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Inhibition of the Lysis of Fungi by Melanins

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Evidence is presented that the resistance of *Aspergillus nidulans* hyphae to lysis by a β-(1→3) glucanase-chitinase mixture results from the presence of melanin in the fungal walls. The resistance of the walls to digestion was directly correlated with the melanin content of the mycelium. A melanin-less mutant of *A. nidulans* was highly susceptible to hydrolysis by the enzyme mixture. Preincubation of a synthetic melanin with the glucanase, chitinase, and a protease, before addition of the substrate, resulted in a marked inhibition of the rate of substrate hydrolysis. Melanin also appeared to combine with and protect at least certain substrates from decomposition, as indicated by the direct relationship between the extent of inhibition of casein hydrolysis by a bacterial protease and the length of time the protein was incubated with the melanin prior to addition of the enzyme. Melanin was found to be highly resistant to microbial degradation, a likely requirement for the polyaromatic to be effective in protecting fungal structures from lysis or decomposition by natural communities of microorganisms.

In addition to containing polysaccharides and amino acid polymers, the cell walls of certain species of filamentous fungi contain pigments related to or identical with the melanins. Evidence has recently been obtained which suggests an ecological significance of the surface-localized melanins or related polyaromatics. Lockwood (6) noted that the dark hyphae of *Helminthosporium sativum* and *Alternaria solani* were more resistant to lysis than the hyaline hyphae. Potgieter and Alexander (10) observed a relation between the melanin content of hyphal walls of selected fungi and their resistance to enzymatic digestion, and they suggested that melanins or melanin-like substances might serve to protect certain fungi from microbial lysis in nature. More recently, it has been reported that the melanin-covered sclerotia of *Sclerotium rolfsii* and the conidia of *Aspergillus phoenicis*, which possess melanin-rich spicules, are resistant to enzymatic digestion, although hyaline hyphae of both species and essentially spicule-free *A. phoenicis* conidia were readily lysed (2). Moreover, albino chlamydospores of *Thielaviopsis basicola*, in contrast with the dark spores, are not resistant to lysis (5).

The evidence favoring a role for melanins in the ecology of fungi and the persistence of fungal forms in nature is still circumstantial. The present study was designed to provide direct evidence that melanins are related to the resistance of fungi to microbial digestion and to determine the basis for the protective effect.

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MATERIALS AND METHODS

Cultures of *Aspergillus nidulans* were provided by A. T. Bull of Bedford College, University of London, London, England. Strain 13 was a melanin producer, and strain 13.1.0L was a non-melanin-producing mutant derived from strain 13. The organisms were grown on a rotary shaker at 30 C in 500-ml Erlenmeyer flasks containing 200 ml of medium. The medium, devised by A. T. Bull, contained: sucrose, 3%; $NaNO_3$, 0.2%; KH_2PO_4, 0.1%; KCl, 0.05%; $MgCl_2 \cdot 6H_2O$, 0.04%; L-tyrosine, 0.01%; $ZnSO_4 \cdot 7H_2O$, 0.001%; $FeSO_4 \cdot 7H_2O$, 0.001%; and $MgSO_4 \cdot 8H_2O$, 0.0008%. The *p*H of the medium was adjusted to 6.5 after autoclaving.

Cell walls, laminarin, and chitin were prepared and purified by the methods of Skujins, Potgieter, and Alexander (13). Exo-β-D-(1 → 3) glucanase was prepared from Basidiomycete QM806 cultures according to the procedure previously described (10). Chitinase obtained either from Worthington Biochemical Corp., Freehold, N.J., or from a culture of *Streptomyces* 3 as described by Skujins et al. (13) was purified by the method of Potgieter and Alexander (10). *Bacillus subtilis* protease and casein were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Before use, 5 mg of the protease preparation was dissolved in 1 ml of 0.033 M phosphate buffer (*p*H 7.7), and the solution was applied to a diethylaminoethyl-Sephadex (A-50) column. The column was

eluted with 0.033 M phosphate buffer (*p*H 7.7), and the eluate was collected in 3-ml fractions. The first protease-containing peak coming off the column was used.

