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Purification and Characterization of a 27,000-M_r Extracellular Proteinase from *Trichophyton rubrum*

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A proteinase of M_r 27,000 with a possible role in the metabolism and invasion of host tissues was purified from the conditioned medium of *Trichophyton rubrum* by concanavalin A and anion-exchange chromatography. Peaks of proteolytic activity were analyzed by substrate gel electrophoresis. The 27,000-M_r proteinase had a pH optimum of 8.0, a calcium dependence of 2 mM, and was inhibited by serine proteinase inhibitors, especially phenylmethylsulfonyl fluoride and Phe-Gly-Ala-Leu-chloromethyl ketone. By polyacrylamide gel electrophoresis, the 27,000-M_r proteinase had a reduced molecular weight of 44,000 and reacted with [^{3}H]diisopropyl fluorophosphate. The proteinase degraded azocoll, type III collagen, type IV procollagen, laminin, fibronectin, and the peptide substrates succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (1,573 M^{-1} s^{-1}) and *t*-butyloxy carbonyl-Ala-Ala-Leu-*p*-nitroanilide (1,614 M^{-1} s^{-1}).

Trichophyton rubrum is the dermatophytic fungus responsible for most ringworm infections in the United States. Because this organism parasitizes keratinized tissues of humans (nails and skin), we propose that *T. rubrum* (and dermatophytes in general) requires proteinases to cleave available proteins into metabolically useable carbon, nitrogen, and sulfur. Keratins, the major constituents of the cornified tissues, are highly disulfide bonded, insoluble in aqueous solutions, and highly resistant to proteolytic attack (9, 13-15, 19). In the stratum corneum, the keratins account for approximately 95% of the protein contained in this layer of the epidermis and require urea and β-mercaptoethanol to be extracted (19). The other 5% of proteins include a number of nonkeratinous proteins of M_r 10,000 to 68,000 that can be easily extracted with aqueous buffers. The water-soluble proteins and nutrients from hair and nails can support the growth of dermatophytes (16). Nevertheless, several investigators have looked for proteinases, elaborated by dermatophytes, that can degrade keratins as well as other proteins.

Early work with the dermatophytes demonstrated that *Microsporum canis*, *M. gypseum*, *Trichophyton schoenleinii*, and *T. rubrum* digested ethylene oxide-sterilized wool, releasing sulfhydryl-containing components into their medium (25, 27). In wool fibers from the *T. rubrum* cultures examined microscopically the cuticles remained intact, whereas the central, medullary region of the hair shaft was dissolved. The wool fibers from the *T. schoenleinii* cultures were randomly fragmented, with erosion of the fiber shaft. When strains of *T. rubrum* and *T. schoenleinii* were separated from radioactively labeled wool by a permeable membrane, the fungi produced a diffusible activity that could degrade the radioactive substrate (26). Although these results were suggestive that dermatophytes could indeed degrade keratin, and that this activity was mediated by a diffusible substance, no attempts were made to characterize the keratinolytic activity.

A proteinase of M_r 48,000 has been purified from the culture medium of *Trichophyton mentagrophytes* (29, 30, 32). In this paper the culture medium into which proteins have been secreted is referred to as conditioned medium (CM). This 48,000-M_r proteinase has a pH optimum of 7.0 and degrades native hair, casein, collagen, elastin, gelatin, and a number of other proteins, amides, and peptides. The class of proteinase cannot be determined from the data presented. The enzymatic activity was somewhat inhibited by EDTA (30% less than controls), but no other inhibitors were tested. In hair treated with the purified keratinase and examined microscopically the cortex contained many fissures, and the medulla was digested. These results suggested that, in the case of *T. mentagrophytes*, a proteolytic enzyme could be purified with keratinolytic activity, and there was no need for direct contact between the cell and its keratin substrate.

Two additional proteinases have been described in *T. mentagrophytes* (31). These cell-bound keratinases were purified from cultures of mycelia that were grown up in a horse hair medium, washed, and then allowed to sit for 1 h in a pH 7.8 phosphate buffer containing 1 M NaCl. The proteinases were purified from this salt wash. Both of these enzymes degrade guinea pig hair, and they have estimated M_rs of 440,000 and 20,300. No attempt was made to determine the class of these proteinases.

A keratinolytic activity with an acidic pH optimum of 4.5 has recently been partially purified from the CM of keratin-grown *T. mentagrophytes* (23). The class of this proteinase is not clear as both phenylmethylsulfonyl fluoride (PMSF) and EDTA are inhibitory. This activity may be due to both a serine proteinase and a metalloproteinase or to a serine proteinase with a requirement for a divalent cation. Keratinolytic activity has also been described in *Trichophyton gallinae* (24) and *M. canis* (20, 22). The enzyme from *M. canis* (M_r, 45,000) is strongly inhibited by 10 mM PMSF. Interestingly, antisera prepared against this proteinase showed that there were immunologically related materials contained in the CM of *M. gypseum*, *T. mentagrophytes*, and *T. rubrum* (21).

Finally, several proteinases have been described and purified from the CM of *T. rubrum*. Meevootisom and Niederpruem (12) have shown that the CM of keratin-grown *T. rubrum* contained proteolytic activity capable of degrading casein, bovine serum albumin, collagen, elastin, guinea pig hair, and keratin. However, the incubations were carried out at 45°C; the physiological significance of substrates degraded at this high temperature is questionable, especially

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for a substrate like collagen with a melting temperature around 37°C. Nonetheless, all of the activities had pH optima of 7.0, with the exception of keratin degradation, which showed a pH optimum of 8.5. All of these activities were inhibited by PMSF. A serine proteinase with a molecular weight of 35,000 has been purified from the CM of Sabouraud dextrose broth-grown *T. rubrum* (18). This enzyme had an alkaline pH optimum and degrades casein, azoalbumin, and α-*N*-benzoyl-L-arginine ethyl ester. This proteinase has no activity against defatted human hair. Last, two proteinases with keratinolytic activity have been purified from the CM of *T. rubrum* (3). These serine proteinases have molecular weights of 93,000 and 71,000 under nonreducing conditions. Both enzymes exist as dimers of a smaller-molecular-weight subunit, M_r 44,000 and 36,000 respectively, and are serine proteinases with a pH optimum of 8.0 and a calcium dependence of 1 mM. They degrade azocoll, elastin, keratin, and synthetic peptide substrates with hydrophobic amino acids in their P-1 site, e.g., succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (pNA).

