JOURNAL OF BACTERIOLOGY, Apr. 1972, p. 354-362

Vol. 110, No. 1
Printed in U.S.A.

# Isolation and Characterization of *Microsporum gypseum* Lysosomes: Role of Lysosomes in Macroconidia Germination

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Received for publication 5 January 1972

Three types of lysosomes containing either acid protease, alkaline protease, or phosphodiesterase were isolated from a *Microsporum gypseum* macroconidial homogenate on Ficoll gradients. The acid protease was contained in an assimilative lysosome since its activity was affected by the complexity of the exogenous nitrogen source. Ultracentrifugation and electron microscopy revealed that the alkaline protease-containing vesicles were associated with the spore coat material prior to macroconidial germination. During macroconidial germination, zones of spore coat hydrolysis were seen surrounding these vesicles. Other larger vesicles, believed to contain the phosphodiesterase, were also observed in the spore coat during macroconidial germination.

The excretion of extracellular enzymes, synthesis of cell wall material, and the budding of yeast cells have been reported to be associated with vesicular bodies of various types (3, 4, 10, 14, 16). The cytoplasmic origin of these vesicles is not entirely clear, but the endoplasmic reticulum has been implicated (16). Lysosomes, also of endoplasmic reticulum origin, are a class of vesicles of wide distribution characterized by their content of lytic enzymes (15).

In the dermatophyte, *Microsporum gypseum*, an alkaline protease has been shown to be necessary for macroconidial germination (7). In ungerminated spores, this protease is associated with the spore coat (7), and, during spore germination, it is released as an extracellular enzyme (19).

In the studies presented here, the cellular localization and vesicular nature of the acid and alkaline proteolytic enzymes of *M. gypseum* were investigated. The possible cytoplasmic origin and probable function of these vesicles in macroconidial germination is discussed.

## MATERIALS AND METHODS

A strain of *M. gypseum* (Bodin; Guiart and Grigorakis, 1928; designated strain R87) originally obtained from F. Blank, Temple University, Philadelphia, Pa., was used in these investigations. This strain was previously designated $SP^+Pig^+$ (8). An asporogenous pleomorphic strain of R87, previously designated $SP_2$ $Pig^-$ (8), also was used in these studies.

**Sporulation medium, macroconidia preparation, and germination.** Spore production and isolation were carried out as previously described (6). Liquid cultures were grown in a nutrient medium which consisted of 1% glucose (w/v), 1% neopeptone (w/v; Difco), and distilled water (*p*H 6.4). Liquid cultures were aerated by shaking a 250-ml Erlenmeyer flask containing 100 ml of medium at 125 rev/min in a R77 Metabolyte shaker water bath (New Brunswick Scientific Co., New Brunswick, N.J.), at 25 C. Spore germination procedures have been previously described (19).

**Cell-free extract preparation and gradient centrifugation.** Cell-free extracts (CFX) were prepared by the liquid nitrogen method of Bleyman and Woese (1). Debris was removed from the liquid nitrogen homogenate by centrifugation at 10,000 × *g* for 10 min at 4 C. Extracts for enzyme determinations were buffered in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (*p*H 7.4).

Spore coat material was prepared for enzyme determinations by grinding isolated macroconidia in liquid nitrogen as described for the CFX preparations. Homogenization was carried to 95% breakage, as estimated with a microscope. The 10,000 × *g* pellet was washed an additional eight times in physiological saline (*p*H 6.5) and resedimented between washes at 500 × *g* for 2 min at room temperature (25 C) in a clinical centrifuge (Clay-Adams Co. Inc., New York).

Extracts for ultracentrifugation were also broken in liquid nitrogen in the presence of a viscous grinding solution containing 0.5 M sucrose and 50%

glycerol (v/v) in 0.01 M Tris (*p*H 7.5). The homogenate was centrifuged at 200 × *g* for 10 min, and the resulting pellet was suspended in grinding solution and resedimented at 200 × *g* for 10 min. The supernatant fractions from both 200 × *g* centrifugations were pooled and centrifuged at 10,000 × *g* for 10 min in a Sorvall SS-1 centrifuge. The 10,000 × *g* supernatant fraction was centrifuged at 30,000 × *g* for 1 hr, and the resulting supernatant fraction was centrifuged at 40,000 × *g* for 2.5 hr in a Spinco model L ultracentrifuge (#30 rotor). All centrifugations were carried out at 4 C, and all pellets were suspended in 0.1 M Tris (*p*H 7.5) for enzyme determinations.

Spore extracts for gradient ultracentrifugation were prepared in liquid nitrogen in the presence of 0.05 M Tris (*p*H 7.5) containing 0.25 M sucrose. Debris was removed from the homogenate by centrifugation at 3,000 × *g* for 20 min at 4 C. Vesicles were isolated by the method of Matile and Wienken (15), by flotation in the presence of 8% Ficoll, (w/v; Pharmacia, Uppsala, Sweden). Vesicles also were isolated by a modification of the method of Brown (2) by sedimentation of 5 ml of homogenate through a 20-ml linear 0 to 20% Ficoll gradient containing 0.25 M sucrose and 0.05 M Tris (*p*H 7.5). The gradients were centrifuged at 10,000 rev/min for 15 min (flotation) or at 30,000 rev/min for 60 min (sedimentation) at 4 C in a Spinco model L ultracentrifuge (#30 rotor). Gradient fractions (1-ml) were collected with an Isco model A fraction collector (Instrument Specialties Co. Inc., Lincoln, Nebraska).

