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Melanins and Resistance of Fungi to Lysis

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Hyphal walls of *Aspergillus phoenicis* and *Sclerotium rolfsii* are composed of large amounts of glucose- and *N*-acetylhexosamine-containing polysaccharides, and the walls are extensively digested by streptomycete culture filtrates or by a mixture of purified chitinase and β-(1 $\rightarrow$ 3) glucanase preparations with the release of the monomeric units. *A. phoenicis* conidial walls also contain polymers of glucose and *N*-acetylhexosamine, but these walls are resistant to digestion by microorganisms or the enzyme combination active on the hyphae. When the melanin-containing spicules were removed from the spore surface, however, the chitinase and glucanase partially digested the underlying structural components. Microorganisms decomposing hyphal walls of *S. rolfsii* did not attack the melanin-covered sclerotia produced by this fungus. No microorganism capable of lysing two fungi, *Rhizoctonia solani* and *Cladosporium* sp., producing hyphae containing abundant melanin was found. The ecological significance of these findings and possible mechanisms for the protective influence associated with melanins are discussed.

Fungal structures which show marked durability under adverse conditions frequently exhibit dark pigmentation. These pigmented structures often are responsible for the prolonged persistence of certain organisms in natural habitats. Recent investigations have shown that the black or brownish-black pigments extracted from several fungi are melanins or are melanin-like (4, 8, 11, 15, 18). Although lysis arising from the destruction of fungal surface structures by bacterial or actinomycete enzymes appears to be an important mechanism by which fungi are destroyed in nature, vegetative hyphae, resting structures, and spores of a number of genera or species are not readily susceptible to enzymatic digestion.

In the present study, a relationship between the presence of melanin in the surface structures of certain of these microorganisms and their resistance to lysis is demonstrated.

MATERIALS AND METHODS

Four fungi which produce pigmented structures were examined. Two of the four species undergo during their growth a morphological change from hyaline hyphae to dark-pigmented resting structures. These two microorganisms, *Aspergillus phoenicis* QM 1005 and *Sclerotium rolfsii*, were grown at room temperature on a rotary shaker in a medium containing 10 g of peptone (Difco), 3 g of yeast extract (Difco), and 20 g of glucose per liter of distilled water. The cultures were obtained from E. T. Reese, Quartermaster Research and Engineering Center, Natick, Mass., and D. F. Bateman, Cornell University. Nonpigmented walls were prepared from the hyaline hyphae thus obtained by disrupting the cells with Superbrite no. 110 glass beads (3M Company, St. Paul, Minn.) in an Omni-mixer homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) at temperatures below 10 C and extensively washing the isolated wall material with cold distilled water. Two fungi which produced hyphae containing melanin, *Rhizoctonia solani* and *Cladosporium* sp., were also employed. They were cultured as previously described (15). Analysis of the hyphal walls of these two organisms showed them to contain 8.5 and 12.8% melanin, respectively (15). Spores of *A. phoenicis* were obtained by growing the fungus in Fernbach flasks on moist pearled rice grains and harvesting the spores in cold distilled water. Sclerotia of *S. rolfsii* were obtained from cultures grown on an agar medium identical to the liquid medium except for a higher (0.4%) glucose concentration. After 7 days, the mature sclerotia were harvested by lightly scraping them from the agar surface.

Autolyzed preparations of *A. phoenicis* spores were prepared by mixing together equal weights of *A. phoenicis* conidia and distilled water and 10% (v/v) of toluene. After incubation at 50 C for 3 days, the residue was centrifuged at 3,000 $\times$ *g* for 10 min and washed several times with distilled water to remove cytoplasmic matter. This treatment did not visibly affect the spiculate surface of the spore. Most of the black spicules on the surfaces of freshly harvested *A. phoenicis* conidia were detached by suspending the spores in distilled water and abrading them with glass beads in the Omni-mixer for periods up to 60 min at a temperature below 10 C. The largely spicule-

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free wall material was separated from the dark pigmented fraction by centrifugation at 2,000 × *g* for 10 min; the wall preparations, now essentially free from spicules, appeared as a light-gray pellet, whereas the spicule-containing material floated to the surface of the liquid.

