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Phosphate-Mediated Alteration of the *Microsporum gypseum* Germination Protease Specificity for Substrate: Enhanced Keratinase Activity

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Inorganic phosphate was found to decrease the caseinolytic and ethyl-esterase activities of the *Microsporum gypseum* germination protease. The germination protease possessed exokeratinase (beta-keratinase) activity immediately after release from the fungal spore. After phosphate treatment of the enzyme, the germination protease also possessed endo-keratinase (alpha-keratinase) activity. Phosphate altered the protease's pH optimum from 9.0 to 7.0 and decreased the molecular weight from 33,000 to 16,000. These values were identical to those found for the keratinase. Alpha- and beta-keratinase activities were stimulated in excess of 200-fold by disulfide reducing agents. Natural and suspected keratin degradation products also enhanced keratinase activity. Cell fractionation and in vitro conversion of the alkaline germination protease into a functional keratinase suggested that the subunits comprising the germination protease and the keratinase were of a common origin.

The keratinolytic activity of dermatophytes has been a subject of interest for several years (4, 26, 28). Purified keratinase alone has been shown to be relatively ineffective in digesting native keratin (12). Mechanical penetration by the mycelial "eroding complex" and disulfide reduction prior to proteolytic attack, however, undoubtedly enhances keratinolysis (7, 13, 19, 25).

The ringworm infection caused by *Microsporum gypseum* usually is transmitted via fungal spores. Germination of these spores is accompanied by the release of a single alkaline protease into the germination medium (14). After germination initiation, the hydrolytic activity of the germination protease toward spore coat substrates is inhibited by inorganic phosphate (21, 23).

The present investigation was carried out to determine if the alkaline protease of *M. gypseum* also functioned as a keratinase and to determine if phosphate mediated changes in the enzyme's physical or activity characteristics. Because native keratin appears to be denatured by "sulfitolysis" prior to proteolytic attack by the keratinase complex (13), alkaline thioglycolate extracts of native keratins (10) were used as enzyme substrates. Also, chemical agents which may denature or be denaturation products of native keratin in vivo were examined to determine if these agents could alter the activity or structure of the germination protease and keratinase. A model for sequential protease and keratinase action in the digestion of native keratin, based on the results of this investigation, is presented. Evidence for a common origin and derivation of the germination protease and keratinase also is described.

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MATERIALS AND METHODS

Organisms and growth conditions. The growth and sporulation characteristics of the strain of *M. gypseum*, designated R87, used in these studies have been described elsewhere (15, 22). A mutant strain B17 of the wild-type strain also was used (15). A pleomorphic strain, R87P1, isolated as a spontaneous mutant of the wild-type strain and asporogenous under normal cultural conditions, was induced to sporulate by increased aeration methods (22).

Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of agar medium composed of glucose, 1% (wt/vol); neopeptone (Difco), 1% (wt/vol); agar, 1.8% (wt/vol); and distilled water (pH 6.5). Sporulation of 4-day pregrown cultures was initiated after 2 days of aeration at 40 cm^3/min, and mature spores were formed after 4 days of total aeration. Control cultures without additional aeration and aerated cultures were harvested daily by scraping the aerial hyphae from the agar surface with a bent glass rod. Three flasks were harvested at each time period to give sufficient material for further studies.

Spore harvesting and germination procedures have been described previously (21, 23). The alkaline germination protease was collected and partially purified by the method of Leighton and Stock (14). Cell-free extract preparation also has been described (21, 23).

Keratinase induction. Keratinase was induced by the method of Yu, Harmon, and Blank (30) by using a medium composed of glucose, 0.09% (wt/vol); and $MgSO_4 \cdot 7H_2O$, 0.06% (wt/vol); in 0.028 M phosphate buffer, pH 7.8. Hair (guinea pig, human, or horse) or chicken feather, cut into 0.5-cm lengths, was washed thoroughly with 0.1% (wt/vol) sodium dodecyl sulfate to remove surface contaminants and then with 1:1 (vol/vol) methanol and water with shaking for 18 h at 27 C. The keratin was filtered through Miracloth (Calbiochem, La Jolla, Calif.) to remove the solvents. The final keratin residue was washed with distilled water, then with 95% ethanol, and air dried. The washed hair or feather (0.5 g) was added to 100 ml of medium in a 250-ml Erlenmeyer flask and routinely was autoclave-sterilized for 15 min at 121 C. The medium was inoculated with 5-day-old liquid-grown mycelia, which has been sheared by VirTis homogenation (Model 23, VirTis Inc., Gardiner, N.Y.) for 30 s at approximately 16,000 rpm. After inoculation, the medium was allowed to stand for 5 days and then was shaken for 7 days at 27 C on a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, Pa.).