**Analytical determinations.** Protein estimations in CFX and spore coat material were made by the method of Lowry et al. (9). Protein estimations in the presence of Ficoll or sucrose were made after precipitation of the sample with 10% trichloroacetic acid and suspension of the pellet in 0.1 N sodium hydroxide. Relative protein concentrations in gradient fractions also were made by measuring absorbance at 280 nm.

Protease activity was measured by the method of McDonald and Chen (11) with 2% buffered casein. Acid protease determinations were made with 0.05 M citrate phosphate buffer (*p*H 5.0), and alkaline protease determinations were made with 0.05 M sodium barbital (Veronal) buffer (*p*H 9.0). Protease specific activity was measured as caseinolytic units per mg of enzyme or CFX protein, where one caseinolytic unit equals the amount of enzyme which will cause an increased absorbance (280 nm) of 0.001 in acid-soluble material per min.

Diaphorase and dihydronicotinamide adenine dinucleotide (NADH) reductase were measured by the method of Mahler by following the reduction of 2,6 dichlorophenol-indophenol at 600 nm (12). Phosphodiesterase was measured by the method of Neu and Heppel, employing bis-paranitrophenol phosphate as the substrate (17), and acid phosphatase was determined by following the release of *p*-nitrophenol at 410 nm (4). All enzyme determinations were carried out at 37 C in a Gilford model 2400 recording spectrophotometer (Gilford Instrument Laboratories.)

**Electron microscopy.** Ungerminated and germinated spores were fixed in 1.5% (w/v) aqueous potassium permanganate, washed in 0.05 M sodium potassium phosphate buffer (*p*H 7.4) and then dehydrated in graded ethanol baths. Following infusion with propylene oxide, infiltration with Epon 812 was continued for 24 hr at 25 C. Samples were embedded in Epon 812 blocks containing 28.6 g of Epon 812, 21.0 g of dodecenyl succinic anhydride (DDSA), 10.53 g of nadic methyl anhydride (NMA), and 1% (w/v) DMP-30 catalyst. Polymerization was carried out at 37 C for 12 hr followed by 60 C for 30 hr. Blocks were sectioned with a LKB ultrotome (LKB-Produkter AB, Stockholm, Sweden). Sections were mounted on carbon-coated grids and poststained with Reynold's lead citrate for 8 min and with 1% uranyl acetate for 30 min. Sections were scanned in a Phillips 300 electron microscope at 60 kv.

**Chemicals.** Substrates for enzyme analysis: paranitrophenol phosphate, bis-paranitrophenol phosphate, and 2,6 dichlorophenol indophenol were obtained from Calbiochem. NADH was obtained from Sigma Chemical Co. Reagents for electron microscopy: DDSA, NMA, and DMP-30 were obtained from Ernest F. Fullame, Inc. Epon 812, ethylenediaminetetraacetic acid, acid-washed casein, and all other reagent grade chemicals were obtained from Fisher Scientific Co., Ltd. (Vancouver, B.C.).

## RESULTS

**Determination of the number of proteases present during sporulation.** To determine the number of proteases active in sporulating *M. gypseum*, protease activity was determined over a wide range of *p*H values (Fig. 1). Comparison of the alkaline germination protease activity curve with the CFX protease activity curve showed that the CFX had more proteolytic activity at acidic *p*H levels than the germination enzyme, suggesting that there was an additional acid protease present in the CFX. Examination of the pleomorphic strain for the acid protease revealed a definite peak (Fig. 2), owing to the low alkaline protease levels in this mutant (Fig. 3). Treatment of sporulating strain R87 with phenyl methyl sulfonyl fluoride (a known inhibitor of the alkaline protease; reference 7), allowed the detection of the acid protease at a level of activity comparable to the pleomorphic strain, suggesting that the acid protease was not involved directly with sporulation. Furthermore, acid protease activity increased when the pleomorphic strain or strain R87 was grown in liquid culture on a complex nitrogen source [1% (w/v) casein], and acid protease activity decreased when the strains were grown on a simple nitrogen source [1% (w/v) Casamino Acids]. The alkaline protease activity level was unaffected by the complexity of the exogenous nitrogen source. These results suggested that the acid protease