Synthetic melanin was prepared from DL-β-3,4-dihydroxyphenylalanine (DOPA) as described by Akino (1). The melanin solution commonly employed contained 250 μg of acid-insoluble materials per ml, as determined by Akino's method. Melanin was extracted from fungal cell walls by the method of Nicolaus et al. (9). Dry weights were determined on samples dried at 105 C.

Labeled melanin was prepared by incubating uniformly labeled tyrosine-^{14}C (5 to 15 μc/μmole) obtained from Volk Radiochemical Co., Chicago, Ill., and 50 μg of polyphenol oxidase (Worthington) in 50 ml of 0.067 M phosphate buffer (*p*H 6.1) for 2 days at 30 C on a shaker. The radioactive CO_2 released from soil treated with labeled tyrosine or synthetic melanin was collected in 0.2 N $Ba(OH)_2$, and the $BaCO_3$ was plated on aluminum planchets and counted with an Abacus Scaler, model 123 (Baird Atomic, Inc., Cambridge, Mass.).

The low alkalinity copper reagent of Somogyi (14) and the arsenomolybdate chromogen of Nelson (8) were employed for the determination of reducing sugars released from laminarin as a result of β-(1 → 3) glucanase activity. Casein was the substrate in assays of proteolytic activity, and the reaction was terminated by addition of 3 ml of 5% trichloroacetic acid. The solution was centrifuged, and 2 ml of 0.5 N NaOH and 0.6 ml of Folin's phenol reagent (Fisher Scientific Co., New York, N.Y.) were added to 1.0 ml of the supernatant fluid. The resulting color intensity was measured at 650 mμ. Chitinase activity was determined by measuring the generation from chitin of *N*-acetylhexosamine by use of the method of Reissig et al. (12). The β-(1 → 3) glucanase assay was performed at 37 C in 0.1 M acetate buffer (*p*H 5.0), the chitinase assay at 37 C in 0.033 M phosphate buffer (*p*H 6.1), and the test for protease activity at 35 C in 0.033 M phosphate buffer (*p*H 7.7). Unless otherwise stated, the enzyme preparations were used at a concentration of 10 μg/ml, and they catalyzed the formation of 395 to 410 μg of reducing sugar per ml in 30 min, 20 to 21 μg of *N*-acetylhexosamine per ml in 30 min, and 67 μg of L-tyrosine equivalents per ml in 15 min for the glucanase, chitinase, and protease, respectively.

The inhibitory effect of melanin was assessed by incubating 1.0 ml of the enzyme solution with 1.0 ml of a melanin-containing solution in the above-named buffers. After 30 min, 1.0 ml of a solution containing 1.0 mg of substrate was added, and the reaction mixtures were incubated either 30 min for the glucanase and chitinase or 15 min for the protease test. The reaction was terminated by boiling for 30 sec (glucanase), by addition of the tetraborate reagent used in *N*-acetylhexosamine determination (chitinase), or by addition of trichloroacetic acid (protease). In other instances, the melanin was preincubated with the substrate, and then the enzyme was added. Suitable controls containing all components except the substrate were routinely examined. Assays were performed in triplicate.

RESULTS

To investigate the relationships between age of culture, melanin content of the hyphae, and the resistance of hyphal walls to enzymatic digestion, cultures of varying ages were harvested by filtration, and the melanin content of one sample of the mycelium thus obtained was determined by extraction of 50-g samples of air-dried mycelium. Cell walls were prepared from the hyphae of the various cultures, and samples of the walls were incubated with the glucanase and chitinase for 4 days at 37 C in the presence of 10^{-7} M Merthiolate. A 15-mg amount of walls was incubated with 20 μg of the β-(1→3) glucanase and 35 μg of the chitinase preparation in 0.1 M acetate buffer (*p*H 5.0). The total volume was 15 ml. After 2 days, second and equal amounts of the two enzyme preparations were added.