Crude preparations of sclerotial rinds were made by light abrasion of the sclerotia with glass beads. Freshly harvested sclerotia were mixed with an equal weight of glass beads and sufficient distilled water to cover them, and were then abraded for 1 min at low speed in the Omni-mixer. The crude rind preparations floated and were readily separable from the sclerotial residues and glass beads. Wall and rind preparations were routinely stained with Lugol's iodine to determine the absence of cytoplasmic contamination.

Acid hydrolysis of the hyphal walls was carried out by heating for 6 hr at 100 C with 6 N HCl. For complete dissolution of spore walls, three successive 6-hr treatments with 6 N HCl at 100 C were required. Enzymatic hydrolyses were performed at *p*H 5.5 and 37 C, either with the culture filtrate of a streptomycete that was grown for 72 hr at 30 C in an inorganic medium (17) containing 0.2% (w/v) *A. phoenicis* hyphae as the sole carbon source or with a mixture containing an exo-β-(1 → 3) glucanase and chitinase. The glucanase was prepared from basidiomycete strain QM806, the chitinase was purified from a commercial enzyme preparation (Worthington Biochemical Corp., Freehold, N.J.), and the walls were hydrolyzed enzymatically by the methods previously described (17). The presence of other glycanases in these enzyme preparations was not determined.

Anthrone-positive components were determined by the procedure described by Bartnicki-Garcia and Nickerson (3), and the values were expressed as glucose. Hexosamine was measured by a modification of the Elson-Morgan method (20) and was expressed as glucosamine. Sugars in the hydrolysates were separated and identified by one-dimensional descending paper chromatography with *n*-butanol-pyridine-water (6:4:3, v/v) as the solvent system; the chromatograms were sprayed with aniline phthalate or ammoniacal silver nitrate (6). The proportion of glucose in the reducing sugar fraction was established by quantitative elution of the spots revealed by aniline phthalate (6). Acetyl units were determined separately by the technique of Ludowieg and Dorfman (12). The quantity of *N*-acetylhexosamine released from the walls by enzymatic hydrolysis was determined by the method of Reissig, Strominger, and Leloir (16), and glucose was determined quantitatively by means of the Glucostat reagent (Worthington Biochemical Corp.).

RESULTS

Analysis of acid hydrolysates of hyphal walls obtained from a 48-hr culture of *A. phoenicis* showed that the walls contained 67.7% anthrone-positive components (calculated as glucose), and glucosamine accounted for 20.9% of the dry weight of the walls. Chromatograms of neutralized hydrolysates showed major spots corresponding to glucose and glucosamine, with smaller spots corresponding to mannose and galactose and a faint spot corresponding to galactosamine. Glucose, measured by quantitative elution of the appropriate spot from paper chromatograms, accounted for approximately 96% of the reducing sugar fraction. The separate acetyl determinations on wall preparations suggested that the glucosamine was fully acetylated (Table 1).

A. phoenicis hyphae were readily degraded by soil microorganisms. Thus, a number of *Bacillus* and *Streptomyces* isolates capable of lysing *A. phoenicis* hyphae were obtained by sprinkling soil crumbs on an agar medium containing hyphae, 1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 1.5% agar (Difco). The activity of the isolates was verified on an agar medium containing inorganic salts (17) and 0.2% hyphal walls. Enzymes in the lytic culture filtrate of one of the isolates, *Streptomyces* S-3, catalyzed the release of glucose and acetylhexosamine from wall preparations. When 3.0 mg of *A. phoenicis* hyphal walls was incubated with 10 ml of the crude culture filtrate at 37 C, 62% of the wall was digested in 10 hr. Glucose was released more readily than the acetylated sugar; e.g., 73% of the bound glucose but only 32% of the bound acetylhexosamine was liberated. Moreover, a mixture containing 66 milliunits of exo-β-(1 → 3) glucanase and 3.0 milliunits of chitinase per ml, used under conditions and at enzyme concentrations previously employed (17), caused solubilization of approximately 92% of the *A. phoenicis* hyphal walls (as measured by weight loss) and 95% of the bound glucose in 3 hr. Thus, components of the *A. phoenicis* hyphal wall, which appears to contain chitin and a glucan, are readily digested, and the mycelium is easily lysed by microorganisms.

