in both standard and gelatin SDS/polyacrylamide-gel electrophoresis, the following M_r standards were run simultaneously: phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soya-bean trypsin inhibitor (21 500) and lysozyme (14 400).

Assay of general proteinase activity

General proteinase activity was assayed with Azocoll (Sigma Chemical Co.) as substrate. Azocoll (5 mg) was incubated with 10–200 μ l of enzyme sample and 1 mM- CaCl_2 /100 mM-Tris/HCl buffer, pH 7.8, for 2–24 h at 37 °C. The final reaction volume was 1 ml. After incubation, unchanged substrate was removed by centrifugation in a Beckman Microfuge for 5 min, and released dye in the supernatant was measured by the absorbance at 540 nm. For inhibition studies, inhibitors were preincubated with enzyme for 20 min at 22 °C before substrate was added. For inhibitors requiring ethanol or dimethyl sulphoxide in stock solutions, an equivalent amount of solvent was run as a control.

Elastase activity

Purified ox ligamentum-nuchae elastin (60 mesh) was labelled with NaB^3H_4 , and degradation was assayed by methods described in detail previously (McKerrow *et al.*, 1985).

Keratinase activity

Epidermal keratin was extracted from newborn rats (Murozuka *et al.*, 1979) and labelled with [^{14}C]acetic anhydride by the method of Gisslow & McBride (1975). [^{14}C]Keratin degradation was assayed by the method of Tzeng *et al.* (1983).

Protein determination

Protein was determined by the method of Bradford (1976), with chymotrypsin as a standard.

Kinetic studies

The rates of hydrolysis of the peptide-pNA substrates were measured at 410 nm on a Gilford model 260 spectrophotometer. The reactions were initiated by adding 10 μ l of the enzyme solution (4–29 nmol) to 0.50 ml of a solution containing substrate in 1 mM- CaCl_2 /0.1 M-Tris/HCl buffer, pH 7.8. Substrate concentrations were varied over a 10-fold range 0.1–1.0 mM. An ϵ value of 8800 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 410 nm was used for the liberated 4-nitroaniline (Erlanger *et al.*, 1961), and the kinetic constants were determined from the initial rates of 4-nitroaniline appearance. The data were plotted, with a least-squares analysis, by the method of Lineweaver & Burk (1934). Five to seven points were measured for each plot and correlation coefficients were calculated. All but three correlation coefficients were greater than 0.99, and no value was lower than 0.97.

The rates of hydrolysis of several peptide-AMC substrates were measured with an Aminco SPF-500 spectrofluorimeter. The cleavage of substrates was measured as the increase in fluorescence of liberated aminomethylcoumarin (Castillo *et al.*, 1979), by using a 380 nm excitation wavelength and monitoring the emission at 460 nm. The fluorescence scale was calibrated with a standard AMC solution (1.3 μM), and the rate of substrate hydrolysis was determined from the continuously monitored slope of the AMC increase in the presence of 20 μ l of the enzyme sample. A non-enzymic control was obtained from the substrate reaction before addition of the enzyme. The AMC substrate solution (20–40 mM in dimethyl sulphoxide) was added to a solution of 1 mM- CaCl_2 /0.1 M-Tris/HCl buffer, pH 7.8, containing dimethyl sulphoxide to give a volume of 2.0 ml, including 100 μ l of total dimethyl sulphoxide. Substrate concentrations were in the range 0.06–1.2 mM. The data were plotted as in the nitroaniline assays.

The rates of hydrolysis of the thioester substrates were measured by adding substrate in dimethyl sulphoxide and 4,4'-dithiodipyridine in dimethyl sulphoxide to 1.8 ml of 1 mM- CaCl_2 /0.1 M-Tris/HCl buffer, pH 7.8, to give a total volume of 2.0 ml (10% dimethyl sulphoxide). A 20 μ l enzyme sample was added to start the reaction. The increase in absorbance at 324 nm was monitored with a Gilford 260 spectrophotometer. An ϵ value of 19800 $\text{M}^{-1}\cdot\text{cm}^{-1}$ was used for the thiopyridone product (Grassetti & Murray, 1967). The substrate concentration range was 0.1–1.0 mM. The data were plotted as in the nitroaniline assays.

