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Isolation of a Keratinolytic Proteinase from *Trichophyton mentagrophytes* with Enzymatic Activity at Acidic pH

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A keratinolytic proteinase with enzyme activity at acidic pH was isolated from culture filtrates of *Trichophyton mentagrophytes*, a major pathogenic fungus of dermatophytosis. The molecular weight of the proteinase was estimated to be 41,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 38,000 by gel filtration. The isoelectric point was determined to be 3.9. The proteinase had a pH optimum of 4.5 for keratin and 5.5 for hemoglobin. This enzyme hydrolyzed the synthetic chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-MCA (K_m, 0.59 mM), and its activity was strongly inhibited by chymostatin. Previously reported proteinases from dermatophytes have had enzyme activities in neutral or alkaline pH; however, healthy skin has a weakly acidic pH. Thus, the purified proteinase which has an optimal activity at acidic pH and hydrolyzes skin constituents could be an important virulence factor in dermatophytosis.

Dermatophytes invade hair, nail, and skin to cause a superficial mycosis. The hydrolysis of keratin by proteinases (keratinases) is very likely an important aspect of fungal pathogenesis. Some extracellular proteinases have been purified from species of *Trichophyton* (2, 4, 14, 21–24) and *Microsporum* (18, 19). However, these proteinases have enzyme activities only at neutral or alkaline pH. Because normal human skin surface has a weakly acidic pH (3), it is doubtful whether those proteinases work as virulence factors. For these reasons we attempted to isolate an extracellular proteinase with enzyme activity at acidic pH from a clinical isolate of *Trichophyton mentagrophytes*.

MATERIALS AND METHODS

Organism and growth conditions. A fresh clinical isolate of *T. mentagrophytes* (strain M-420) was collected from a patient with tinea pedis. It was identified as *T. mentagrophytes* by standard morphological and physiological studies (12, 13, 16). Liquid culture medium, which was sterilized by membrane filtration (GS, 0.22-μm pore size; Millipore Corp., Bedford, Mass.), had the following constituents per liter: 12 g of yeast carbon base (Difco Laboratories, Detroit, Mich.), 0.05 g of inositol, 0.01 g of thiamine, and 0.01 g of pyridoxine (Sigma Chemical Co., St. Louis, Mo.). To induce enzyme production, insoluble keratin powder from human plantar skin (as previously described [6, 11]) was added at a concentration of 4 mg/ml of culture medium. The keratin powder was sterilized separately with ethylene oxide gas. A stock culture was cultured in Sabouraud dextrose broth, and one loop of the organism was added to the proteinase-inducing culture medium. Erlenmeyer flasks (500 ml) containing 200 ml of the keratin-supplemented medium were shaken at 100 cycles per min for 5 days at 27°C. The culture broth was collected after filtration through a 0.22-μm-pore-size membrane filter and was used for subsequent proteinase purification.

Assay of proteinase activity. Proteinase activity was assayed with the above-mentioned insoluble keratin powder [6, 11]. Keratin (5.0 mg) in 50 mM citric acid–100 mM disodium phosphate buffer (pH 4.5) was incubated with 0.2 ml of enzyme solution (total volume, 1.0 ml) for 2 h at 37°C. Incubation was terminated by the addition of 1.0 ml of 10% trichloroacetic acid (TCA) solution. After centrifugation, TCA-soluble peptides in the supernatant were measured by the method of Lowry et al. (9). A_{750} was measured by a Hitachi spectrophotometer (model 100-40), and 1 U of proteinase activity was defined as an increase of 0.10 corrected absorbance value per h. For characterization of purified proteinase, denatured hemoglobin (Sigma H-2625) was also used as a substrate. Protein concentration was determined by the method of Lowry et al. (9).

Purification of extracellular proteinase. All the following procedures were performed at 4°C. The culture filtrate was concentrated by ultrafiltration by using a YM-5 membrane filter (Amicon Corp., Lexington, Mass.) and dialyzed against 20 mM sodium phosphate buffer (pH 7.0). After centrifugation, the supernatant was applied to a DEAE-Sepharose CL-6B column (1.2 by 11 cm) which had been equilibrated with phosphate buffer. The column was flushed with equilibrating buffer, and a 0 to 0.6 M NaCl linear gradient elution was performed in the same buffer at a rate of 25 ml/h; 2.8-ml fractions were collected. Fractions with proteinase activity were concentrated to 2 ml by ultrafiltration (Amicon YM-5) and chromatographed on a Sephacryl S-200 column (1.6 by 100 cm) at the same flow rate. A molecular weight estimate for the proteinase was performed by gel filtration with calibration standards (Pharmacia Inc., Piscataway, N.J.).