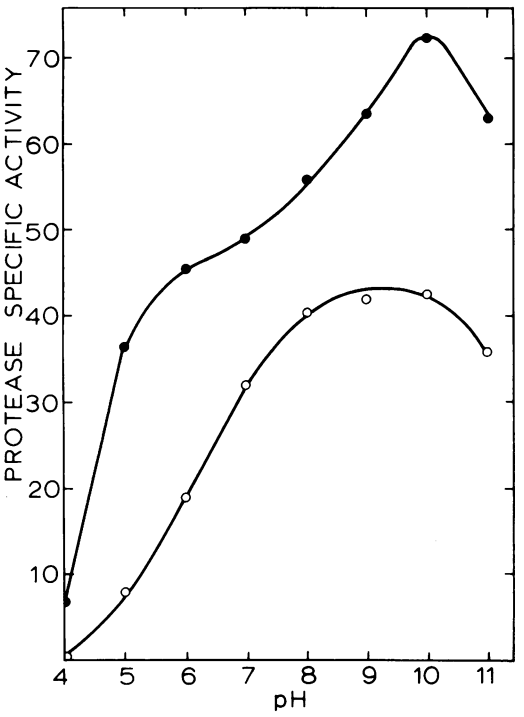


FIG. 1. *Comparison of strain R87 CFX protease and germination protease activity versus* pH. *Specific activity = caseinolytic units per milligram of protein.* (●) *5-day-old strain R87 sporulating culture CFX;* (○) *germination enzyme.*

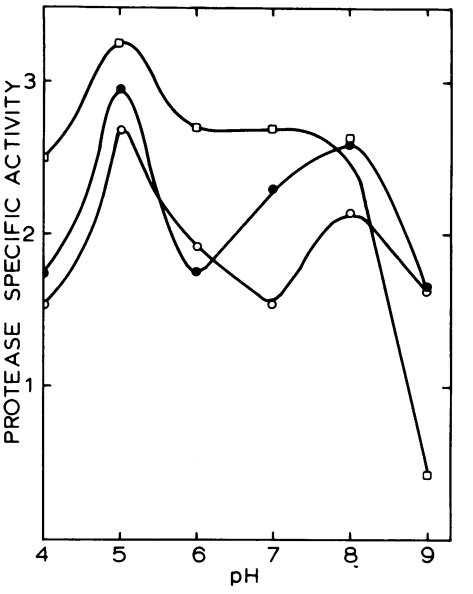


FIG. 2. *Presence of acid and alkaline protease in cell-free extract of strain R87. Specific activity = caseinolytic units per milligram of protein.* (●) *5-day-old strain R87 sporulating culture CFX +* $10^{-3}$ M *phenyl methyl sulfonyl fluoride (preincubated for 30 min at 37 C);* (○) *5-day-old strain R87 sporulating culture CFX +* $5 \times 10^{-3}$ M *phenyl methyl sulfonyl fluoride (preincubated for 30 min at 37 C);* (□) *5-day-old pleomorphic strain culture CFX.*

was assimilative and involved with mycelial growth.

**Occurrence of the alkaline protease during sporulation and germination.** During sporulation of strain R87, alkaline protease levels increased, whereas in the asporogenous pleomorphic strain and the liquid-grown strain R87 (nonsporulating in liquid culture), the protease levels were much lower than those in the sporulating strain R87 (Fig. 3). The increase of 21 protease activity units from 4 to 5 days in sporulating strain R87 was entirely accounted for in the isolated spore CFX plus spore coat homogenate, and the apparent decrease of 17 protease activity units from 5 to 7 days in sporulating strain R87 was accounted for in the spore coat material (Table 1). The protease activity associated with the spore coat material appeared as a decrease in Fig. 3 since the spore coat material sedimented at 10,000 × *g* and was discarded routinely in CFX preparation.

**Stability of the alkaline protease binding to the spore coat.** Fractionation of ungerminated and germinated macroconidia by ultracentrifugation (Table 2) confirmed the previous observation of the association of the alkaline protease with spore coat material (7). In ungerminated spores, the protease is tightly bound to the spore coat and little activity was removed by washing with 0.05 M Tris buffer (*p*H 7.4; 200 × *g* wash supernatant fraction). After germination and release of 56% of the protease into the germination medium, the remaining activity was less tightly bound to the spore coat and was removed in the 200 × *g* wash supernatant fraction (Table 2).

Washing ungerminated spore coat material with various solvents indicated that the protease was not covalently bound to the spore coat since 97% of the activity was removed by washing with 8 M urea or with 10% sodium dodecyl sulfate after 24 hr. The protease was unlikely to be associated with the spore coat as a free enzyme since it was not totally removed from the coats by any of the solvents after 24 hr. The presence of 10 to 39% of the original protease activity after 24 hr of washing with either 0.05 M Veronal buffer or a solution with high salt concentration (6 M ammonium chlo-