RESULTS

Purification of *T. rubrum* proteinases

Crude culture filtrate had both Azocoll-lytic and elastase activity. Azocoll-lytic activity was inhibited 80%

by 1 mM-phenylmethanesulphonyl fluoride and 95% by 100 μ g of α_1 -proteinase inhibitor/ml. The absence of inhibition with 2 mM-1,10-phenanthroline or 5 mM-*N*-ethylmaleimide suggested that the proteolytic activity was primarily due to one or more serine proteinases.

To determine how many proteolytic species were actually present in the fungal filtrate, we subjected crude filtrate to electrophoresis on a gelatin/polyacrylamide substrate gel. Three major bands of proteolytic activity

were observed at approximate M_r values of 90 000, 71 000, and 27 000 (Fig. 1). Sephadex G-100 chromatography separated a broad peak of proteolytic activity in the filtrate from most of the non-proteolytic proteins, which were eluted in the void volume (Fig. 2). Assay of elastase activity showed that a minor peak of activity (fractions 70–80, Fig. 2) could be resolved from the major peak of activity (fractions 90–130). A gelatin/polyacrylamide substrate gel of this minor peak showed the M_r -27 000 species (results not shown). Proteolytic activity from the major Sephadex peak was further resolved by Chromatofocusing on a Mono P column eluted with a gradient of pH 8.5. Three major peaks of proteolytic activity were separated (Fig. 3). The first was eluted with the void volume (proteins with pI > 8). The second was eluted at approx. pI 7.8 and the third at an approx. pI 6.5. SDS/polyacrylamide-gel electrophoresis of these peaks is shown in Fig. 4. Peaks 2 and 3, which bound to the column, each showed single bands on a silver-stained gel at approx. M_r 44 000 and 36 000 respectively. Peak 1 was a mixture of several proteins, including one with an M_r identical with that of the protein in peak 3. Electrophoresis of these peaks on gelatin/polyacrylamide substrate gels confirmed the separation of two distinct proteolytic species with approx. M_r 90 000 (peak 2) and 71 000 (peak 3) (Fig. 1). Since the substrate gels were run in non-reducing conditions without boiling, it appeared that both proteolytic species existed as disulphide-linked dimers in the 'native' state. This was confirmed by also running the standard SDS/polyacrylamide-gel electrophoresis under non-reducing conditions and observing that the subunit M_r values were approximately doubled.

Table 1 summarizes the purification data for the proteolytic activity of *T. rubrum* culture filtrate in the Azocoll-degradation assay. Although both proteolytic species degraded elastin and keratin as well as Azocoll, that of peak 3 had lower specific activity than that of peak 2 against all three substrates. Peak 2 had a specific activity of 200 mg of keratin degraded/h per mg of protein, and peak 3 32 mg degraded/h per mg of protein, at 37 °C. Peak 2 had 59 000 units of elastase activity/mg, and peak 3 had 19 000 units/mg (1 unit = 1 μ g of elastin degraded/h). Our next set of experiments was designed to test whether the two proteolytic species of M_r 36 000 (peak 3) and 44 000 (peak 2) were distinct proteinases or represented different forms of a single enzyme.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of crude *T. rubrum* culture filtrate and fractions from purification steps on gels containing gelatin substrate

The 4% stacking/10% running gels were used as described in the Materials and methods section. Proteinase activity appears as clear bands. Lane C, crude culture filtrate; lane S, pool of all active fractions from Sephadex G-100 chromatography; lanes 1, 2 and 3, peaks 1, 2 and 3 respectively from Chromatofocusing (see Fig. 3).

Fig. 2. Sephadex G-100 chromatography of crude *T. rubrum* culture filtrate

Conditions are described in the Materials and methods section. —, A_{280} ; ●, Azocoll hydrolysis; ○, elastase activity. Bar indicates fractions pooled for Chromatofocusing.

Fig. 3. Chromatofocusing of pooled fractions from Sephadex G-100 chromatography

Conditions are described in the Materials and methods section. ●, pH; —, A_{280} ; ○, Azocoll hydrolysis; ▲, elastase activity.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of fractions from purification steps

Conditions are described in the Materials and methods section. Lane C, crude culture filtrate; lanes 1, 2 and 3, peaks 1, 2 and 3 respectively from Chromatofocusing. The 4% stacking/10% running gels were used and stained with silver.

pH optima

Fig. 5 shows the effect of pH on the Azocoll-lytic activity of peaks 2 and 3. The curves were similar, with optima near pH 8.