Estimation of keratin percent utilization. Keratin utilization was estimated by the method of Chesters and Mathison (4). First, the total weight of hairs covered with mycelium was estimated by filtration and desiccation in vacuo over calcium chloride. Then the dry weight of the mycelium was estimated after dissolution of the hair by treatment with 50 ml of 10% (wt/vol) NaOH at 96 C for 10 min. The value thus obtained for the weight of mycelium after extraction was corrected for mycelium weight loss due to NaOH extraction by multiplying by the coefficient 1.60. This value was obtained treating *M. gypseum* strain R87 liquid-grown hyphae in a similar manner with NaOH and calculating the hyphal weight loss. The weight of hair remaining after hyphal growth was, therefore, calculated as the weight of (hair + mycelium) − (weight of mycelium), and the percentage of utilization of keratin was calculated as the (original weight of hair − weight of hair remaining) ÷ original weight of hair × 100%. All estimations were done in triplicate.

Keratin extraction and fractionation. Natural keratin sources (hair or feather) were fractionated by a method utilizing alkaline thioglycolate extraction (10) and the fractionation scheme of Corfield et al. (6). The beta-keratin and whole-hair fractions (1 mg dry weight/ml) were resuspended in 0.05 M phosphate buffer, pH 9.0, for routine enzyme assays. The protein concentration of the alpha-keratin was estimated by the method of Lowry et al. (17), and then was suspended at 0.5 mg of protein/ml of 0.05 M phosphate buffer, pH 7.0.

Enzyme assay procedures. Guinea pig hair routinely was used as a source of alpha- and beta-keratin for enzyme assays. Germination protease was measured by using 1% (wt/vol) casein in 0.05 M Veronal (sodium barbital) buffer, pH 9.0 (21, 23).

The standard enzyme reaction mixture contained 50 μg of enzyme protein in 0.5 ml of buffer and 0.5 ml of either alpha-keratin, beta-keratin, or casein substrate. The mixture was incubated at 37 C for 60 min. The reaction was stopped by the addition of 1.0 ml of 10% (wt/vol) perchloric acid (PCA), and the resultant precipitate was removed by centrifugation at 1,000 × g for 2 min at 27 C. Blanks contained the complete reaction mixture, but were acidified with PCA prior to incubation. Assays involving the effects of cations, anions, or urea on enzymatic activity were preincubated with the enzyme for 30 min at 37 C. One unit of activity was equal to an optical density change of 0.001 at 280 nm of enzyme per ml per min. Specific activity was calculated as units per milligram of enzyme protein.

The determination of pH profiles was made by assays by using either 0.05 M Veronal or 0.05 M phosphate buffers adjusted with HCl or NaOH for the pH 5.5 to 9.0 range. Activities at pH 9.0 to 10.0 also were assayed by using 0.05 M borax-NaOH or 0.05 M borax-NaOH-phosphate buffers.

Esterase activity was measured by using benzoyl-L-tyrosine ethyl ester (BTEE) as substrate (9). The inhibiting effects of inorganic phosphate and phenyl methyl sulfonyl fluoride (PMSF) (14) were followed spectrophotometrically with a Gilford Model 2400 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Sephadex chromatography. Columns (45 by 1.5 cm) containing either Sephadex G100 or G200 gels were used routinely in these studies. Either 0.05 M phosphate buffer, pH 7.8, or 0.05 M veronal buffer, pH 9.0, was used for gel rehydration and elution. The columns were calibrated by the method recommended for Sephadex gels ("Gel filtration in theory and practice", Pharmacia Fine Chemicals, Uppsala, Sweden), by using crystalline ribonuclease A, chymotrypsin, ovalbumin and aldolase. Enzyme samples applied to the columns contained 0.3 to 0.4 optical density 280 nm units/ml. The column drop-former was constructed from a 50-μliter disposable pipette, and the flow rate was 1 drop/10 s (or 1 ml/6 min). Fractions of 85 drops or approximately 2.5 ml were collected on a Warner-Chilcot Model 1205-E2 fraction collector (Canal Industrial Corp., Rockville, Md.). Forty fractions were collected before application of a new sample. Protein concentration in each fraction was estimated as the optical density at 280 nm. All columns were run at 25 C.

Enzyme concentration. Alkaline protease and keratinase were concentrated either by dialysis against 20% (wt/vol) polyethylene glycol (PEG) or 20% (wt/vol) Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), in 0.05 M phosphate or Veronal buffers. Other methods of concentration were flash evaporation at 25 C and Diaflow dialysis concentration in an Amicon Model 52 Ultrafiltration cell (American Corp., Lexington, Mass.). Dialysis concentration in the presence of reducing agents was conducted after adding 45 mM cysteine or 5 mM dithiothreitol (DTT) to the 20% PEG solution (5).

RESULTS

Phosphate stimulation of keratinase activity. When the germination protease from a 10-h germinated spore supernatant was assayed for activity at different pH levels, a peak of activity at pH 7.0 was detected that was not present in 3-h germinated spore supernatants (Fig. 1). The 10-h germination protease, assayed for activity in the presence of phosphate buffer, showed decreased protease activity at pH 9.0 and a marked increase in protease activity at pH 7.0 (Fig. 1).