Electrophoresis. (i) SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (7). Samples were treated with 1% SDS, 1% 2-mercapthoethanol, and 2 min of boiling at 100°C. Electrophoresis was done with a 4.5% stacking gel and a 12% running gel. The protein was stained with silver by the method of Merril et al. (10). Sigma SDS-7 calibration proteins were used for molecular weight estimation.

(ii) Isoelectric focusing. Isoelectric focusing was performed in a 5% polyacrylamide slab gel with 6.25% Pharmalyte (Pharmacia; pH 3–10) by modifying the manufacturer's

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instructions. The pH gradient profiles were estimated by cutting a gel run in parallel into 4-mm pieces.

pH optimum. pH optimum of the enzyme was assayed with 50 mM citric acid–100 mM disodium phosphate buffer (pH 3.0 to 7.5) and 50 mM Tris-maleic acid buffer (pH 8.0 to 10.0).

Effect of proteinase inhibitors and metal ions. Stock solutions (0.1 ml) of proteinase inhibitors or metal ions were preincubated with 0.65-ml enzyme solutions in proteinase assay buffer for 15 min at 37°C. Then, 0.25 ml of 20-mg/ml denatured hemoglobin solution was added (total volume, 1.0 ml) and proteinase activities were assayed by the method described above. Residual activity was compared with that of the reaction mixture which did not contain any inhibitor. The following stock solutions of proteinase inhibitors and metal ions were prepared: soybean trypsin inhibitor, diisopropyl fluorophosphate, *N*-ethylmaleimide, iodoacetamide, EDTA, $CaCl_2$, $SrCl_2$, $MgCl_2$, or $ZnCl_2$ (Sigma) in distilled water; chymostatin (Peptide Institute, Inc., Tokyo, Japan) in 0.01 N acetic acid; pepstatin (Peptide Institute) in 0.01 N sodium hydroxide; and phenylmethylsulfonyl fluoride (Sigma) in 2-propanol. An equivalent amount of solvent was run as a control.

Determination of kinetic constants. Kinetic constants of the purified enzyme were measured for hemoglobin and synthetic peptide substrates. Denatured hemoglobin (0.25 to 2.0 mg) in proteinase assay buffer (pH 5.5) was incubated with 20 μl of an enzyme solution (0.5 μg) at 37°C (total volume, 1.0 ml). Every 10 min the reaction was stopped by the addition of 1.0 ml of 10% TCA solution, and the TCA-soluble peptides were assayed by the method of Lowry et al. (9). The synthetic substrates Bz-Arg-MCA (25), Suc-Ala-Ala-Pro-Phe-MCA (15) (Peptide Institute), Cbz-Ala-Arg-Arg-4MNA (17), and Bz-Arg-Gly-Phe-Phe-Pro-4MNA (1) (Bachem) were stored in 100% dimethyl sulfoxide. Enzyme solution (0.5 μg) was reacted with 0.05 to 0.5 mM of each substrate in proteinase assay buffer (pH 5.5) at 25°C. Released compounds were monitored continuously with a Hitachi fluorescence spectrophotometer (model 850). Kinetic constants for each substrate were determined by Lineweaver-Burk analysis (8).

Natural substrate reactivity. The hydrolysis of natural substrates, including some constituents of skin, was measured as the amount of TCA-soluble peptides by the Lowry method (9). Each (5.0 mg) of the following substrates was dissolved in proteinase assay buffer: human stratum corneum, hemoglobin, α-casein (Sigma), bovine serum albumin (Sigma A-7030), type I insoluble collagen (Sigma), and defatted human hair (11). The substrates were incubated with 0.5 μg of purified enzyme solution at 37°C for 2 h (total volume, 1.0 ml).