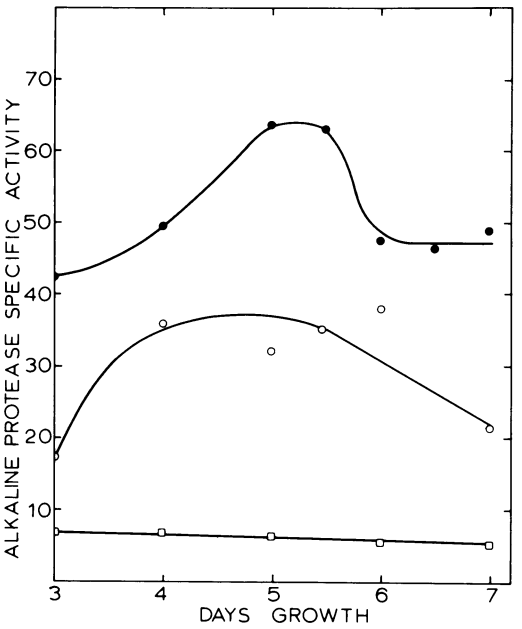


FIG. 3. *Changes in protease enzyme activity during growth. Specific activity = caseinolytic units per milligram of protein. (●) Strain R87, sporulating culture (solid growth medium); (○) strain R87, nonsporulating culture (liquid growth medium); (□) pleomorphic strain (solid growth medium).*

TABLE 1. *Localization of the alkaline protease in spore coat material*

| CFX material | Growth time (days) | Alkaline protease specific activity[a] |
|---|---|---|
| Spores + mycelia[b] | 4–5 | +21 |
| Spores + mycelia[b] | 5–7 | −17 |
| Spore CFX + spore coats | 7 | 22.5 |
| Spore coats | 7 | 15 |
| Spore CFX | 7 | 7.5 |

[a] In terms of caseinolytic units per milligram of protein at *p*H 9.0, 37 C. + indicates increased enzyme activity; − indicates decreased enzyme activity.

[b] Data derived from Fig. 3.

TABLE 2. *Binding of alkaline protease with spore coat material before and after spore germination*

| Fraction | Total alkaline protease units[a] (%) | |
|---|---|---|
| | Ungerminated spores | Germinated spores |
| 200 × *g* wash pellet[b] | 86.9 | 18.2 |
| 200 × *g* wash supernatant[b] fraction | 10.5 | 21.3 |
| 10,000 × *g* pellet | 0.4 | 2.6 |
| 30,000 × *g* pellet | 2.0 | 0.19 |
| 40,000 × *g* pellet | 0.0 | 7.7 |
| 40,000 × *g* supernatant fraction | 0.0 | 0.0 |
| Germination medium | 0.0 | 56.0 |

[a] Calculated as = (units of protease activity per fraction/units of protease activity per unfractionated homogenate) × 100%, where 1 unit of activity = (Δ OD 280 nm × $10^3$) per min.

[b] Pellet (200 × *g*) washed with 0.05 M Tris buffer (*p*H 7.4) and resedimented at 200 × *g* for 10 min.

TABLE 3. *Stability of the alkaline protease-binding with the spore coat*

| Solvent[a] | Untreated spore coat alkaline protease specific activity[b] (%) | |
|---|---|---|
| | After 24 hr of washing | After 48 hr of washing |
| 6 M $NH_4Cl$ | 38.7 | 5.2 |
| 8 M Urea | 2.4 | 0.0 |
| 10% Sodium dodecyl sulfate | 3.1 | 0.0 |
| $10^{-2}$ M Ethylenediaminetetraacetic acid | 4.3 | 1.2 |
| 0.85% NaCl | 5.3 | 0.0 |
| 0.85% NaCl (37 C) | 4.4 | 0.0 |
| 0.05 M Veronal buffer (*p*H 9.0) | 10.1 | 0.0 |

[a] All solvent washes carried out at 25 C.

[b] Specific activity = units per milligram of spore coat material (dry weight). Unit = (Δ optical density at 280 nm × $10^3$) per minute.

ride), respectively, suggested that these conditions must stabilize the binding of the protease to the spore coat (Table 3).

**Separation of acid and alkaline proteases in Ficoll gradients.** Acid protease-containing vesicles were demonstrable by the method of Matile and Wienken (15), by flotation in the presence of 8% Ficoll; however, by this procedure, the alkaline protease activity sedimented with the spore coat material. As shown in Fig. 4, the alkaline protease also was contained in vesicles which could be separated from the acid protease-containing vesicles by flotation in 0 to 20% Ficoll gradients by the modified method of Brown (2). Further clarification of the protease activity bands was obtained by high-speed sedimentation (30,000 rev/min for 60 min at 4 C), through a 0 to 20% Ficoll gradient. Under these conditions, the acid protease was present as one peak (A), and the al-

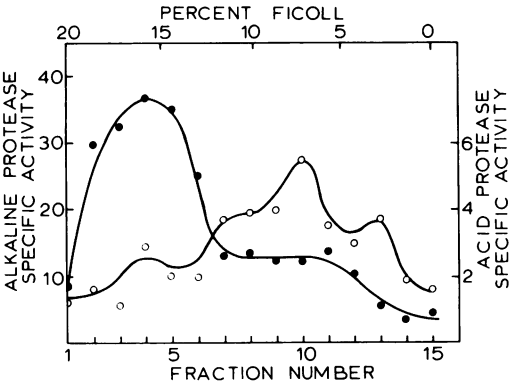


FIG. 4. *Separation of acid and alkaline protease activities by flotation in the presence of Ficoll. Specific activity = caseinolytic units per absorbance at 280 nm per fraction.* (O) *Acid protease activity;* (●) *alkaline protease activity.*