Stability

When stored at 4 °C for 3 weeks, both peaks retained 85% of activity. When stored for 3 weeks at -20 °C, peak 2 retained 92% and peak 3 retained 85% of its activity.

Inhibition profile

Table 2 shows an inhibition profile for both peaks. The findings were consistent with the data on the activity of the crude filtrate; both species were inhibited by a number of inhibitors of serine proteinases, but neither was inhibited by *N*-ethylmaleimide or 1,10-phenanthroline. Three peptidyl chloromethane derivatives were tested as inhibitors. Ala-Ala-Pro-Ala-CH₂Cl is an excellent inhibitor of leucocyte and pancreatic elastase (Powers & Tuhy, 1973). Ala-Ala-Pro-Leu-CH₂Cl and Phe-Gly-Ala-Leu-CH₂Cl are excellent inhibitors of subtilisin, but will also inhibit chymotrypsin (Powers *et al.*, 1973). Although Phe-Gly-Ala-Leu-CH₂Cl was the most effective inhibitor for both proteinase peaks, the absolute inhibition of peak 2 was significantly greater than that of peak 3. Both species showed a Ca²⁺-dependence with maximum activity at 1 mM-CaCl₂, but peak 3 was inhibited less than peak 2 by 10 mM-EDTA.

Kinetic studies

On the basis of the results of experiments with peptidyl chloromethane inhibitors, we chose to test a number of synthetic peptide substrates with hydrophobic amino acids at the P-1 site. Table 3 shows that, for a group of peptide-pNA, peptide-AMC and peptide-SBzl substrates, phenylalanine or methionine at the P-1 site gave the highest k_{cat}/K_m . β -Branching of the P-1 side chain decreased activity significantly in the peptide-pNA substrates. However, activity with valine at the P-1 site could be measured with the use of a more-reactive *S*-benzyl derivative. Although the specific activity of peak 2 was greater than that of peak 3, the general trends of k_{cat}/K_m for the various peptides were similar. Three peptide-AMC substrates were also assayed with peak 2. These were significantly less reactive than the peptide-pNA or the *S*-benzyl-peptide substrates.

Table 1. Summary of purification of proteolytic activity from *T. rubrum* culture filtrate

For experimental details see the Materials and methods section. One unit of enzyme activity is defined as that amount of enzyme needed to give a change in A_{540} of 0.1/h in the Azocoll-hydrolysis assay.

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification factor	Yield (%)
Culture filtrate [(NH ₄) ₂ SO ₄ fraction]	66	302	19932	1	100
Gel filtration (Sephadex G-100)	12	972	11664	3.2	58.5
H.p.l.c. Chromatofocusing	0.26* 1.01†	12820* 735†	4075	42* 2.5†	20.4
*Peak 2. †Peak 3.					

Fig. 5. pH optima of two proteinase species with Azocoll (5 mg/ml) as substrate

The buffer in the pH range 4–6 was 1 mM-CaCl₂/0.1 M-acetate/HCl, and that in the pH range 7–10 was 1 mM-CaCl₂/0.1 M-Tris/HCl. ●, Peak 2; ○, peak 3.

DISCUSSION

We have purified the major proteolytic enzymes from *T. rubrum* culture filtrate. These enzymes are chymotrypsin-like serine proteinases that can degrade denatured type I collagen (Azocoll), elastin and keratin. The two proteinases have subunit M_r values of 44000 and 36000. They exist as dimers in the absence of reducing agents. Since most of their properties are similar, the most rational interpretation is that they represent different forms of the same enzyme. The lower- M_r species could be an autoproteolysis form that has lost a 6000- M_r polypeptide. A conformational change giving lower specific activity because of loss of the hydrophobic binding site at P-4 would explain the differences between the two proteins in inhibition by the peptide Phe-Gly-Ala-Leu-CH₂Cl. A third proteolytic species of M_r 27000 was seen in crude filtrates and was resolved as a minor peak of proteolytic activity on Sephadex G-100 chromatography. It has also been shown to be a serine proteinase inhibited by the same tetrapeptidyl chloromethane derivatives as were the other two species (J. A. Apodaca, unpublished work).