RESULTS

Purification of an extracellular proteinase. After 5 days of growth, the culture filtrate of the fungus was collected. The pH of the culture filtrate was 6.5. Figure 1 shows the pH profile of the proteinase activity in the culture filtrate. Proteolytic activity was observed as a sharp peak in acidic pH and as a broad peak in neutral and alkaline pH. Since neutral proteinases have been isolated (4, 21), isolation of the proteinase with enzyme activity in acidic pH was attempted. The elution pattern of the proteinases in a DEAE-Sepharose CL-6B column can be seen in Fig. 2. Three peaks of proteinase activity at acidic pH were observed. Of these, the third peak showed the greatest proteinase activity in spite of a decreased protein content. The first and second peaks had extremely high proteinase activities at pH 8.5, while the third peak lacked activity at alkaline pH (data not shown). Thus, the third peak fractions (tubes from 25 to 27) were pooled and subjected to Sephacryl S-200 gel filtration (Fig. 3). Proteinase activity at pH 4.5 was observed in a single peak which coincided with a peak of A_{280}. The purification protocols are summarized in Table 1. Purification and recovery of the enzyme were 17-fold and 1.6%, respectively.

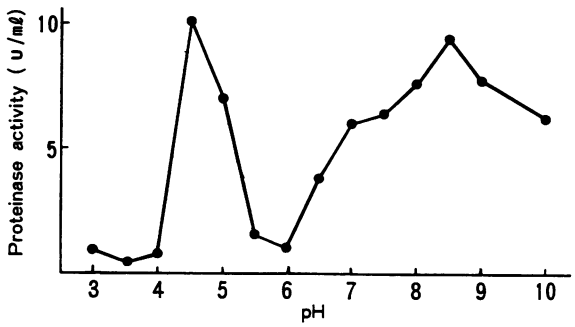

FIG. 1. pH profile of proteinase activities in the culture filtrate. After 5 days of cultivation, proteinase activity of the culture filtrate was assayed with insoluble keratin powder as a substrate. TCA-soluble peptides were assayed by the Lowry method (9). One unit of proteinase activity was defined as an increase of 0.10 corrected absorbance value per h.

Characteristics of the purified proteinase. (i) Stability. The purified proteinase was unstable and lost activity during the purification; therefore, the quantity recovered was not large (Table 1). The proteinase was routinely stored at −70°C, which permitted studies for at least 2 months.

(ii) Purity. The purified proteinase migrated as a single protein band in both SDS-PAGE (Fig. 4) and isoelectric focusing. Protein loaded on the gel could be stained by silver, but not by Coomassie blue.

(iii) Molecular weight. Molecular weight of the purified

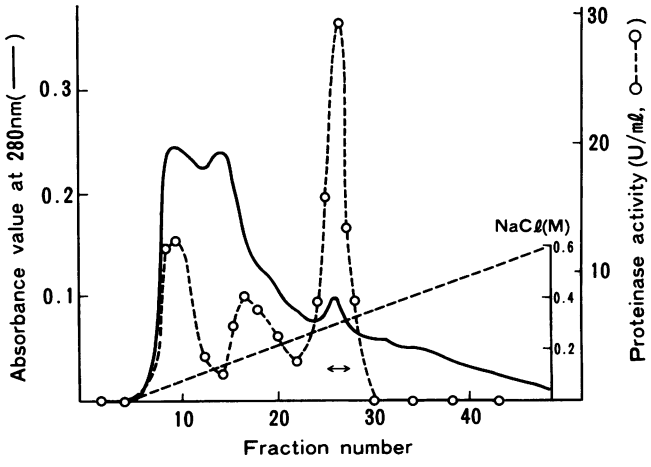

FIG. 2. DEAE-Sepharose CL-6B column chromatography. Culture filtrate, applied to a DEAE-Sepharose CL-6B column (1.2 by 11 cm), was eluted by the NaCl linear gradient method, and 2.8-ml fractions were collected. Proteinase activity was assayed at pH 4.5 with insoluble keratin powder as a substrate. The third peak fractions of proteinase activities were collected for subsequent gel filtration.