Proteinases appear to be important in several aspects of dermatophytosis. For the organism, proteinases could free up nutrients from their nutrition-poor environment. The proteinase described in this study is of particular interest because it is expressed when the organism is starved for sulfur (2; G. Apodaca and J. H. McKerrow, J. Cell Biol. **107**:183, 1989). The elaborated proteinases could also be important in the pathogenesis of dermatophytosis. Invasive granulomatous infections by *T. rubrum* have been described in individuals with lymphomas, Cushing's syndrome, abnormalities of carbohydrate metabolism, and immune impairment (17). Invasiveness is thought to depend in part on the ability of the fungus to degrade extracellular macromolecules through the elaboration of proteinases. The subject of this communication is the purification and characterization of a major proteinase in *T. rubrum* CM with a putative role in both the invasion and metabolism of host tissues.

MATERIALS AND METHODS

Culture of *T. rubrum*. *T. rubrum* IFO 9185 is a stock culture from Kyushu University, Fukuoka, Japan, and was obtained from M. Asahi, Kyushu University. It was routinely maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants stored at 4°C. The fungus was subcultured by streaking it onto the same medium every 2 months. *T. rubrum* spore suspensions were also maintained as stock cultures. To obtain the spore suspensions the fungus was grown on Sabouraud dextrose agar slants at 30°C, and when the fungus completely covered the surface of the slant the tube was flooded with approximately 5 ml of sterile, distilled water. The water was pipetted rapidly up and down to dislodge the spores, but careful attention was paid to not disrupt the integrity of the actual agar surface. If necessary, this spore suspension was filtered through sterile gauze to remove bits of agar and mycelium. These spore suspensions were stored at ambient temperature in tightly stoppered, sterile containers.

To obtain stationary-phase cultures of this fungus a sample of the spore suspension, typically 500 μl, was added to 100 ml of Sabouraud dextrose broth in 75-cm^2 plastic tissue culture flasks (Costar, Cambridge, Mass.) and incubated at 30°C for approximately 3 to 4 weeks. After this amount of time, the fungus forms a thick white mat on the surface of the medium, and it has been determined that the fungus is in stationary phase (2). The CM from these cultures was retained for purification of proteinases.

Purification of the 27,000-M_r proteinase. To purify the 27,000-M_r proteinase, proteins in stationary-phase CM (1,100 ml) of *T. rubrum*, grown in Sabouraud dextrose broth, were precipitated with 70% saturated $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, Mo.), collected by centrifugation (model RC-5 centrifuge, GSA rotor; Ivan Sorvall, Inc., Wilmington, Del.) at 20,000 × *g* for 20 min at 4°C, suspended in 15 ml of water, dialyzed against 4 liters of water overnight, and then lyopholized. The freeze-dried samples (20 to 50 mg of protein) were dissolved in 4 ml of loading buffer (20 mM Tris hydrochloride [pH 7.4], 500 mM NaCl, 0.02% NaN_3; all from Sigma) and centrifuged in a microfuge to remove particulate matter, and the supernatant was applied to a 11-ml disposable Econo column (Bio-Rad Laboratories, Richmond, Calif.) packed with 8 ml of concanavalin A–Sepharose 4B (Sigma) that had been washed with 10 column volumes of the above buffer. The mannose-containing molecules were allowed to interact with the column for 25 to 30 min before the column was washed with 100 ml (2.5 ml per fraction) of the same buffer at a flow rate of 45 ml/min. After the 40th fraction, the loading buffer was changed to the elution buffer (20 mM Tris hydrochloride [pH 7.4], 500 mM NaCl, 200 mM α-methyl mannoside [Sigma], 0.02% NaN_3), and an additional 15 fractions were collected. Active fractions from the flowthrough of the concanavalin A column (typically fractions 1 through 10) were pooled and then concentrated and dialyzed against H_2O in a Pro-Di-Con apparatus (Pierce Chemical Co., Rockford, Ill). The concentrated proteins (in 0.5 ml) were mixed with an equal volume of 50 mM bis-Tris hydrochloride (pH 5.8) and applied to a Polyanion SI column (Pharmacia Fine Chemicals, Piscataway, N.J.) that had previously been equilibrated with the same buffer. Samples were eluted at a flow rate of 0.5 ml/min, and 1-ml fractions were collected. After 6 ml of the 50 mM bis-Tris hydrochloride (pH 5.8) buffer had flowed through the column, proteins that interacted with the column were eluted off the matrix by a 30-ml linear gradient of salt from 0 to 500 mM NaCl in the same buffer. From fractions 36 to 40 the concentration of salt was linearly increased to 1 M, and it was maintained at this concentration for an additional 10 fractions. All procedures were carried out at 4°C.

Purification was monitored by sodium dodecyl sulfate (SDS; Bio-Rad)-polyacrylamide gel electrophoresis (7). A 4% polyacrylamide stacking gel and a 10 to 14% polyacrylamide resolving gel were used. The sample buffer was the same as that used in the substrate gel analysis, except that it contained 5% (vol/vol) β-mercaptoethanol (Sigma) or 100 mM dithiothreitol (Sigma), and samples were boiled for 5 min before electrophoresis. Protein bands were identified by staining with silver (28). Molecular weight standards (Bio-Rad) were myosin (200,000), β-galactosidase (116,300), phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,500).

Protein was determined by the method of Bradford (4) with chymotrypsin (Sigma) as a standard.