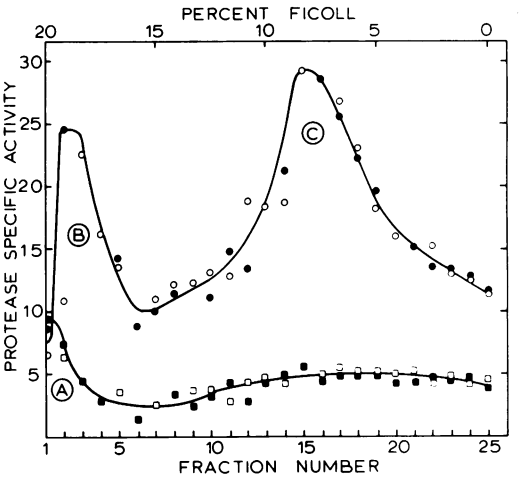


FIG. 5. *Separation of acid and alkaline protease activities by sedimentation on a Ficoll gradient. Specific activity = caseinolytic units per absorbance at 280 nm per fraction.* (O; ●) *Duplicate experiments, protease activity, pH 9.0;* (□; ■) *duplicate experiments, protease activity, pH 5.0.*

kaline protease was present in two major peaks (B and C). The sedimentation of peak B suggested that these vesicles were very dense, whereas the peak C vesicles were more buoyant (Fig. 5).

**Enzymes associated with the acid and alkaline protease vesicles.** Table 4 shows that maximal acid phosphatase activity was associated with the acid protease vesicles (peak A). The alkaline protease vesicles (peaks B and C) contained no acid protease activity and had less acid phosphatase activity than peak A. Phosphodiesterase activity was concentrated in peak B with some activity present in peak C. The fractions were free of mitochondrial contamination as judged by the absence of NADH reductase activity.

**Electron microscopic examination.** The macroconidia of *M. gypseum* appeared multiseptate and elongate under examination with the light microscope. The germ tube arises terminally as previously noted (19). In the 2-hr germinating spore, thin cross sections revealed a thick spore coat of at least three layers. Small vesicles were visible inside the spore coat layer next to the plasmalemma (Fig. 6). Spores which had been shaken for 4 hr in germination medium had peripheral membrane-bound bodies which were either large and electron-dense (DV) or small and electron-transparent (TV) visible within the spore coat material. The inner layer of the spore coat appeared less granular around the TV than around the DV (Fig. 7). Vesicles appearing in spores germinated after 7 hr were predominantly of the large, electron-dense type and were seen within the areas of the inner spore coat which had become less granular (Fig. 7 and 8).

TABLE 4. *Enzymes present in isolated vesicles*

| Determination | Enzyme specific activity | | | |
|---|---|---|---|---|
| | Unfractionated CFX | Peak A[a] | Peak B[a] | Peak C[a] |
| Alkaline protease[b] | 76.28 | 9.25 | 34.41 | 154.78 |
| Phosphodiesterase[c] | 64.39 | 0.0 | 114.28 | 37.01 |
| Acid protease[b] | 2.38 | 28.32 | 0.0 | 1.96 |
| Acid phosphatase[c] | 29.77 | 177.61 | 70.93 | 57.40 |
| Diaphorase[d] | 74.41 | 25.92 | 34.44 | 22.59 |
| NADH reductase[d] | 29.52 | 0.0 | 0.0 | 0.0 |

[a] Peaks A, B, and C of Fig. 5.

[b] Specific activity = caseinolytic units per milligram of protein.

[c] Specific activity = [Δ optical density (OD) at 410 nm × $10^3$] per minute per milligram of protein.

[d] Specific activity = (Δ $OD_{600}$ × $10^2$) per minute per milligram of protein.

## DISCUSSION

The results of this investigation have shown that *M. gypseum* mycelia contained two cytoplasmic proteases. During sporulation, an alkaline protease was incorporated into the spore coat. The alkaline protease was not associated with the spore coat as a soluble or covalently bound enzyme but was localized in vesicles.