This shift in pH optimum was accompanied by a change in specificity for substrate, when compared with the intracellular alkaline protease (Table 1). Further examination of the apparent increased preference for keratin substrates showed that the 10-h germination protease hydrolyzed whole keratin and beta-keratin (exo-keratinase activity), and that activity against alpha-keratin (endo-keratinase activity) was increased by phosphate ions (Table 2). Figure 2 shows how inorganic phosphate dramatically decreased caseinolytic activity at pH 9.0, and at the same time increased alpha-keratinolytic activity at pH 7.0. Phosphate also decreased the BTEE esterase activity of the 3-h germination protease (Fig. 2B). Neither the 10-h germination protease nor a

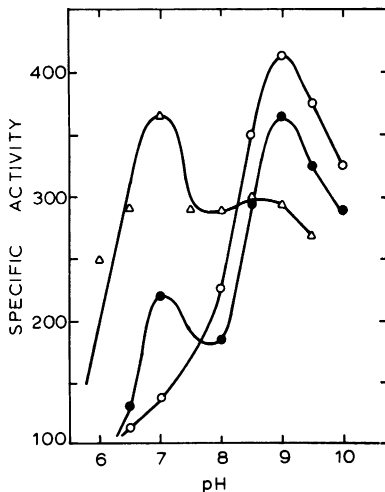

FIG. 1. *Alteration in the germination protease pH optimum on casein in the presence of inorganic phosphate. Germination protease activity was measured after 3 h of germination (○) and after 10 h of germination (●), in 0.05 M Veronal buffer. Protease collected at 10 h also was assayed in the presence of 0.05 M phosphate buffer (△). Specific activity = ΔOD at 280 nm × 10³ per min per mg of enzyme protein.*

TABLE 1. *Comparison of intracellular and extracellular alkaline protease specificity for substrate*

Substrate[a]	Relative sp act[b]	
	Intracellular alkaline protease[c]	Extracellular alkaline protease[d]
Casein	1.0	1.0
Native albumin	0.03	0.005
Heat-denatured albumin	0.58	0.82
Bovine serum albumin	0.08	0.06
Hemoglobin	0.25	0.20
Gelatin	0.23	0.20
Protamine	0.40	0.20
Keratin[e]	0.09	0.50

[a] All substrates suspended at 1% (wt/vol), in 0.05 M Veronal buffer, pH 8.0.
[b] Specific activity = μg of protein released per minute per milligram of enzyme protein.
[c] MW 33,000 from Sephadex G100 chromatography.
[d] From 10-h germinated spores supernatant.
[e] Whole guinea pig hair.

TABLE 2. *Relative activity of 10-h germination protease on keratin fractions*

Enzyme mixture	Enzyme relative activity[a]		
	Unfractionated hair[b]	Alpha-keratin[b]	Beta-keratin[b]
Germination protease	1.0	0	4.8
Germination protease plus 10^{-5} inorganic phosphate	0.8	3.68	3.32
Germination protease plus 10^{-3} M thiosulfate	1.32	5.0	4.12
Keratinase[c]	1.0	8.0	10.48

[a] Specific activity = ΔOD at 280 nm × 10³ per min per mg of enzyme protein. (All values are relative to germination protease activity on unfractionated hair).
[b] From guinea pig hair.
[c] Induced by growing on guinea pig hair.

keratinase preparation possessed detectable BTEE esterase activity.

Figures 3 and 4 show the pH activity profiles of the 10-h germination protease and the extracellular *M. gypseum* keratinase using alpha- and beta-keratins as substrate. The germination protease did not display exceptional activity prior to phosphate addition. After phosphate addition, however, the germination protease showed enhanced activity toward both keratin sources and displayed sharper pH optima at pH 7.0 for alpha-keratin and at pH 9.0 for beta-

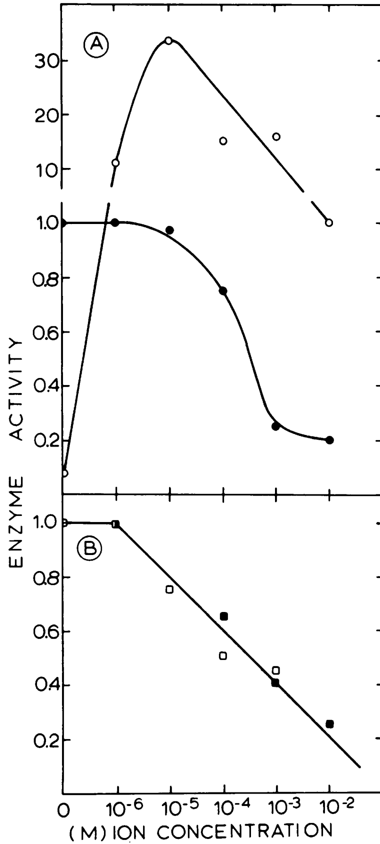

FIG. 2. *Figure 2A shows the inhibitory effect of inorganic phosphate on the 3-h germination protease caseinolytic activity in 0.05 M Veronal buffer, pH 9.0, (●) and the stimulatory effect of inorganic phosphate on the 3-h germination protease alpha-keratinolytic activity in 0.05 M Veronal buffer, pH 7.0, (O). Figure 2B shows the inhibitory effect of inorganic phosphate (■) and PMSF (□) on the BTEE esterase activity of the 3-h germination protease. Specific activity of protease = ΔOD at 280 nm × 10^3 per min per mg of enzyme protein. Specific activity of esterase equals micromoles of substrate used per minute per milligram of enzyme protein.*

keratin. Additional phosphate concentrations also enhanced the beta-keratinolytic activity of the keratinase.