The enzymes that we have purified and characterized from *T. rubrum* may be related to proteinases reported in other *Trichophyton* fungi. Yu *et al.* (1969) purified two

Table 2. Inhibition profile of two proteolytic species purified from *T. rubrum* culture filtrate

For experimental details see the Materials and methods section.

Inhibitor	Final concentration	Activity remaining (% of control)	
		Peak 2	Peak 3
α_1 -Antitrypsin	75 μ g/ml	0.6	0.6
Phenylmethanesulphonyl fluoride	1 mM	3.1	0.1
Trasylol (aprotinin)	150 μ g/ml	3.9	3.5
EDTA	10 mM	33.9	84.7
N-Ethylmaleimide	5 mM	95.9	85.3
1,10-Phenanthroline	1 mM	97.4	76.0
MeOSuc-Phe-Gly-Ala-Leu-CH ₂ Cl	0.05 μ M	7.3	57.4
	0.10 μ M	4.2	46.2
Suc-Ala-Ala-Pro-Leu-CH ₂ Cl	0.10 μ M	43.2	60.7
	0.20 μ M	28.6	38.3
Suc-Ala-Ala-Pro-Ala-CH ₂ Cl	1.0 μ M	72.9	55.7
	2.0 μ M	54.7	46.8

Table 3. Kinetic studies of the hydrolysis of tetrapeptide substrates by the two proteolytic species

Assay conditions are described in the Materials and methods section.

Proteolytic species	Substrate	K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Peak 2	Suc-Ala-Ala-Pro-Phe-pNA	0.35	9.46	27028
	Suc-Ala-Ala-Pro-Met-pNA	0.73	11.21	15354
	Suc-Ala-Ala-Pro-Nle-pNA	0.52	4.67	8974
	Suc-Ala-Ala-Pro-Ile-pNA	No activity*		
	Suc-Ala-Ala-Pro-Val-pNA	No activity*		
Peak 3	Suc-Ala-Ala-Pro-Phe-pNA	0.71	2.72	3837
	Suc-Ala-Ala-Pro-Met-pNA	0.24	0.38	1580
	Suc-Ala-Ala-Pro-Nle-pNA	0.41	0.56	1360
	Suc-Ala-Ala-Pro-Ile-pNA	No activity*		
	Suc-Ala-Ala-Pro-Val-pNA	No activity*		
Peak 2	MeOSuc-Ala-Ala-Pro-Val-SBzl	0.56	3.04	5410
Peak 3	MeOSuc-Ala-Ala-Pro-Val-SBzl	0.80	1.12	1408
Peak 2	MeOSuc-Ala-Phe-AMC	0.40	0.16	400
	Suc-Ala-Ala-Pro-Phe-AMC	1.13	0.31	274
	Suc-Phe-Gly-Ala-Leu-AMC	0.33	0.028	86

* $< 0.002A/10$ min at 1 mM substrate.

proteolytic enzymes of M_r 38000 and 48000 from *Trichophyton mentagrophytes*. The 48000- M_r 'keratinase' had a macromolecular substrate specificity similar to that of the enzyme that we have described. However, the inhibitor data reported were insufficient for unequivocal determination of its proteinase class, although it did cleave dipeptides containing leucine and phenylalanine. A less-well-characterized proteinase of M_r 34300 has also been reported in culture medium of *Trichophyton granulosum* (Day *et al.*, 1968).

The enzymes that we have purified probably account for the serine-proteinase-like activity reported in crude *T. rubrum* filtrates by Meevootisom & Niederpruem (1979) and by Chattaway *et al.* (1963). Most intriguing, however, is the fact that Takiuchi *et al.* (1983) purified a 45000- M_r proteinase from *M. canis* and produced antiserum against it. This antiserum cross-reacted with material in culture filtrates of *T. rubrum*, *Microsporum gypsum* and *T. mentagrophytes*. This suggests that structurally similar proteinases may be expressed by many, if not all, dermatophytes.

The primary function of the extracellular proteinase we have purified is probably to degrade keratin at the skin surface as a nutrient source for the fungi. There are reports of *T. rubrum* producing deeper destructive lesions of skin (Mikhail, 1970), particularly with strains from tropical regions (Rippon, 1982). The presence of significant elastase activity suggests that *T. rubrum* could grow and cause tissue destruction in the elastin-rich dermal layer of skin.

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