Glucose and glucosamine were the major carbohydrate components identified by paper chromatography of acid hydrolysates of *A. phoenicis* spore walls essentially freed from the spicules which are normally embedded in the external surfaces of the conidia. Spots corresponding to mannose and galactose were also noted, and a faint spot appeared with R_F corresponding to

TABLE 1. *Acetyl content of hyphal and spore walls of Aspergillus phoenicis and Sclerotium rolfsii*

Fungus	Source of walls	Acetyl content of walls	Moles of acetyl/ moles of hexosamine N
		%	
A. phoenicis	Hyphae	5.3	0.97
	Spores	8.0	0.98
S. rolfsii	Hyphae	12.7	0.82

that of galactosamine. Although the walls of the spores and hyphae exhibit a qualitatively similar carbohydrate composition, analysis of acid hydrolysates of spore walls revealed that glucose accounted for 51.3% and glucosamine for 32.1% of the weight of the walls. Acetyl determinations indicated that the glucosamine was probably fully acetylated (Table 1).

Soil enrichments, prepared as described above, designed to favor the growth of organisms capable of using autolyzed spores of *A. phoenicis* as substrate, failed to yield any isolate capable of digesting the spore walls. Autolyzed spore preparations likewise did not serve as substrates for any of the lytic organisms active against the hyphae. Detectable amounts of glucose and *N*-acetylglucosamine were not released from the spore autolysates incubated either with the lytic filtrate of *Streptomyces* S-3 grown on a hyphae-salts medium or the β-(1 $\rightarrow$ 3) glucanase and chitinase combination. When a spicule-free spore wall preparation, which was essentially nonpigmented, was incubated with the mixture containing 66 milliunits of glucanase and 3.0 milliunits of chitinase per ml, 23% of the bound glucose and 15% of the bound *N*-acetylhexosamine were released, in marked contrast to the lack of enzymatic hydrolysis of the spicule-containing walls. These results suggest that the pigmented particles contribute to the resistance of spores to lysis.

The pigmented material obtained from the spicules of *A. phoenicis* spores was soluble in 1 N KOH but not in 1 N HCl, and it was slowly solubilized by hot concentrated H_2SO_4. It was bleached by 5% sodium hypochlorite and 30% (v/v) H_2O_2, properties indicating a kinship of the fungal product with the melanins. Hydrolysis of the isolated spicules with 6 N HCl did not lead to the release of detectable quantities of anthrone-positive substances, but 12 ninhydrin-positive spots appeared on two-dimensional paper chromatograms of the hydrolysates prepared with *n*-butanol-acetic acid-water (25:6:25, v/v) and phenol saturated with 0.2 M K_2HPO_4 in water (3). One of the spots had the chromatographic characteristics of glucosamine, as indicated by its reaction with the acetyl acetone-dimethylaminobenzaldehyde reagent (6). A residual pigmented material, which was soluble in 1 N KOH but not in HCl, accounted for 82 to 85% of the dry weight of the particles. Neither a crude keratinase prepared from a strain of *Streptomyces fradiae* (14) nor a trypsin preparation released ninhydrin-positive materials when incubated for 12 hr at 37 C with the isolated pigmented particles.