Evidence for germination protease subunit structure. The molecular weight of the 3-h germination protease has been estimated previously at 30,000–33,000 by disk gel electrophoresis (21). This value also was obtained when G100 or G200 Sephadex chromatography was used (0.05 M Veronal buffer, pH 7.8) (Fig. 5A). However, when the enzyme was chromatographed in the presence of phosphate or was collected at later germination times (6 to 10 h),

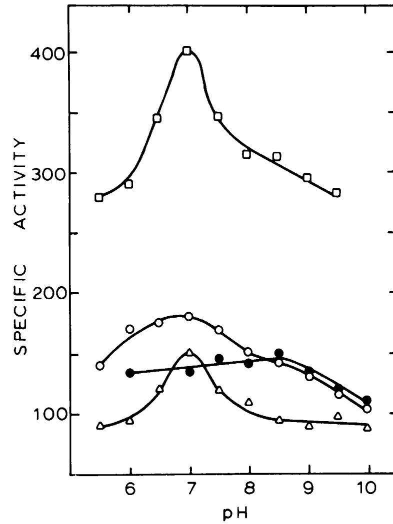

FIG. 3. *pH activity profiles of the 10-h germination protease in 0.05 M Veronal buffer (●), 0.05 M phosphate buffer (O), 0.1 M phosphate buffer (Δ), and the extracellular keratinase in 0.05 M phosphate buffer (□), on alpha-keratin substrate. Specific activity = ΔOD at 280 nm × 10^3 per min per mg of enzyme protein.*

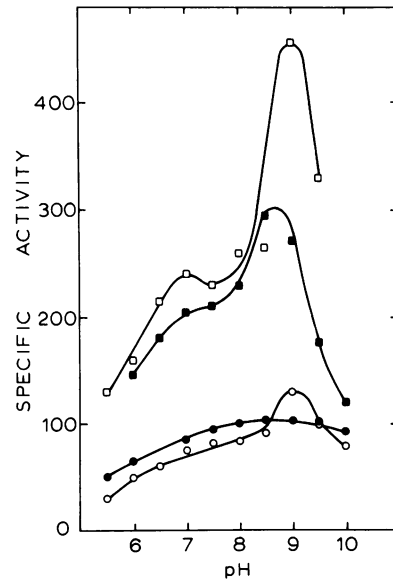

FIG. 4. *pH activity profiles of the 10-h germination protease in 0.05 M Veronal buffer (●), and in 0.05 M phosphate buffer (O), and the extracellular keratinase in 0.05 M Veronal buffer (■), and in 0.05 M phosphate buffer (□), on beta-keratin substrate. Specific activity = ΔOD at 280 nm per min per mg of enzyme protein.*

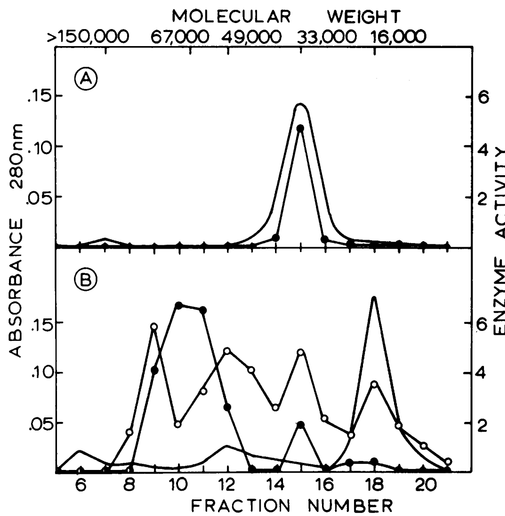

FIG. 5. *Elution of the 3-h germination protease from a Sephadex G100 column with 0.05 M Veronal buffer, pH 9.0 (A), and elution of the 3-h germination protease from a Sephadex G100 column with 0.05 M phosphate buffer, pH 7.8 (B). Alkaline protease activity (●) and alpha-keratinase activity (O) were estimated in each fraction; beta-keratinase activity was congruent with the alkaline protease peaks. Fraction 9 in part B corresponds to a MW of 98,000. Units of activity = ΔOD at 280 nm per min per ml of fraction. The solid line represents protein per fraction.*

the bulk of the protein chromatographed at 16,000 molecular weight (MW), and the enzyme activity was dispersed through the column fractions (Fig. 5B). Thus, it was considered that phosphate affected the stability of the germination protease, possibly splitting the 33,000 MW enzyme into 16,000 MW subunits which lacked alkaline protease activity, but possessed limited keratinase activity. The enzyme peaks in Fig. 5B were considered to be aggregates of the dissociated protease, as they all ran at MW multiples of 16,000.