Substrate gel analysis. General proteinase activity was assayed in SDS-polyacrylamide gels copolymerized with substrate (3), in which proteinases can be detected and molecular weights can be determined simultaneously. The resolving gel (10 or 12% polyacrylamide) was polymerized in the presence of 0.12% pig skin type I gelatin (Sigma). The samples were not boiled or reduced, and the stacking gel (4% polyacrylamide) did not contain substrate. The gels (0.75 mm thick) were prepared in a Hoefer (San Francisco, Calif.) small-slab-gel apparatus. CM was diluted with an equal

volume of 2× sample buffer (0.25 M Tris hydrochloride [pH 6.8], 20% [vol/vol] glycerol, 0.2% [wt/vol] SDS, and 0.005% [wt/vol] bromophenol blue), and 15 to 25 μl was loaded on the gel. After electrophoresis, the gels were washed in 100 ml of 2.5% (vol/vol) Triton X-100 (Sigma) for 30 min to remove the SDS. The gels were then incubated for 12 to 24 h at 37°C in the same buffered solution used in the azocoll assay. It was later found that this incubation period could be cut down to a 2 to 4-h period with no significant loss of visualization of proteinase activity. The gels were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad) in water-methanol-acetic acid (5:5:1, vol/vol) for 30 min and then destained in 45% (vol/vol) methanol–3% (vol/vol) acetic acid until the proteolytic bands could be visualized. In some experiments CM was preincubated with 10 mM PMSF, as described below for the inhibition assays, before electrophoresis. The same concentration of PMSF was included in the incubation buffer. Protein standards were the same as those used for SDS-polyacrylamide gel electrophoresis.

Detection of proteolytic activity with substrate-impregnated cellulose acetate membranes. Samples of concentrated crude *T. rubrum* CM (10 μl) were mixed with the 2× nonreducing sample buffer described above and electrophoresed on 12% SDS-polyacrylamide gels. Colored molecular weight markers were run in adjacent lanes (rainbow markers; Amersham Corp., Arlington Heights, Ill.). The gel was soaked in 2.5% (vol/vol) Triton X-100 for 30 min and then overlaid with a cellulose acetate membrane impregnated with succinyl-Ala-Ala-Phe-aminofluorocoumarin (EOM membrane; Enzyme System Products, Livermore, Calif.) that had been prewetted with 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$ buffer. After a 10 to 20-min incubation at 37°C, the positions of the molecular weight markers were marked on the membrane, and proteolysis of the substrate was detected by shining a hand-held longwave UV lamp (Fisher Scientific Co., Pittsburgh, Pa.) over the surface of or underneath the membrane. A light blue fluorescence was detected wherever the substrate had been cleaved, releasing UV-absorbing, free aminofluorocoumarin.

[^{3}H]diisopropyl fluorophosphate labeling of the 27,000-M_r proteinase. A 150-μl sample of the purified 27,000-M_r proteinase (about 5.2 μg of protein) was mixed with 10× concentrated buffer (1 M Tris hydrochloride [pH 8.0], 10 mM $CaCl_2$) and water to give a final reaction volume in 1× buffer of 300 μl. A reaction with no enzyme was run as a control. To each of the reaction mixtures 25 μCi of ^{3}H-labeled diisopropyl fluorophosphate (5 mCi/ml, 3.5 C/mmol; Amersham) was added, and the reaction was allowed to proceed for 2 h at 37°C. A 12-μl amount of each sample was mixed with 2× sample buffer (with or without dithiothreitol and boiling) and electrophoresed on a 14% polyacrylamide gel. Labeled proteinase was visualized by autoradiography of the En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.) fluorographed gels. ^{14}C-labeled standards (Amersham) were run in lanes adjacent to the samples. These standards have the same molecular weights as those used in SDS-polyacrylamide gel electrophoresis.

Inhibition assays. Inhibitors were preincubated with enzyme for 20 min at ambient temperature before azocoll substrate was added to the reaction mixture. PMSF, 1,10-phenanthroline, and *N*-ethylmaleimide (all from Sigma) were made up as 150 mM stocks in ethanol, whereas the chloromethylketone inhibitors (Enzyme System Products) were made up as 1-mg/ml stocks in dimethyl sulfoxide (Sigma). α_1-Proteinase inhibitor (Sigma) was made up as a 2-mg/ml stock in H_2O. For inhibitors requiring ethanol or dimethyl sulfoxide in stock solutions, an equivalent amount of solvent was run as a control.

Azocoll degradation. CM or column fractions, typically 50 to 100 μl, were incubated with 3 to 5 mg of azocoll (Sigma or Calbiochem, La Jolla, Calif.) in 100 mM Tris hydrochloride (pH 8.0)–mM $CaCl_2$ buffer in 1.6-ml Eppendorf tubes for 4 to 24 h at 37°C. The final reaction volume was 1 ml. After incubation, the samples were centrifuged for 5 min in a microfuge (Beckman Instruments, Inc., Palo Alto, Calif.). Degradation of azocoll was measured by determining the A_{520} of the supernatant in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). One unit of azocoll-degrading activity released 0.1 A_{520} unit per h.

Elastin degradation. Purified ox ligamentum nuchae elastin (60 mesh; Elastin Products Corp., Pacific, Mo.) was labeled with NaB[^{3}H]$_4$ and assayed as described by McKerrow et al. (11). The radiolabeled elastin was suspended to a final concentration of 2 mg/ml in a 300 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$ buffer. A 100-μl sample was mixed with 50 to 100 μl of enzyme solution and sufficient H_2O to bring the reaction volume up to 300 μl and incubated at 37°C for 18 to 24 h. After this incubation period the undigested elastin was sedimented by centrifugation for 5 min in a microfuge, 100 μl of the supernatant was mixed with 7 ml of Opti-Fluor (Packard Instrument Co., In., Downers Grove, Ill.) scintillation fluid, and the radioactivity was counted in a liquid scintillation spectrophotometer (Beckman model LS 100C). Control incubations with water were included with each assay. The total amount of radioactive elastin available for degradation was determined by adding 10 μl (160 μg) of 2× crystallized porcine pancreatic elastase (Sigma) to a control reaction. One unit of elastase activity will degrade 1 μg of elastin per h.

Keratin degradation. Keratin degradation was assayed by using keratin azure (Sigma), a dyed wool product. Purified 27,000-M_r proteinase (approximately 1 μg) was incubated with 5 mg of keratin azure in a 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$ buffer for 48 to 72 h at 37°C. The final reaction volume was 1 ml. After incubation, the reaction was spun in a microfuge for 5 min. The degradation of keratin azure was measured by determining the A_{595} of the supernatant in a spectrophotometer. A change of 0.01 A_{595} unit per h equalled 1 U of keratinase activity.