The hyphal walls of *S. rolfsii* were examined by the methods used with *A. pheonicis*. Glucose and glucosamine were the major components on paper chromatograms of acid hydrolysates of the hyphal walls; these accounted for 18.3 and 61.0% of the weight of the walls, respectively, prepared from 72-hr cultures. Faint spots corresponding to mannose and galactose were also present, as well as a very faint spot at the R_F of authentic galactosamine; the quantity of these sugars was not determined. Three separate assays of the acetyl content of the walls gave values indicating 81 to 84% acetylation of the hexosamine, with a mean of 82% (Table 1).

The culture filtrate from *Streptomyces* S-7, isolated from soil, as well as a combination of purified β-(1 $\rightarrow$ 3) glucanase and chitinase, caused extensive degradation of *S. rolfsii* hyphal walls, releasing both *N*-acetylglucosamine and glucose; the reaction mixture contained 44.0 milliunits of glucanase, 2.0 milliunits of chitinase, and 0.33 mg of walls (dry weight) per ml. The filtrate of S-7, without further concentration or purification, solubilized 87% of the wall preparations in 10 hr at 37 C. The combination of purified enzymes solubilized 93% of the wall in 3 hr under the standard conditions (17), releasing 98% of the bound glucose and, assuming 82% acetylation of the hexosamine fraction, 86% of the bound acetylhexosamine. A small amount of free glucosamine was noted on paper chromatograms of the enzymatic hydrolysates of the wall components. Lytic filtrates of *Streptomyces* S-7, capable of digesting *S. rolfsii* hyphal walls, also degraded mycelial walls of *Fusarium solani*, which contain appreciable quantities of chitin and a β-(1 $\rightarrow$ 3) glucan (17), solubilizing 83% of the wall material in 10 hr at 37 C.

The sclerotium formed by *S. rolfsii* is characterized by a brown-pigmented outer layer or rind. Townsend and Willetts (19) have shown that these sclerotia are formed by the coalescence of vegetative hyphae, followed by the development of several thickened wall layers around the hyphal core. Toluene-treated whole sclerotia did not serve as a substrate for the lytic organisms which digested *S. rolfsii* hyphae. Crude preparations of the rinds were incubated with either the lytic filtrate of *Streptomyces* S-7, after growth of the organism in a hyphae-mineral salts medium, or with the purified glucanase and chitinase preparation which degraded the hyphal wall of this fungus. Examination of the supernatant fluid for free amino acids and sugars, by paper chromatography, and determination of possible weight losses showed that digestion had not occurred.

A brown pigment obtained from the *S. rolfsii* sclerotial rinds was soluble in both hot 1 N HCl and 1 N KOH. The pigmented material was ex-

tracted from the crude rind preparation with 1 N HCl at 100 C under an atmosphere of nitrogen. This pigment-containing fraction was precipitated from the solution by adjusting the *p*H to 6.5 with 1 N KOH, and the dark residue was washed with water, dissolved in 1 N HCl and reprecipitated. Two-dimensional chromatography of 6 N HCl hydrolysates of the pigmented rind fraction revealed 13 ninhydrin-positive spots; one of the major spots on the chromatograms stained purple with acetyl acetone-dimethylaminobenzaldehyde reagent, suggesting that it was glucosamine (6). The brown residue remaining after hydrolysis at 100 C was soluble in 1 N KOH but not in 1 N HCl; this residue accounted for 5 to 6% of the dry weight of the crude rind and ca. 55% of the extracted pigmented complex. The latter pigmented material had the properties of a typical melanin, that is, solubility in alkali and bleaching by hypochlorite and H_2O_2. A melanin-type pigmented complex, soluble in both dilute acid and alkali, has been found in the spore walls of *Mucor rouxii* (4).

Attempts were made to isolate microorganisms from soil which could lyse hyphae of *R. solani* and *Cladosporium* sp., fungi which contain melanin pigments in their hyphal walls. Several elective culture techniques were employed: (i) incubating soil crumbs on the surface of mineral salts-agar media containing 2.0% (w/v) autoclaved hyphae of the appropriate fungus and 0.05% (w/v) Difco yeast extract, hyphae plus 0.05% (w/v) yeast extract, and 0.1% (w/v) glucose or hyphae alone; and (ii) incubating 0.1-g soil samples in shallow layers of a solution having the same composition as the agar media described. The enrichments were incubated at 28 C for up to 3 weeks, but no organisms active on purified walls of the fungi could be isolated. This agrees with failures in earlier attempts to obtain an organism lysing *R. solani* (15).