When the intracellular alkaline protease from sporulating hyphae was examined by G200 Sephadex chromatography, the 33,000 MW species was found to be the major peak at 5 days, during spore maturation (Table 3). However, lower MW alkaline proteases were found in abundance at 1, 2, and 4 days in immature spores. An apparent gradient from low MW (8,000) at 1 day (hyphae only) to higher MW (33,000) at 5 days was evident. Very high species (120,000) also were obtained at 2, 4, and 5 days. The first appearance of these higher MW species coincided with the first appearance of the 16,000 MW protease species and with the onset of sporulation at 2 days.

Strain R87 hyphae, harvested at various stages of sporulation, showed that the intracellular keratinase activity peak preceded the intracellular alkaline protease activity peak (Fig. 6A). This result was consistent with the data reported in Table 3, which showed that the 16,000 MW protease species preceded the appearance of the 33,000 MW alkaline protease species. The pleomorphic strain (R87P1) of *M. gypseum* was found to be devoid of both alkaline protease and keratinase activity (Fig. 6A and B). Shortly after forced aeration and initia-

TABLE 3. *Molecular species of the alkaline protease during sporulation*

Colony age (days)	Sp act[a]			
	8,000 MW	16,000 MW	33,000 MW	120,000 MW
1	1280	7.4	0	0
2	63	920	26.7	475
4	46	300	380	300
5	16	6.4	920	385

[a] Specific activity = ΔOD at 280 nm × 10^2 per min per mg of CFX protein.

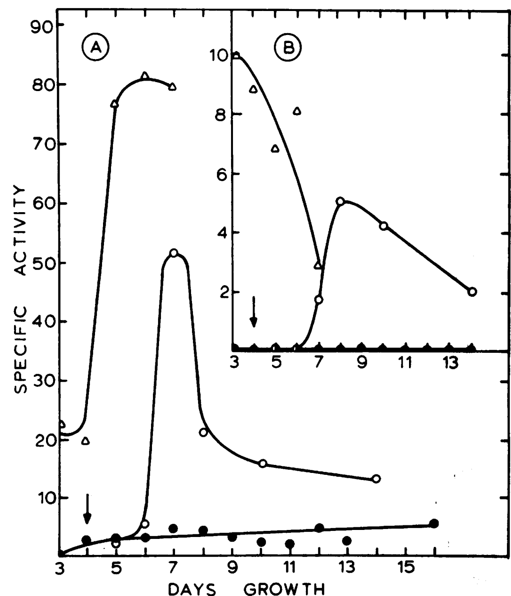

FIG. 6. *Comparison of the intracellular alkaline protease activity (A), and the time of appearance of intracellular keratinase activity (B), in strain R87 during sporulation (Δ), the pleomorphic strain R87P1 under normal growth conditions (●), and the pleomorphic strain R87P1 under increased aeration (O). Specific activity = ΔOD at 280 nm × 10^3 per min per mg of CFX protein.*

tion of sporulation in pleomorphic cultures, the appearance of the alkaline protease preceded the keratinase activity peak. As the pleomorphic strain initially has no protease activity, the early protease peak may have represented induction of the 8,000 MW species.

Keratinase molecular weight and aggregation. *M. gypseum* possessed definite keratinase activity when grown on keratin substrates as the sole carbon and nitrogen source (Table 4). Mutant strain B17 and the pleomorphic strain R87P1, which have altered germination protease activity (15), also had lower keratinolytic activity than the wild-type strain R87.

When the extracellular keratinase was collected, concentrated by flash evaporation, and chromatographed, most of the protein and alpha-keratinolytic activity was found to elute at 16,000 MW (although much of the enzyme's activity was lost during partial purification). Keratinase concentration by dialysis against PEG, Ficoll, or by Diaflow ultrafiltration, followed by chromatography, resulted in the inactivation of the 16,000 MW peak and increased elution of the enzyme protein at 33,000 and >150,000 MW.

Aggregation of the enzyme protein also was obtained when isolated Sephadex G100 enzyme preparations were rechromatographed on Sephadex G100. Rechromatography of isolated 16,000 MW keratinase gave an array of peaks, with fractions eluting at 16,000, 33,000, 65,000, and >150,000 MW. When the 65,000 MW keratinase was isolated and rechromatographed, some of the enzyme protein eluted at 16,000 MW; however, most eluted at >150,000 MW. Very little protein eluted at 65,000 MW. Similarly, when isolated keratinase of 33,000 MW was rechromatographed on G100, almost all of the enzyme protein eluted at 65,000 rather than 33,000 MW. These results suggested that in the process of rechromatography or dialysis, some factor necessary for the activity and integrity of the 16,000 MW unit was lost.