Degradation of macromolecular substrates. Five micrograms of type I collagen (from human fetal membrane; Calbiochem), type III collagen (from human fetal membrane; Calbiochem), type IV collagen (from EHS tumor; Collaborative Research, Inc., Lexington, Mass.), type V collagen (from human fetal membrane; Calbiochem), laminin (murine; Collaborative Research), or fibronectin (human; Collaborative Research) was incubated with 25 μl of the purified 27,000-M_r proteinase (1.1 μg of protein). In some experiments the enzyme was preincubated for 20 min with 1.0 μM Phe-Gly-Ala-Leu-CH_2Cl before it was added to the reaction mixture. The reaction volume (50 μl) was brought up to a final concentration of 100 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–1 mM $CaCl_2$ and incubated at 34°C for 18 h. A sample (10 μl) was mixed with an equal volume of 2× reducing sample buffer and boiled, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis on 4% polyacrylamide stacking gels and 7% polyacrylamide resolving gels and stained with silver.

R-22 assay. [^{3}H]proline-labeled R-22 cell extracellular matrix was prepared and analyzed for degradation of its glycoprotein, elastin, and collagen components as previously described (6, 10). Concentrated *T. rubrum* CM from station-

ary-phase cultures (25 μl; 250 μg of protein) or purified 27,000-M_r proteinase (25 μl; 1μg of protein) was added to wells with 475 μl of 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$ and incubated for 18 h at 37°C. The degradations of the various constituents of the matrix were compared with those in control wells that contained only buffer in the original proteinase digestion. Eight wells of each sample were digested, and the data were analyzed by a computer program (R-22 degradation) designed by William Keene, University of California, Berkeley.

Hydrolysis of peptide substrates. The rates of hydrolysis of the peptide-*p*NA substrates (succinyl-Ala-Ala-Pro-Phe-*p*NA was obtained from Vega, Tucson, Ariz.; other succinyl-peptide-*p*NA substrates were kindly provided by C. Largman, Veterans Administration Hospital, Martinez, Calif.) were measured by A_{410} on a Gilford spectrophotometer fitted with a model 6050 chart recorder. The reactions for the 27,000-M_r proteinase were initiated by adding 30 μl (25 pmol) of the enzyme to 0.5 ml of a solution containing substrate in 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$. Substrate concentrations were varied over a 10-fold range of 0.1 to 1.0 mM. Because of solubility problems associated with *t*-butyloxy-carbonyl (Z)-Ala-Ala-Leu-*p*NA (Peninsula Laboratories, Belmont, Calif.), the conditions were different for this substrate. For these reactions, 200 μl of substrate in dimethylformamide (Sigma) was added slowly to 925 μl of 100 mM Tris hydrochloride (pH 8.0)–1 mM $CaCl_2$, and then 75 μl of the 27,000-M_r proteinase (75 pmol) was added to this buffered solution of substrate. The substrate, made up as a 0.5-mg/ml stock in dimethylformamide, was varied over a 10-fold range of 0.02 to 0.2 mM. An ε value of 8,800 mol cm^{-1} at 410 nm was used for the liberated 4-nitroaniline (5), and the kinetic constants were determined from the initial rates of 4-nitroaniline appearance. The data were plotted, with least-squares analysis, by the method of Lineweaver and Burk (8). Five to seven points were measured for each plot, and correlation coefficients were calculated. Correlation coefficients were no lower than 0.97.

RESULTS

Because *T. rubrum* parasitizes the keratinized tissues of humans, we hypothesized that this organism would secrete proteinases able to cleave available proteins into readily metabolized sources of carbon, nitrogen, and sulfur. There are several proteinases, detectable by substrate gel electrophoresis, that are expressed by *T. rubrum* (3) (see Fig. 2, lane C). Two of the major proteolytic species (M_r, 93,000 and 71,000) found in *T. rubrum* CM have been purified (3). We were interested in purifying the other abundant gelatinolytic activity in *T. rubrum* CM, the 27,000-M_r proteinase, to determine its substrate specificity, its relationship to the previously purified enzymes, and its role in fungal metabolism.

Purification of the 27,000-M_r proteinase. Fungal CM was concentrated by $(NH_4)_2SO_4$ precipitation and loaded onto a concanavalin A column (Fig. 1). The flowthrough of this column contained the majority of A_{280}-absorbing material. Analysis on gelatin substrate gels indicated that these fractions contained 124,000-, 71,000-, and 27,000-M_r activities (Fig. 2A). A 53,000-M_r activity was detected when the concanavalin A column was developed with α-methyl mannoside (Fig. 2A), as were the 124,000-, 71,000-, and 27,000-M_r activities. The flowthrough fractions (typically numbers 1 through 10) of the concanavalin A column were concentrated and then loaded on a Polyanion SI anion-exchange column