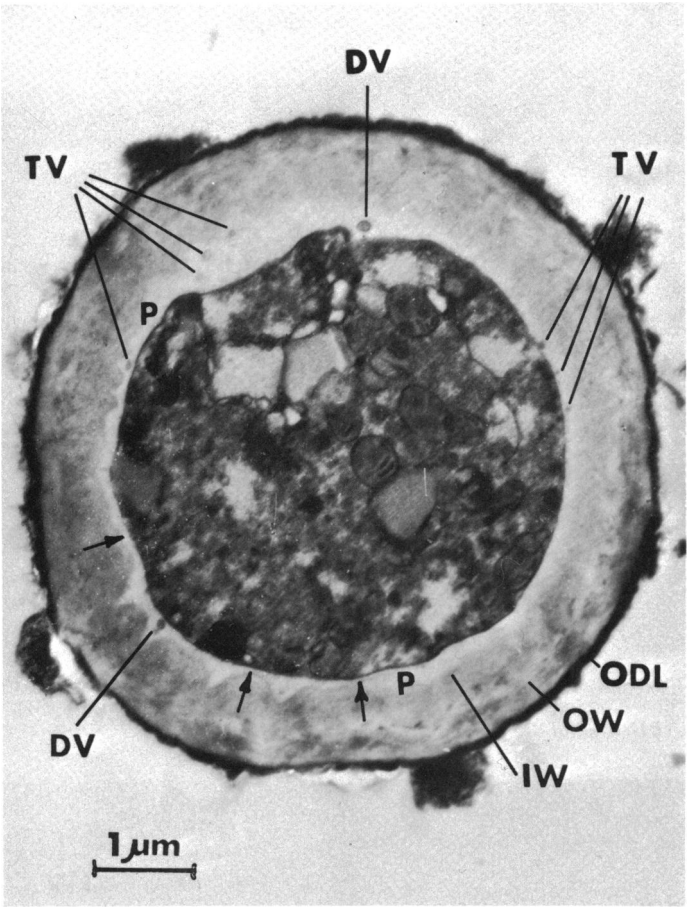


FIG. 6. *Cross-sectional view of a macroconidium after 2-hr germination. The spore coat is composed of three layers: an inner granular layer (IW), an outer lamellar layer (OW), and an outer electron-dense layer (ODL). Numerous peripheral vesicles are located within the inner wall layer and are either small electron-transparent vesicles (TV) or large electron-dense vesicles (DV). The inner wall layer is in close contact (arrows) with the plasmalemma (P), where the vesicles are absent.*

These vesicles were seen in electron micrographs of germinating *M. gypseum* macroconidia and were isolated by Ficoll gradient centrifugation.

Morphologically, the alkaline protease-containing vesicles resembled those isolated from *Neurospora crassa*, where the protease-containing vesicles cross the plasmalemma as intact particles by possible invagination of the plasmalemma (14). During *M. gypseum* macroconidial germination, the vesicles, which probably contained the alkaline protease, appeared electron transparent, and the spore coat around them became less granular, probably due to proteolysis by released alkaline protease. The spore coat layer most affected by hydrolysis was that next to the plasmalemma. This layer has been reported to be a peptido-glucan in *Saccharomyces cerevisiae* (5) or a peptido-glucan-chitin complex in *Candida utilis* (18). It is quite likely that the hydrolysis observed during *M. gypseum* macroconidial germination also involved a peptido-glucan layer, as preliminary experiments have shown that the germination protease hydrolyzed the peptido-glucan isolated from *M. gypseum* spore coats more rapidly than the spore coat peptido-mannan (W. Page, *unpublished data*). Furthermore, the observation that phosphatase and phosphodiesterase activity was associated with the alkaline protease-containing vesicles confirmed an earlier hypoth-

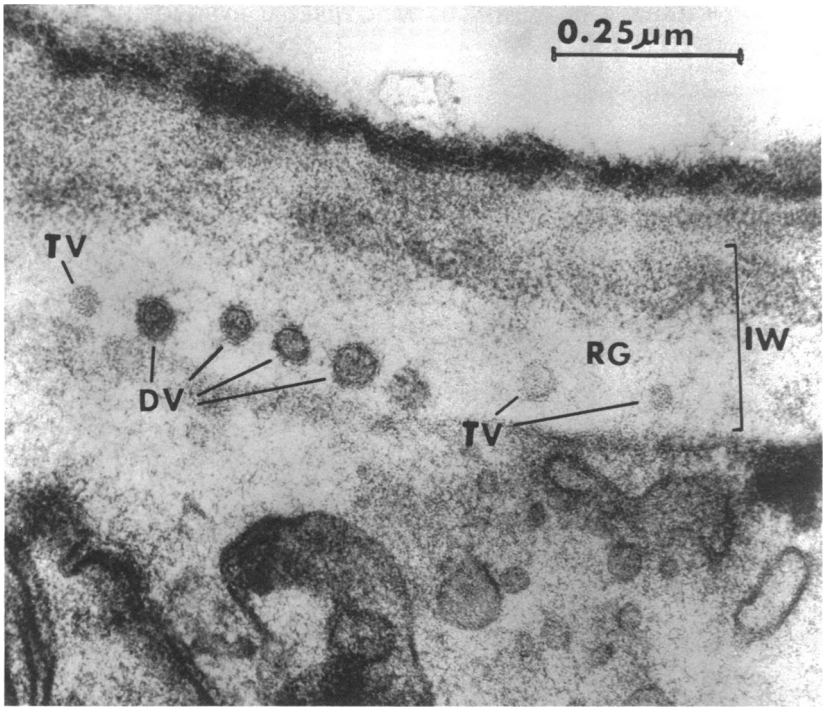


FIG. 7. *Longitudinal view of a macroconidium after 4 hr of germination. Both electron-transparent vesicles (TV), and electron-dense vesicles (DV) are seen within the spore coat inner wall (IW). The inner wall has reduced granularity (RG) around the electron transparent vesicles.*