DISCUSSION

The biochemical basis for the persistence of certain microbial structures in natural ecosystems is unknown. Many fungi produce filaments that are readily colonized by other microorganisms, and these hyphae are usually digested quickly by components of the mixed microbial community of natural habitats. The lysis-susceptible mycelium of the species that have been examined to to date contain chitin and a glucan with a preponderance of β-(1 $\rightarrow$ 3) linkages (15, 17; K. Horikoshi, H. Koffler, and H. R. Garner, Bacteriol. Proc., p. 95, 1961), whereas other fungi whose hyphae likewise seem prone to digestion by enzymes produced by mycolytic bacteria and actinomycetes contain glucans with a preponderance of β-(1 $\rightarrow$ 4) or β-(1 $\rightarrow$ 2) linkages (2, 13).

A. phoenicis and *S. rolfsii* likewise develop vegetatively by means of hyphae which contain chitin and a glucose-containing polymer, possibly also a glucan with an abundance of β-(1 $\rightarrow$ 3) bonds. These two species produce structures, a conidium and a sclerotium, typical of the fungal bodies which persist for long periods in terrestrial environments in the presence of a heterogeneous microflora which undoubtedly contains a multitude of species liberating extracellular enzymes catalyzing the degradation of cell wall constituents. Indeed, the sclerotia of many soil-borne phytopathogenic fungi, such as *S. rolfsii*, have achieved a degree of notoriety because it is these structures which often account for the prolonged viability of the pathogens in the absence of their hosts (10). Likewise, hyphae of certain species resist decomposition in nature, and frequently these hyphae, as in the instance of *R. solani* (5), are not readily decomposed and retain their viability for long periods, even in the absence of a utilizable substrate.

The evidence presented herein suggests that the resistance of at least certain fungal structures is correlated with the presence in the external surface of melanin or a melanin-like material. Thus, the dark hyphae of *R. solani* and *Cladosporium* sp., both of which possess significant amounts of melanin, were not found to be suitable as sole carbon and energy sources for any soil microorganism. Likewise, sclerotia containing a melanin-rich rind and conidia containing melanin-rich spicules on their outer surfaces were not lysed, whereas digestion did occur when the external pigmented material was removed from the spores. Potgieter and Alexander (15) have already shown that *R. solani*, although possessing glucose-rich walls, was largely unaffected by β-(1 $\rightarrow$ 3) glucanase and chitinase.

Although the evidence indicates a correlation between the presence of melanin and resistance to microbiological lysis, no direct test of the hypothesis has been made. More direct evidence, however, has been obtained with a lysis-resistant strain of *Aspergillus nidulans* and a susceptible, non-melanin-producing mutant derived from it (Kuo and Alexander, *unpublished data*). Should the melanin indeed be directly responsible for the resistance, the polyaromatic material may serve to inhibit enzymes degrading those cell wall or surface-localized constituents which maintain structural integrity, or the melanin may combine with or overlay the lysis-susceptible surface components in such a way as to protect them or to prevent the enzymes from combining with

their potential substrates. Akino (1) has presented evidence that a melanin does indeed serve as an enzyme inhibitor. On the other hand, a precedent for the protection afforded to a polysaccharide by the physical barrier associated with the presence of a polyaromatic material is found in the observations of the influence of lignin on cellulose hydrolysis (9). The fact that complex polyaromatic materials like lignin and humus are among the natural products most resistant to decomposition in terrestrial and aquatic habitats and that synthetic melanins are resistant to microbial deggradation (7) lends credence to the suggestion that melanins localized in the external surfaces of microorganisms may confer on the fortunate species the ability to survive in environments containing heterogeneous, biochemically active microfloras.

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