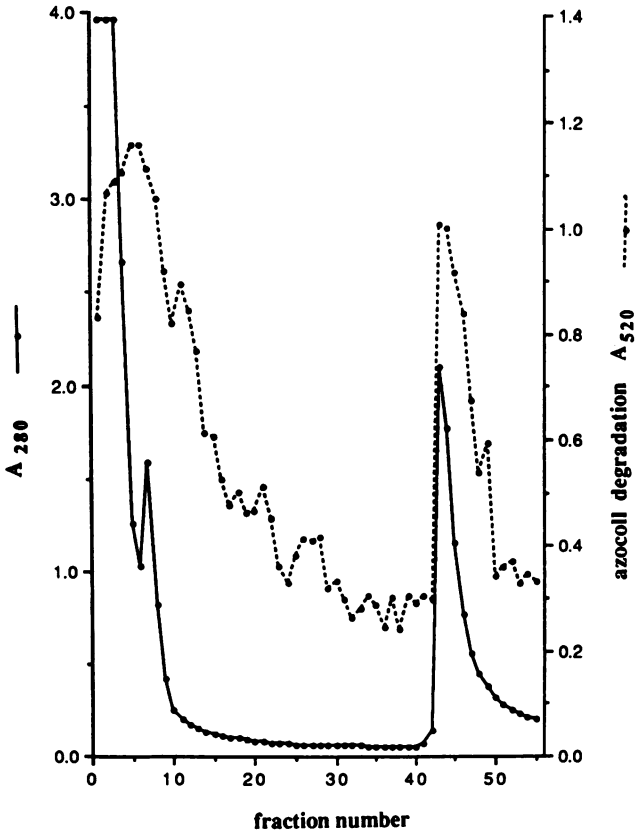

FIG. 1. Concanavalin A affinity chromatography of *T. rubrum* CM. Proteins in fungal CM (20 to 50 mg) were dissolved in 100 mM Tris hydrochloride (pH 7.4)–500 mM NaCl–0.02% NaN_3 and applied to a 11-ml disposable column packed with 8 ml of concanavalin A-Sepharose 4B equilibrated with the same buffer. The proteins were allowed to interact with the column for 30 min and then unbound proteins were eluted with the loading buffer. After fraction 40 (100 ml), the bound proteins were eluted with 100 mM Tris hydrochloride (pH 7.4)–500 mM NaCl–200 mM α-methyl mannoside–0.02% NaN_3, and a sample was assayed for A_{280} and for degradation of azocoll. Samples were eluted at a flow rate of 45 ml/h, and 2.5-ml fractions were collected.

equilibrated with 50 mM bis-Tris (pH 5.8). The column was developed with a linear gradient of salt from 0 to 500 mM NaCl. Three peaks of azocollytic activity were resolved (Fig. 3). The flowthrough contained higher-molecular-weight activities, including the 124,000-M_r proteinase. The second peak contained the 71,000-M_r proteinase, and in the third peak the 27,000-M_r activity was detected (Fig. 2B). The salt wash contained the 27,000-M_r activity and two faster migrating species of M_r 25,000 and 23,000. When the material that adsorbed to the concanavalin A column was chromatographed on the Polyanion SI column, a similar elution profile was observed, with one exception: there was an additional peak of activity around fraction 24 that contained the 53,000-M_r proteinase. However, this activity was always contaminated with the 27,000-M_r species. The 53,000-M_r activity, like all *T. rubrum* gelatin-degrading proteinases, was inhibited by PMSF on gelatin substrate gels and was able to cleave the substrate succinyl-Ala-Ala-Phe-aminofluorocoumarin impregnated in cellulose acetate membranes (data not shown). The purification of the 27,000-M_r proteinase is summarized in Table 1. A purification of approximately

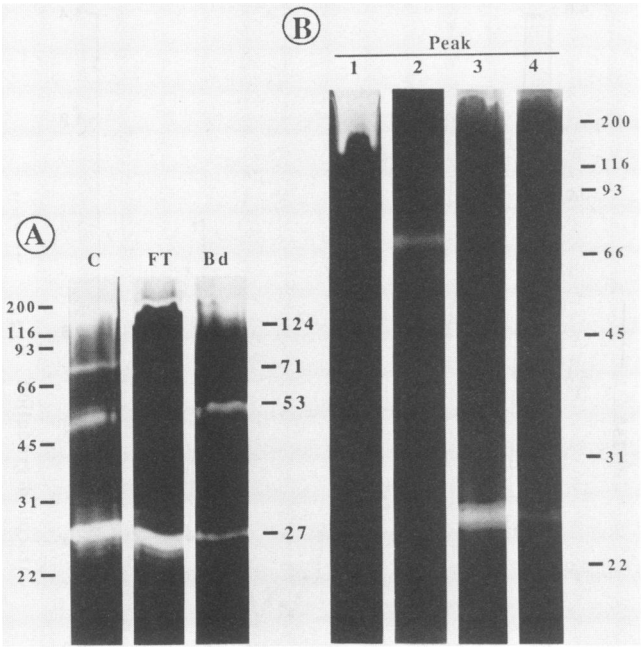

FIG. 2. Gelatin substrate gel of fractions from the purification of the 27,000-M_r proteinase. (A) The crude CM (C) and samples from the flowthrough (FT) and bound (Bd) fractions of a concanavalin A column were analyzed on gelatin-containing substrate gels. Molecular weight markers (in thousands) are indicated at the left, and relative molecular weights (in thousands) of the major proteolytic species are indicated at the right. (B) Lanes 1, 2, 3, and 4 contained peaks I, II, III, and IV, respectively, of a Polyanion SI column analyzed on gelatin substrate gels. Molecular weight markers (in thousands) are indicated at the right.

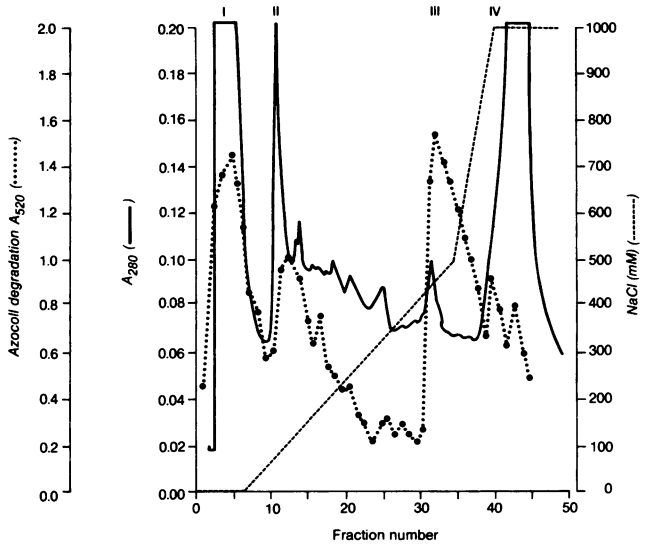

FIG. 3. Anion-exchange chromatography of pooled fractions from the flowthrough of a concanavalin A column. Active fractions from the flowthrough of a concanavalin A column (typically fractions 1 through 10) were pooled, concentrated, and dialyzed against water. The concentrated proteins (in 0.5 ml) were mixed with an equal volume of 50 mM bis-Tris hydrochloride (pH 5.8) and applied to a Polyanion SI column that had previously been equilibrated with the same buffer. After 6 ml of the start buffer had flowed through the column, proteins that interacted with the column were eluted off the matrix by a 30-ml linear gradient of salt (– – –) from 0 to 500 mM NaCl in the same buffer. Samples were eluted at a flow rate of 0.5 ml/min, and 1-ml fractions were collected. Samples from each fraction were assayed for azocollytic activity (●).