Neither rechromatography nor dialysis concentration of the 16,000 MW germination protease unit, however, promoted formation of the >150,000 MW complex. This indicated that although the keratinase and the germination protease plus phosphate units had the same molecular weight, some additional alteration had affected the 16,000 MW keratinase species that rendered it unstable to rechromatography or dialysis.

Stimulation of keratinase activity with autoclaved feather supernatant. During the investigation of keratinase digestion of various natural keratin sources, it was found that autoclaved chicken feathers served as an excellent substrate, but steamed or ethanol-sterilized feathers were utilized poorly (Table 5). The nearly complete utilization of autoclaved feathers was not entirely due to heat denaturation of the native protein structure, as autoclaved and resuspended feather was digested to the same degree as the steam-sterilized feather substrate. Similarly, isolated beta-keratin was utilized just as well as the steamed feather substrate. Isolated alpha-keratin proved to be a poor growth substrate.

The results in Table 5 suggested that a soluble factor present in the autoclaved chicken feather supernatant (AFS) was enhancing the utilization of the chicken feather substrate. When AFS was added to the isolated keratinase, the enzyme's activity was increased. Dilution of the >150,000 MW keratinase caused a decrease in alpha- and beta-keratinase activities and an increase in alkaline protease activity (Fig. 7A). Dilution of the same keratinase

TABLE 4. *Percentage of utilization of keratin sources by strains of M. gypseum*

Keratin source	Utilization after 13 days of growth (%)		
	Strain R87	Strain B17	Pleomorphic strain R87P1
Human hair	39.3	21.4	9.3
Horse hair	53.1	38.9	10.9
Guinea pig hair	53.1	42.0	8.0
Chicken feather[a]	24.5	13.5	6.3

[a] Steamed at 100 C for 15 min.

TABLE 5. *Effect of the method of sterilization on the percentage of utilization of chicken feather keratin by M. gypseum*

Treatment	Utilization after 13 days of growth (%)
Autoclaved[a]	96.4
Steamed[b]	24.5
Ethanol[c]	9.7
Autoclaved, filtered and resuspended	28.0
Beta-keratin fraction (autoclaved)	31.8
Alpha-keratin fraction (autoclaved)	<5.0

[a] Feather suspended in medium and autoclaved at 121 C for 15 min.

[b] Feather steamed at 100 C for 15 min and then suspended in sterile medium.

[c] Soaked in 95% ethanol for 18 h and then filtered, air dried, and suspended in sterile medium.

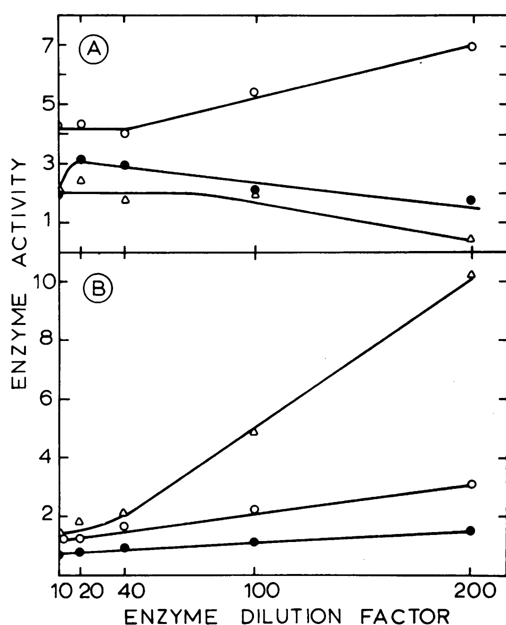

FIG. 7. *Comparison of the effect of dilution on keratinase (>150,000 MW), alkaline protease (O), alpha-keratinase (●), and beta-keratinase (Δ), activity in the absence (A), and presence (B) of added AFS material. Activity units = ΔOD at 280 nm × 10^3 per min per ml of enzyme.*

preparation in the presence of AFS enhanced the activity of the beta-keratinase by 2.56-fold and decreased the activity of the alkaline protease by 2.45-fold (Fig. 7B).

When the AFS was slurried with H^+ Dowex, or was dialyzed, it lost its ability to enhance keratinase activity. This result did not occur, however, when the AFS was slurried with Cl^- Dowex or was deproteinized with PCA. This suggested that the factor in AFS, which enhanced the keratinase activity, was a small, negatively charged, nonprotein molecule.

Stimulation of keratinase activity with reducing agents. Dilution of the keratinase in the presence of 45 mM cysteine or 5 mM sulfite, bisulfite, or DTT also enhanced the activity of the keratinase, when compared with Fig. 7A. A 100-fold dilution of enzyme protein, in the presence of cysteine, stimulated alpha-keratinase by 129-fold, beta-keratinase by 162-fold, and alkaline protease by 31-fold. The presence of sulfite enhanced alpha-keratinase by 205-fold, beta-keratinase by 790-fold, and alkaline protease by 32.4 fold. DTT enhanced alpha-keratinase by 273-fold, beta-keratinase by 290-fold, and alkaline protease by 25-fold. Stimulation of the keratinase activities also was obtained when 10^{-3} M thiosulfate was added to the enzyme mixture (Table 2). Overall, sulfur-reducing agents were shown to have a definite stimulatory action on the activity of both the alpha- and beta-keratinases.