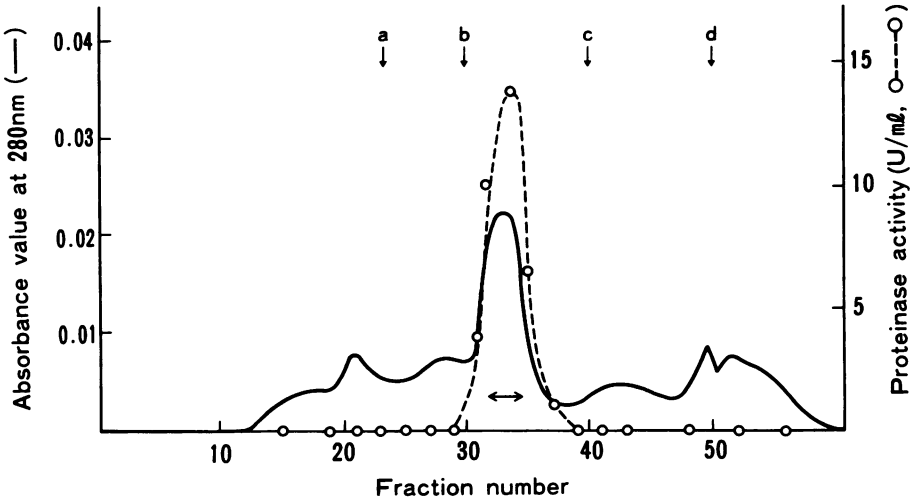

FIG. 3. Sephacryl S-200 gel filtration. Samples, chromatographed on a Sephacryl S-200 column (1.6 by 100 cm), were eluted in 2.8-ml fractions. Proteinase activity was assayed at pH 4.5 with insoluble keratin powder as a substrate. Peaks (molecular weight): a, bovine serum albumin (67,000); b, ovalbumin (43,000); c, chymotrypsinogen A (25,000); d, RNase A (13,700).

proteinase was estimated to be 38,000 by gel filtration and 41,000 by SDS-PAGE (Fig. 4).

(iv) Isoelectric point. Isoelectric point of the purified proteinase was estimated to be 3.9.

(v) pH optimum. The pH optimum for the substrate keratin was 4.5, while the pH shifted to 5.5 for hemoglobin (Fig. 5) and other synthetic substrates. Purified proteinase lost activity at pH values less than 3.0 and greater than 7.0 for all the substrates.

Effect of proteinase inhibitors and metal ions. The effects of proteinase inhibitors and metal ions on enzyme activity can be seen in Table 2. The serine proteinase inhibitors phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate strongly inhibited the activity, but soybean trypsin inhibitor did not. The chymotrypsin inhibitor chymostatin also strongly inhibited the proteinase activity. Both the cysteine proteinase inhibitors *N*-ethylmaleimide and iodoacetamide and a carboxyl proteinase inhibitor, pepstatin, failed to affect the proteinase activity. The divalent cations Ca^{2+} and Sr^{2+} increased the proteinase activity, while the chelating agent, 5 mM EDTA, mildly decreased the activity.

Kinetic constants. Hemoglobin was an excellent substrate for the proteinase (K_m, 1.84×10^{-5} mM). A synthetic substrate for cathepsin D, Bz-Arg-Gly-Phe-Phe-Pro-4MNA, a trypsin substrate, Bz-Arg-MCA, and a cathepsin B substrate, Cbz-Ala-Arg-Arg-4MNA, were not hydrolyzed by the proteinase. Contrary to what we expected, the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-MCA offered an excellent kinetic constant (K_m, 0.59 mM).

Natural substrate reactivity. The quantities of TCA-soluble peptides from casein, bovine serum albumin, type I insoluble collagen, and hemoglobin were compared with that of human stratum corneum, which was calculated as 100%. The purified proteinase hydrolyzed casein (60%), bovine serum albumin (81%), and type I insoluble collagen (144%) as well as hemoglobin (1,550%). Human hair was not hydrolyzed even when the incubation time was extended to 24 h.

DISCUSSION

Dermatophytosis is a colonization of hair, nails, and skin by dermatophytic fungi. Lesions are generally restricted to the nonliving cornified layers. Keratinolysis by fungal proteinases has been considered a likely part of fungal pathogenicity. Day et al. (4) and Yu et al. (21–24) purified two extracellular and two cell-bound keratinases from the same isolate of *T. mentagrophytes*. They used a growth medium (pH 7.8) that contained defatted hair as a nitrogen source. One of the extracellular proteinases had a molecular weight of 48,000 with an optimal pH of 7.0 and pI 9.4. The second proteinase had a molecular weight of 34,000 and an optimal pH of 9.5–9.8. Reactivities of both of the proteinases were mildly activated by Ca^{2+}, Mg^{2+}, and Ba^{2+} and blocked by EDTA. On the other hand, the two cell-bound keratinases had molecular weights of 440,000 and 20,300, respectively, and a pH optimum of 7.0. The extracellular proteinases of *T. rubrum* reported by Asahi et al. (2) and Sanyal et al. (14) also have optimal proteolytic activity at neutral or alkaline pH and serine proteinase-like characteristics. In this study, we isolated a proteinase that had optimal enzyme activity at acidic pH. Our purified proteinase had an approximate molecular weight of 40,000, an optimal pH of 4.5 for keratin