32-fold with a yield of 9.4% was achieved. The yield and purification are artificially low, as there are many activities in *T. rubrum* CM that degrade azocoll and some of the proteinases have a higher specific activity for azocoll than the 27,000-M_r proteinase (e.g., the 93,000-M_r enzyme).

Characterization of the purified 27,000-M_r proteinase. When samples of the fractions containing the 27,000-M_r proteinase were electrophoresed on a nonreducing SDS-polyacrylamide gel, a single band at approximately M_r 27,000 was visualized (Fig. 4). Interestingly, when reduced, the 27,000-M_r proteinase ran with a relative molecular weight of 44,000. To confirm this result, the 27,000-M_r proteinase was labeled with [^{3}H]diisopropyl fluorophosphate and electrophoresed under reducing and nonreducing conditions. Again, under nonreducing conditions the proteinase ran as a 27,000-M_r protein, and under reducing conditions a protein of M_r 44,000 was detected (Fig. 5). No labeling was detected in control reactions that contained buffer and no enzyme. This proteinase was also inhibited by other inhibitors of serine proteinases, including 1 mM PMSF (94%) and 0.2 μM Phe-Gly-Ala-Leu-CH_2Cl (92%) (Table 2). There was inhibition by the thiol proteinase inhibitor *N*-ethylmaleimide (53%) but little by the metalloproteinase inhibitor 1,10-phenanthroline (7%). There was significant inhibition by chloromethylketone inhibitors that contained a large, hydrophobic amino acid next to the chloromethyl group, whereas Ala-Ala-Pro-Ala-CH_2Cl had little inhibitory activity.

Degradation of macromolecular substrates by the purified 27,000-M_r proteinase. The purified proteinase degraded azocoll (2,520 U/mg), elastin (30.2 U/mg), and keratin azure (77 U/mg). Additionally, this enzyme degraded several components of the extracellular matrix, including laminin, fibronectin, type III collagen, and type IV procollagen (Fig. 6). This degradation was prevented by the addition of 1.0 μM Phe-Gly-Ala-Leu-CH_2Cl to the reaction mix, except in the case of the degradation of fibronectin. The degradation of type III collagen was never complete; residual protein could always be detected in the enzyme plus substrate incubations. There was no degradation of types I and V collagen.

The ability of the purified enzyme to degrade connective tissue macromolecules in an interactive matrix was also assessed. The purified enzyme was able to degrade 20.8 ± 2.1% of an in vitro model of the dermis, the R-22 matrix. This matrix contains ^{3}H-labeled glycoprotein, elastin, and collagen components that can be assayed for by their sensitivity to trypsin, elastase, and collagenase. A significant amount of the trypsin-sensitive proteins (43.9 ± 2.8%), a small amount of the elastase-sensitive material (14.0 ± 5.0%), and a variable amount of the collagenase-sensitive molecules (6.2 ± 6.3%) were degraded. Crude fungal CM was able to degrade 57.7 ± 2.2% of the total available radioactivity. All components of the extracellular matrix were degraded, including 24.9 ± 7.1% of the trypsin-sensitive material, 58.0 ± 3.8% of the elastase-sensitive material, and 88.9 ± 14.4% of the collagenase-sensitive material. Control incubations with buffer alone released less than 2.3 ± 0.8% of the total radioactivity present in the matrix.

pH optimum and calcium dependence of the 27,000-M_r proteinase. The 27,000-M_r proteinase had a pH optimum at pH 8.0 (Fig. 7) and a calcium dependence of 2 mM.

Degradation of peptide-pNA substrates by the purified enzyme. The chloromethylketone inhibition data indicated that the 27,000-M_r activity was a serine proteinase that might

TABLE 1. Summary of purification of the 27,000-M_r proteinase from the CM of stationary-phase *T. rubrum* cultures

Purification step	Total protein (mg)	Total activity[a] (U)	Sp act (U/mg)	Purification factor (fold)	Yield (%)
Culture filtrate [70% $(NH_4)_2SO_4$ fraction]	45.90	3,660.1	79.7	1.0	100.0
Affinity chromatography (concanavalin A column)	8.32	1,004.5	120.7	1.5	27.4
Anion exchange (Polyanion SI)	0.14	352.8	2,520.1	31.6	9.6

[a] Azocoll was used as the substrate.

hydrolyze peptide substrates with a leucine or a phenylalanine in the P-1 site. Like the 93,000- and 71,000-M_r proteinases the 27,000-M_r activity cleaved peptide substrates with hydrophobic amino acids in the P-1 site (Table 3). The highest k_{cat}/K_m was for the Z-Ala-Ala-Leu-*p*NA substrate (1,614 M^{-1} s^{-1}). There was neither activity against a pancreatic elastase substrate, succinyl-Ala-Ala-Ala-*p*NA, nor against a trypsin substrate, Z-Lys-Glu-Arg-*p*NA.

DISCUSSION

A major proteolytic species of M_r 27,000 was purified from the CM of *T. rubrum*. The 27,000-M_r proteinase shares a number of characteristics with two proteinases, of M_r 93,000 and 71,000, previously purified from *T. rubrum* CM (3). It is a serine proteinase with a pH optimum of 8.0, it degrades azocoll, and it binds and hydrolyzes peptide substrates with hydrophobic amino acids in the P-1 site. Although the 93,000- and 27,000-M_r proteinases share a similar molecular weight under reducing conditions, they do not appear to be the same enzyme. A summary of the characteristics of the three enzymes that have been purified can be found in Table 4. The 27,000-M_r proteinase is a poor elastase and keratinase, has a pI less than 5.0 (as determined by chromatography on a Mono P chromatofocusing column; G. Apodaca, Ph.D. thesis, University of California, San Francisco, 1989), cleaves the peptide substrate succinyl-Ala-Ala-Pro-Phe-*p*NA with a specific activity 18-fold less than that of the 93,000-M_r species, and is not formed by the dimerization of two smaller subunits.