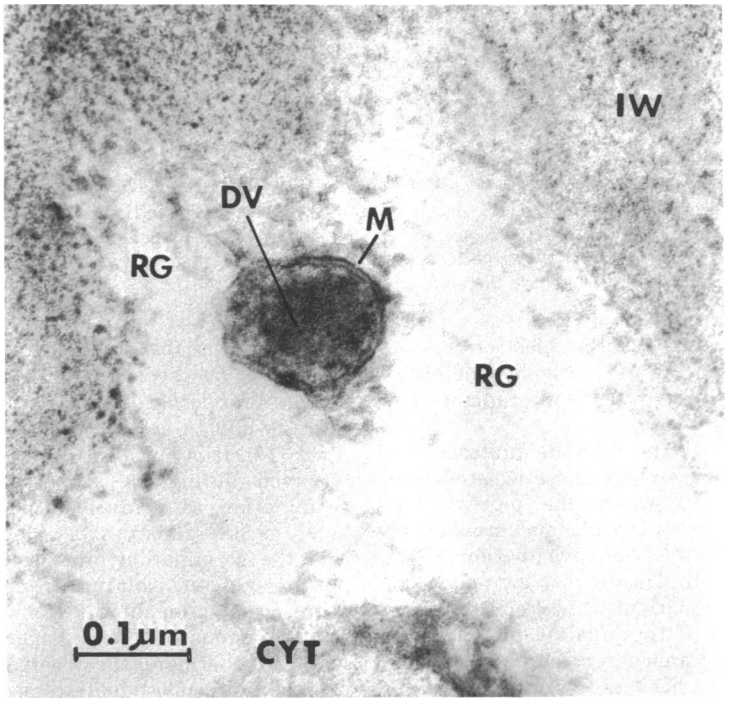


FIG. 8. *Longitudinal view of a germinating macroconidium apical tip after 7 hr in germination medium. The spore coat inner wall (IW) granularity is reduced (RG) around the cytoplasmic material (CYT) and extends into the inner wall material. The apical vesicle (DV) is membrane-bound (M) and contains electron-dense material.*

esis that the source of phosphate which inactivated the alkaline protease after germination was spore coat phosphates or phosphodiesters (19).

The acid protease-containing vesicle was shown to be an assimilative lysosome. Acid protease activity increased as the exogenous nitrogen source became more complex, indicating the lytic-assimilative role of these vesicles. Acid phosphatase activity was associated with the acid protease, as is true for other assimilative lysosomes (2, 15). Also, the behavior of the vesicles during isolation suggested that they were similar to assimilative lysosomes isolated from *S. cerevisiae* and rat livers (2, 15). The acid protease was not believed to be involved in sporulation since it was found at an equal level of activity in both the R87 strain during sporulation and the asporogenous pleomorphic strain. The origin of the acid protease-containing vesicles from the endoplasmic reticulum (ER) was suggested by the presence of diaphorase activity (13).

The origin of the alkaline protease-containing vesicles was less obvious than that of the acid protease-containing vesicles. The presence of diaphorase activity suggested that the alkaline protease-containing vesicles also were derived from the ER, and the distribution of alkaline protease and phosphodiesterase activity in peaks B and C (Fig. 5) suggested that the vesicles were of the same origin, possibly a multivesicular form of vesicle as suggested by Marchant (13). We have shown previously that during *M. gypseum* macroconidial germination, alkaline protease was released from 0 to 2 hr with phosphate release and that subsequent alkaline protease inactivation was most pronounced from 4 to 8 hr (19). It has been shown here that the majority of the alkaline protease was associated with the spore coat prior to germination. These observations suggested that the buoyant vesicles (peak C), containing the most alkaline protease activity, are inserted into the spore coat prior to germination, and the denser vesicles (peak B), containing the greatest phosphodiesterase activity are inserted into the spore coat after initial germination and release of alkaline protease. This would permit spore coat hydrolysis to precede germination protease inactivation. Further evidence supporting this proposal was the observation of two types of vesicles within the inner spore coat layer. The electron transparent vesicles were the predominant type in spores germinated in 2 hr, with the DV appearing and becoming the predominant type in spores germinated 4 to 7 hr. The possible origins of the alkaline protease-containing vesicles are summarized in Fig. 9, where: (I) the alkaline protease-containing vesicles and, (II) the phosphodiesterase-containing vesicles are derived independently from the ER, or (III) the alkaline protease-containing vesicles and the phosphodiesterase-containing vesicles are derived from a common multivesicular body which has been formed directly from the ER. We favor the latter interpretation since it ac-