TABLE 1. Purification of an extracellular proteinase from *T. mentagrophytes*

Step	Total volume (ml)	Total protein (mg)	Total activity (U)	Recovery (%)	Sp act (U/mg)	Purification (fold)
Culture filtrate	800	197	6,080	100	30.8	1
Amicon YM-5	103	16.1	1,220	20	75.8	2.5
DEAE-Sepharose CL-6B	8.4	0.37	160	2.6	432	14
Sephacryl S-200	8.4	0.19	97	1.6	511	17

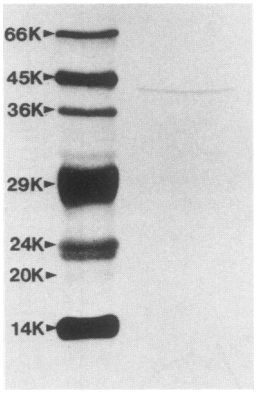

FIG. 4. SDS-PAGE of the purified proteinase. SDS-PAGE was performed with a 12% running gel. A 0.3-μg sample of purified proteinase was loaded and stained with silver.

and 5.5 for hemoglobin, and pI 3.9. These characteristics of the proteinase are quite different from those of the previously reported proteinases. The first and second peak of proteinase activity in DEAE-Sepharose CL-6B chromatography (Fig. 2) could possibly coincide with the proteinases purified by Day et al. (4) and Yu et al. (21–24). It seems important that the purified proteinase has enzyme activities at acidic pH since healthy skin has a weakly acidic pH (10). However, there has been no information about the pH of skin at actual sites of dermatophytosis. No data in this study indicated which proteinases are really responsible for the growth of fungi in vitro. As each proteinase has broad reactivity for pH, we can speculate that the neutral and acidic proteinases work cooperatively, dependent upon pH conditions or affected sites.

The activity of the purified proteinase was strongly inhibited by serine proteinase inhibitors except for soybean trypsin inhibitor. The kinetic constants for the synthetic chymotrypsin substrate and the inhibitor suggested that this proteinase could be a chymotrypsinlike enzyme in spite of its optimal activity at acidic pH. Chymotrypsins generally have optimal activities at around pH 8.0 and are stabilized by Ca^{2+} (5, 20). However, to our knowledge, a chymotrypsin-like enzyme with optimal enzyme activity in acidic conditions has never been purified from bacteria and fungi.

Induction of this proteinase was a consequence of the use of insoluble stratum corneum as a nitrogen source. We could not detect acidic proteinase activity when we used Sabouraud broth or a liquid medium containing defatted hair as a nitrogen source (unpublished data). The quantity and kind of proteinases produced by this organism may well depend on the nitrogen source and environmental pH. However, the mechanism triggering the production of extracellular proteinases is uncertain. Because skin constituents, such as keratin and collagen, were hydrolyzed, the proteinase could enable the organism to invade the stratum corneum by breaking down the insoluble keratin ahead of the organism invasion and supplying the products as nutrients.

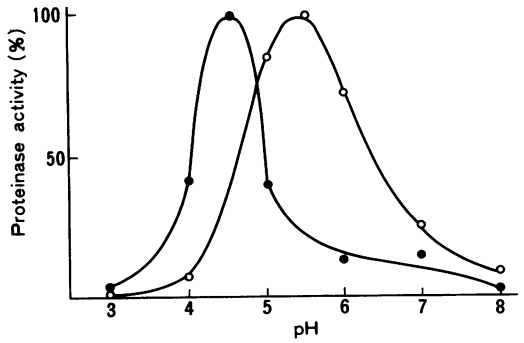

FIG. 5. pH profiles of the purified proteinase. Activities of the purified enzyme were assayed with insoluble keratin powder (●) or denatured hemoglobin (○) as a substrate.

TABLE 2. Effects of proteinase inhibitors and metal ions on proteinase activity

Reagent[a]	Concn	Residual enzyme activity (%)
None		100
PMSF	1 mM	0.0
DFP	1 mM	0.0
SBTI	100 μg/ml	103
Chymostatin	100 μg/ml	0.0
N-Ethylmaleimide	1 mM	137
Iodoacetamide	1 mM	95
Pepstatin	100 μg/ml	119
α_2-Macroglobulin	100 μg/ml	12
Ca^{2+}	1 mM	220
Sr^{2+}	1 mM	238
Mg^{2+}	1 mM	67
Zn^{2+}	1 mM	44
EDTA	5 mM	59

[a] PMSF, Phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor.

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