The 27,000-M_r proteinase appears to result from the tight folding of a 44,000-M_r protein, since its nonreduced molecular weight is less than the reduced molecular weight. This folding could be mediated by intrachain disulfide bonds. This is one mechanism thought to account for the faster migration of nonreduced proteins, as compared with their reduced counterparts, and has been described for several proteins, including pig skeletal muscle actin and kappa light chains (1).

The other proteolytic species of M_r 23,000, 25,000, 53,000, and 124,000 and the high-molecular-weight activities that have been identified have unknown substrate specificities and relationships with the other proteinases that have been purified. The smaller species could represent proteolytic cleavages of the 27,000-M_r proteinases. The high-molecular-weight smear could represent multimers of lower-molecular-weight proteinases or distinct, large proteinase species. It appears that the 53,000-M_r proteinase is a glycoprotein, because it interacted strongly with the concanavalin A column. It was not found in the flowthrough of the column but was eluted with α-methyl mannoside. An alternative explanation would be that it interacts weakly with an inhibitor that is a glycoprotein. However, this would have to be a very specific inhibitor of the 53,000-M_r proteinase, because, like other *T. rubrum* proteinases that have been characterized, this enzyme is a serine proteinase that cleaves substrates with a hydrophobic amino acid in the P-1 site. The purification of this enzyme would allow for the characterization of not only its interaction with lectin columns but its general substrate specificities and regulation as well. It is not clear whether the other proteinases are glycoproteins. Their elution with the concanavalin A-binding material may represent a nonspecific interaction with the large number of

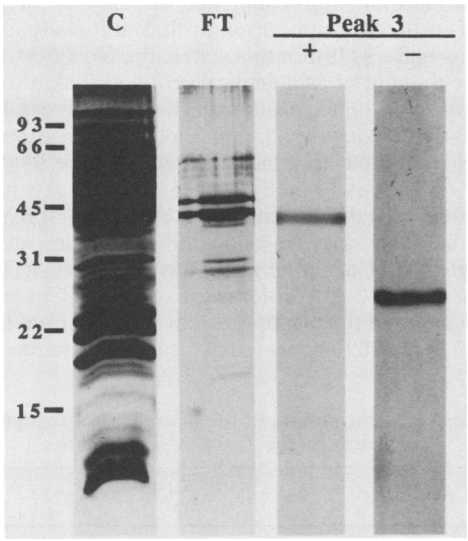

FIG. 4. SDS-polyacrylamide gel electrophoresis of fractions from the purification of the 27,000-M_r proteinase. Fractions from the purification steps were resolved on a 14% polyacrylamide gel. The gel was stained with silver. Lanes: C, crude CM; FT, flowthrough from the concanavalin A column; Peak 3, fractions from the Polyanion SI column reduced (+) or left unreduced (−) before being resolved by electrophoresis. Molecular weight markers (in thousands) are indicated at the left.

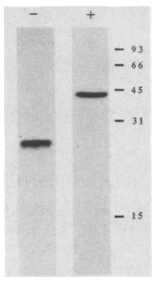

FIG. 5. [^{3}H]diisopropyl fluorophosphate labeling of the 27,000-M_r proteinase. The purified 27,000-M_r proteinase was labeled with [^{3}H]diisopropyl fluorophosphate as described in Materials and Methods. The reduced (+) or unreduced (−) proteinase was analyzed on a 4% polyacrylamide stacking gel with a 14% polyacrylamide resolving gel. The gels were fluorographed, and labeled proteinase was detected by autoradiography. Molecular weight markers (in thousands) are indicated at the right.

TABLE 2. Inhibition profile of the 27,000-M_r proteinase

Inhibitor	Final concn	% Inhibition[a]
α_1-Proteinase inhibitor	75 μg/ml	69
PMSF	1 mM	94
N-Ethylmaleimide	5 mM	53
1,10-Phenanthroline	1 mM	7
Phe-Gly-Ala-Leu-CH_2Cl	0.2 μM	92
Ala-Ala-Pro-Leu-CH_2Cl	0.2 μM	77
Ala-Ala-Pro-Phe-CH_2Cl	0.2 μM	59
Ala-Ala-Pro-Ala-CH_2Cl	0.2 μM	6

[a] Data are reported as percent inhibition of activity as compared with a positive enzyme control. For inhibitors requiring ethanol or dimethyl sulfoxide in stock solutions, an equivalent amount of solvent plus enzyme was run as a control.

proteins that are contained in these fractions. It could also represent a nonspecific interaction with the column. The 27,000-M_r proteinase and a high-molecular-weight activity (around M_r 200,000) were detected in all of the fractions that came after the void. These proteinases could contain sterically hindered mannose residues that would prevent them from interacting as strongly with the column as those of the