The effect of reducing agents on the molecular weight of the protease and keratinase. To determine the effect of AFS and anions on the molecular weight of the keratinase, the enzyme was concentrated separately against PEG both in the absence or presence of either cysteine, DTT, or AFS, and then chromatographed on Sephadex G100. The effect of all of these agents was to dissociate the >150,000 MW aggregate into 16,000 MW units. In the cases of cysteine and DTT, the activity of the alpha-keratinase was maintained well in the 16,000 MW peak.

As previously mentioned, the phosphate-treated germination protease which eluted at 16,000 MW from G100 would not form >150,000 MW aggregates after dialysis against PEG or Ficoll. However, when the phosphate-treated germination protease was reacted with 5 mM DTT, then dialyzed against PEG in the absence of DTT, and chromatographed on G100, the bulk of the enzyme protein eluted at >150,000 MW. Treatment of the phosphate-treated germination protease species with 5 mM DTT or 45 mM cysteine and then immediate G100 chromatography without concentration against PEG resulted in enzyme protein elution in a single 16,000 MW band with good alpha-keratinase and caseinolytic activity.

Although a detailed kinetic analysis of the effect of reducing agents on the keratinase or the phosphate-treated protease was not attempted, some information was obtained by comparing the urea-denaturation curves for the 3-h germination protease and the keratinase (16,000 MW). The keratinase was much more sensitive to urea than the germination protease (Fig. 8). Treatment of the protease with phosphate gave a denaturation curve similar to that of the protease alone, but with lower activity, as would be expected. Treatment of the germination protease with phosphate, and then with thiosulfate, sulfite, or bisulfite gave a denaturation curve identical to that of the keratinase.

DISCUSSION

In order for significant hydrolysis of native keratin to occur, the outer barrier of the keratinized cell membranes must be penetrated. These membranes are composed of beta-keratin which resists extreme conditions (exposure to 5 N NaOH, 8 M urea, 8 M urea plus thioglycolic acid at pH 10.0, or 10% sodium sulfite), but are

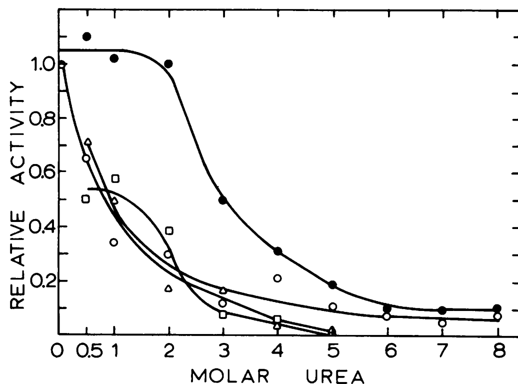

FIG. 8. *Comparison of the sensitivity of the 3-h germination protease (●) and the keratinase (O) to urea inactivation. Treatment of the 3-h germination protease with 10^{-5} M inorganic phosphate (□) and 10^{-3} M thiosulfate (Δ) decreased the enzyme's activity in the presence of urea (0.05 M Veronal buffer, pH 9.0). Activity was calculated as ΔOD at 280 nm per min per mg of enzyme protein. Relative activity was calculated as activity in the presence of urea divided by activity without urea.*

susceptible to proteolytic enzymes like trypsin (18). The inner component of these cells, the alpha-keratin fibrils, resist trypsin action, but are sensitive to the other treatments, giving keratin a "complementary" structure. The other barrier to complete hair digestion is the high concentration of disulfide bonds which cross-link the alpha-keratin fibrils and the beta-keratin membranes (8, 18).

Digestion of keratin by dermatophytes is accompanied by the elaboration of complex "eroding mycelia" which promote penetration and erosion of the hair structure (7). These structures have been shown by Kunert (13) to produce sulfite which possibly reduces the keratin disulfides by "sulfitolysis", exposing the keratin proteins to enzymatic attack. Reduction of disulfides prior to keratinolytic attack also has been observed in the digestion of wool by the clothes moth (*Tineola bisselliella*) larva (24, 25).

It appears reasonable that the germination protease should possess beta-keratinolytic activity, as germinating spores on keratinized tissues would encounter the beta-keratin first. The chymotrypsin-like preference of the germination protease for aromatic amino acids has been reported previously (14, 15). The resistance of the beta-keratin is thought to be due to cross-linking between tyrosine residues; thus, treatments which are active against tyrosine would solubilize the beta-keratin fraction (1).