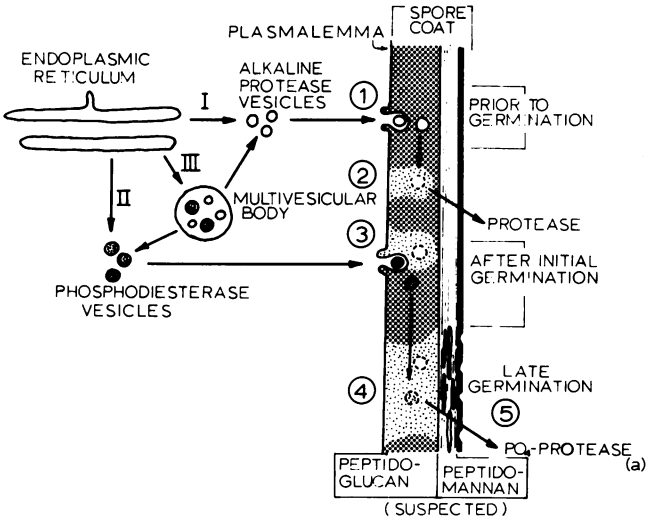


FIG. 9. *Proposed schematic portrayal of the origins, distribution, and function of germination lysosomes of M. gypseum. (a) Inactivation of the germination protease by inorganic phosphate has been established previously (19).*

counts for the presence of both phosphodiesterase and alkaline protease activities in both peaks B and C.

The role of these lysosomes in macroconidial germination could, therefore, provide an ordered sequence of events: (1) insertion of alkaline protease-containing lysosomes into the spore coat prior to germination, (2) release of protease and proteolysis of the spore coat, (3) insertion of phosphodiesterase-containing vesicles into the spore coat, (4) release of phosphodiesterase, diester cleavage in the spore coat, and release of inorganic phosphate and, (5) phosphate inactivation of the alkaline protease in the germination medium.

## ACKNOWLEDGMENTS

We thank Teresa E. Walters for excellent technical assistance with electron microscope procedures.

This investigation was supported by a grant from the Medical Research Council, Ottawa, Canada.

## LITERATURE CITED

1. Bleyman, M., and C. Woese. 1969. Ribosomal ribonucleic acid maturation during bacterial spore germination. J. Bacteriol. **97**:27-31.
2. Brown, D. H. 1968. Separation of mitochrondria, peroxisomes, and lysosomes by zonal centrifugation in Ficoll gradients. Biochim. Biophys. Acta **162**:152-153.
3. Grove, S. N., and C. E. Bracker. 1970. Protoplasmic organization of hyphal tips among fungi: vesicles and spitzenkörper. J. Bacteriol. **104**:989-1009.
4. Iten, W., and P. Matile. 1970. Role of chitinase and other lysosomal enzymes of *Coprinus lagopus* in the autolysis of fruiting bodies. J. Gen. Microbiol. **61**:301-309.
5. Kidby, D. K., and R. Davies. 1970. Invertase and disulfide bridges in the yeast wall. J. Gen. Microbiol. **61**: 327-333.
6. Leighton, T. J., and J. J. Stock. 1969. Heat-induced macroconidia germination in *Microsporum gypseum*. Appl. Microbiol. **17**:473-475.
7. Leighton, T. J., and J. J. Stock. 1970. Biochemical changes during fungal sporulation and spore germination. I. Phenyl methyl sulfonyl fluoride inhibition of macroconidial germination in *Microsporum gypseum*. J. Bacteriol. **101**:931-940.
8. Leighton, T. J., and J. J. Stock. 1970. Isolation and preliminary characterization of developmental mutants from *Microsporum gypseum*. J. Bacteriol. **104**:834-838.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. McClure, W. Katherine, D. Park, and P. M. Robinson. 1968. Apical organization in the somatic hyphae of fungi. J. Gen. Microbiol. **50**:177-182.
11. McDonald, C. E., and L. L. Chen. 1965. The Lowry modification of the Folin reagent for determination of proteinase activity. Anal. Biochem. **10**:175-177.
12. Mahler, H. R. 1955. Diaphorases, p. 707-708. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
13. Marchant, R., and A. W. Robards. 1968. Membrane systems associated with the plasmalemma of plant cells. Ann. Bot. (London) **32**:457-471.
14. Matile, P., M. Jost, and H. Moor. 1965. Intrazellulare lokalsation proteolytischer enzyme von *Neurospora crassa*. Z. Zellforsch. Mikrosk. Anat. **68**:205-216.
15. Matile, P., and A. Wienken. 1967. The vacuole as the lysosome of the yeast cell. Arch. Mikrobiol. **56**:148-155.
16. Moor, H. 1967. Endoplasmic reticulum as the initiator of bud formation in yeast (*Saccharomyces cerevisiae*). Arch. Mikrobiol. **57**:135-146.
17. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685-3692.
18. Novaes-Lédien, M., and C. Garcia-Mendoza. 1970. Biochemical studies on walls synthesized by *Candida utilis* protoplasts. J. Gen. Microbiol. **61**:335-342.
19. Page, W. J., and J. J. Stock. 1971. Regulation and self-inhibition of *Microsporum gypseum* macroconidia germination. J. Bacteriol. **108**:276-281.