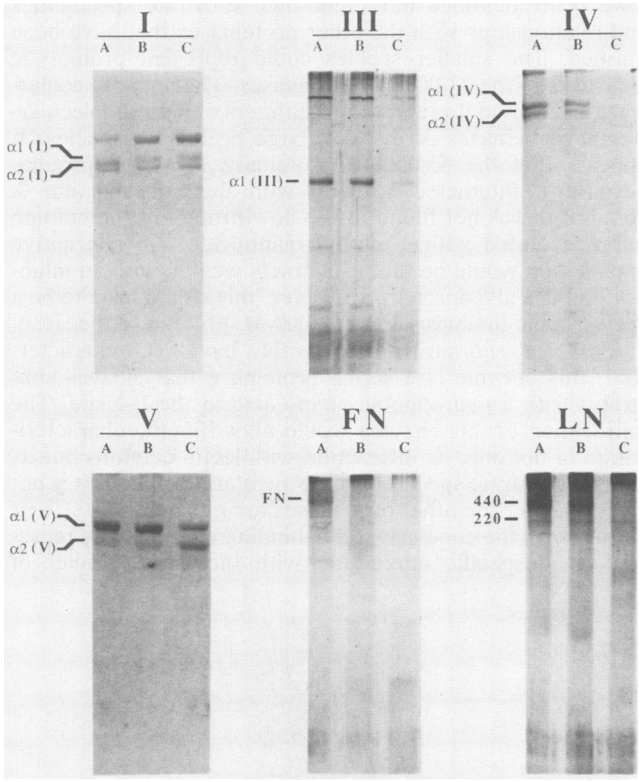

FIG. 6. Degradation of macromolecular substrates by the purified 27,000-M_r proteinase. Samples (5 μg) of the following substrates were incubated with 1.1 μg of the 27,000-M_r proteinase in 100 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–mM $CaCl_2$ at 34°C for 18 h: type I collagen (I), type III collagen (III), type IV procollagen (IV), type V collagen (V), fibronectin (FN), and laminin (LN). A sample from the reactions was reduced, the proteins were resolved by SDS-polyacrylamide gel electrophoresis on 4% polyacrylamide stacking gels and 7% polyacrylamide resolving gels and stained with silver. Lanes: A, substrate alone; B, substrate plus enzyme preincubated with 1.0 μM Phe-Gly-Ala-Leu-CH_2Cl; C, substrate plus enzyme. The locations of the collagen chains and the subunits of laminin are indicated at the left of each substrate.

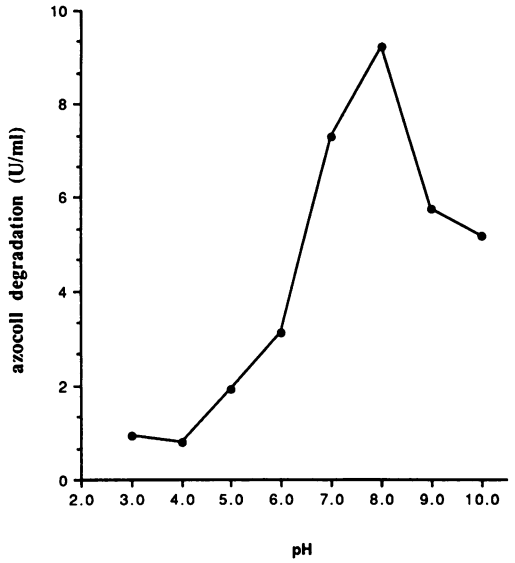

FIG. 7. pH optimum of the 27,000-M_r proteinase. The buffer in the pH range of 3 to 6 was 100 mM sodium acetate hydrochloride–1 mM $CaCl_2$, that in the pH range of 7 to 8 was 100 mM Tris hydrochloride–1 mM $CaCl_2$, and that in the pH range of 9 to 10 was 100 mM glycine-NaOH–1 mM $CaCl_2$. Azocoll was used as the substrate.

53,000-M_r proteinase, or the interaction could be due to the weak binding of the concanavalin A with other carbohydrate moieties.

The primary function of the proteinases that have been purified is probably to degrade the keratins, and other proteins associated with the stratum corneum and nails, into carbon, nitrogen, and sulfur sources easily assimilated by the fungus. The 93,000- and 71,000-M_r proteinases are excellent keratinases (3). Although the 27,000-M_r activity is a poor keratinase, it still could act on the 5% of proteins contained in the stratum corneum that are easily extracted by aqueous buffers (19). In this sense, the 27,000-M_r proteinase can be thought of as a general proteinase akin to trypsin or chymotrypsin. This proteinase could also catalyze the hydrolysis of keratins that had previously been attacked and denatured by actual keratinases, including the 93,000- and 71,000-M_r activities. Finally, these proteinases have the ability to degrade components of the extracellular matrix and could mediate the tissue invasion and destruction seen when *T. rubrum* infects an immunocompromised host (17). The 71,000- and 93,000-M_r proteinases are excellent elastases and could degrade the elastin present in the dermal layers of the skin. The 27,000-M_r proteinase can degrade fibronectin,

TABLE 3. Kinetic studies of the hydrolysis of tetrapeptide substrates by the 27,000-M_r proteinase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Succinyl-Ala-Ala-Pro-Phe-*p*NA	4.68	7.36	1,573
Succinyl-Ala-Ala-Pro-Ile-*p*NA	No activity[a]		
Succinyl-Ala-Ala-Pro-Val-*p*NA	No activity		
Z-Ala-Ala-Leu-*p*NA	1.01	1.63	1,614
Succinyl-Ala-Ala-Ala-*p*NA	No activity		
Z-Gly-Gly-Gly-*p*NA	No activity		
Z-Lys-Glu-Arg-*p*NA	No activity		

[a] <0.01 A_{410} unit per 10 min in 1 mM substrate.

TABLE 4. Summary of the characteristics of three proteinases purified from *T. rubrum* CM

Characteristic	Results for the following proteolytic enzyme:		
	M_r 93,000[a]	M_r 71,000[a]	M_r 27,000
M_r under reducing conditions	44,000	36,000	44,000
pI	7.8	6.5	<5.0
pH optimum	8.0	8.0	8.0
Ca^{2+} dependence (mM)	1.0	1.0	2.0
Class	Serine	Serine	Serine
Ala-Ala-Pro-Phe-*p*NA degradation (M^{-1} s^{-1})	27,028	3,837	1,573
Substrate degraded (U/mg)			
Azocoll	128,000	735	2,520
Elastin	59,000	19,000	30
Keratin[b]	200	32	77

[a] The 93,000- and 71,000-M_r proteinases have been purified previously from *T. rubrum* CM (3).

[b] All assays are comparable except the keratin degradation assays, in which a different substrate was used for the 93,000- and 71,000-M_r proteinases (3).

laminin, type III collagen, and type IV collagen. Although this proteinase does not degrade type I collagen, there is probably an activity secreted by *T. rubrum* that can. Crude CM from this fungus was able to degrade the majority of collagen contained in an in vitro model of the dermis, the R-22 matrix. The identification of the actual collagenase(s) will require further experimentation.

A role for the 27,000-M_r proteinase in fungal metabolism is suggested by recent experiments that show that expression of this enzyme is repressed by both inorganic and organic sources of sulfur (2; Apodaca and McKerrow, J. Cell Biol., 1989). When log-phase cultures of *T. rubrum* are starved for a source of sulfur, expression of general proteolytic enzymes, including the 27,000-M_r proteinase, is derepressed. This strongly suggests that the 27,000-M_r proteinase has an important function in nutrition gathering by this organism.

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