Limited beta-keratin digestion may expose disulfide linkages which then induce sulfite formation and the production of the "eroding mycelium" complex. Mechanical penetration of the beta-keratin membranes by the eroding mycelia would expose the underlying alpha-keratin fibrils to further keratinolytic action. This sequence would require the alpha-keratinase to tolerate a disulfide-reducing environment. The alpha-keratinase would be derived most economically from the germination protease, which is the only protease released upon spore germination and outgrowth (14, 21).

The immediate effect of inorganic phosphate release was to inhibit the esterase and caseinase activity of the germination protease. This action presumably controlled the degree of spore coat hydrolysis during germination, preventing over-hydrolysis of the spore coat and lysis of the germling (21, 23). The action of inorganic phosphate was not entirely inhibitory, however, as phosphate enhanced the keratinolytic activity of the germination protease. Other effects of phosphate on the germination protease were the change in pH optimum from pH 9.0 to 7.0 and the reduction in molecular weight from 33,000 to 16,000. A similar phosphate-induced dissociation of chymotrypsin has been reported previously (29). The lower molecular weight and pH optimum corresponded to those of the keratinase induced when *M. gypseum* was grown on keratin substrates.

The phosphate-binding sites of the germination protease possibly were associated with the esterase-active site, which also was shown to be sensitive to phosphate and PMSF inhibition. In this case, the phosphate may have been bound through serine residues (20). Phosphate also appeared to dissociate the 33,000 MW protease, but the resultant 16,000 MW subunits were not identical to the 16,000 MW keratinase units, as the former would not aggregate to form the >150,000 MW complex when concentrated by dialysis. After disulfide reduction, however, the phosphate-treated protease unit was rendered more active against keratin substrates, perhaps owing to an ensuing conformational change after the release of the disulfide constraints (2). This reduced unit now formed the large MW complex when concentrated by dialysis. These observations were consistent with the preceding model, which suggested that the alpha-keratinase must tolerate a disulfide-reducing environment and would probably exist in a reduced state. Hydrogen bonding or hydrophobic bonding may be involved in the enzyme's new configuration, as evidenced by the denaturant effects of urea (3, 11, 16). The results also showed that thiosulfate, the product of "sul-

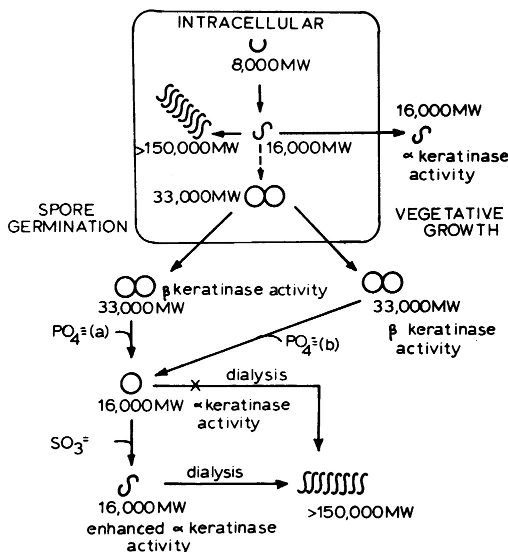

FIG. 9. *Summary of the proposed M. gypseum protease-keratinase molecular interconversions. Phosphate may be (i) released by germinating spores or (ii) released by the growing hyphae (M. Kavanaugh, unpublished data). Further details appear in the Discussion.*

fitolysis," stimulated keratinase activity, as did cysteine, a possible keratin hydrolysis product.

A summary of the proposed germination protease-keratinase molecular interconversions is presented in Fig. 9. The intracellular events were derived from Table 3. Conversion of the 16,000 MW intracellular unit into the 33,000 MW intracellular unit probably involves many steps. The intracellular 16,000 MW protease unit, which appeared after 2 days of growth, was probably the same as the alpha-keratinase which may be released during vegetative growth. The results also showed that the appearance of large intracellular complexes coincided with the appearance of the 16,000 MW unit. Recently, a mutant strain (B6) of *M. gypseum*, which was defective in spore formation beyond the day 2 stage, has been shown to possess an active alpha-keratinase, but neither alkaline protease nor beta-keratinase (N. Wong, unpublished data). This observation and the results of this investigation suggest that alpha-keratinase was present in mycelial extracts immediately after protease induction, but prior to germination protease formation. Further proof of the origin of the keratinase from the alkaline protease may be obtained with future comparison of protein composition and sequence data (27).

The dry surface environment, which a dermatophyte would encounter in a superficial dermatomycosis situation, necessitates the specific, localized elaboration and release of disulfide-reducing agents and keratinases. This inhospitable environment also necessitates the least expensive and most rapid switch from a spore germling outgrowth mode of activity to an invasion and exogenous substrate utilization mode of activity. The derivation of the keratinase from the germination protease, mediated by factors which control spore germination (inorganic phosphate) and by factors which precede keratinolysis (sulfite, etc.), is an attractive and logical model. This model would allow *M. gypseum* germlings to penetrate the hair structure immediately after germ tube emergence, thus insuring the survival of this fungus on the superficial keratinized tissues of a host.

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