The results show the markedly different quantities of melanin or melanin-like materials produced by the parent and mutant *A. nidulans* strains (Table 1). Although the apparent melanin content of hyphae of both the mutant and parent strains increased with age, the maximal amount of melanin-like materials extracted from the mutant was equivalent to 0.0276% of the hyphal weight, while the mycelium of the parent strain contained 4.90% on the 10th day. At 2 days, the hyphae of the wild type were white, but the mycelium became cinnamon color at 4 days and gradually turned light brown and then dark brown with time. By contrast, hyphae of the mutant remained hyaline for the entire period of incubation, although occasionally hyphae in certain flasks became cinnamon colored. By the 10th day, the culture supernatant fluid of the mutant appeared cinnamon in color.

The results also demonstrate that the walls became more resistant to solubilization by the action of β-(1→3) glucanase and chitinase as the culture aged. This increase in wall resistance is correlated with the increase in fungal melanin formation (Fig. 1). It is clear that walls of the parent culture when young and not yet producing melanin are also susceptible to enzymatic destruction.

The inhibition of hydrolysis by DOPA melanin was examined in two ways, by preincubating the polyaromatic with the enzyme for 30 min before adding the substrate and by preincubating the melanin for a like time with the substrate before adding the enzyme. The mixture containing melanin, enzyme, and substrate was then incubated for another 30 min in the glu-

TABLE 1. *Effect of age of Aspergillus nidulans on melanin content of hyphae and resistance of walls to enzymatic digestion*

Age of culture	*A. nidulans* 13		*A. nidulans* 13.1.0L	
	Digestion of wall	Melanin extracted from 50 g of hyphae	Digestion of wall	Melanin extracted from 50 g of hyphae
days	%	*mg*	%	*mg*
2	93.2	1.6	96.4	<1
4	69.9	62.7	94.2	<1
6	46.6	312	91.9	3.3
8	28.0	1,320	89.2	7.6
10	18.7	2,450	86.1	13.8

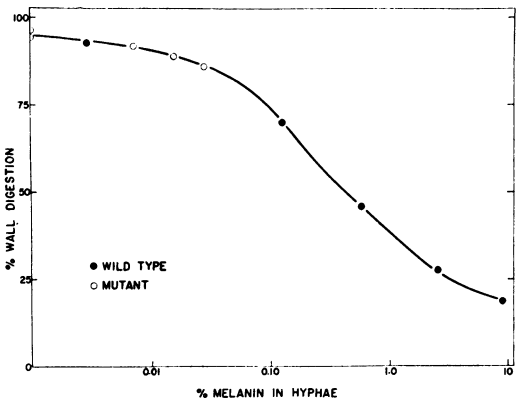

FIG. 1. *Relation between melanin content and resistance of Aspergillus nidulans walls to digestion by β-(1 → 3) glucanase and chitinase.*

canase and chitinase assays or 15 min for the protease determinations. The absorbancy of melanin alone was subtracted from that of the colored substances generated by the reagents used to detect products of enzymatic activity.

When DOPA melanin was incubated for 30 min with the enzymes prior to addition of the substrate, a marked inhibition of activity was observed (Table 2). The synthetic polyaromatic inhibited the three enzymes to different degrees. Chitinase was particularly sensitive to the melanin, and acetylhexosamine formation was totally prevented at the highest melanin concentration employed. The protease was not as sensitive, and glucanase action was the least affected.

The inhibition was less marked when melanin was preincubated with the substrates rather than with the enzymes (Table 2). In this instance, casein hydrolysis was the reaction most sensitive to melanin inhibition. Although it has been suggested that phenolic compounds may protect

TABLE 2. *Inhibition by melanin of the enzymatic hydrolysis of β-(1 → 3) glucan, chitin, and casein*

Melanin added	Inhibition (%)		
	β-(1 → 3) Glucanase	Chitinase	Protease
μg/ml			
Melanin preincubated with enzyme			
50	22.4	33.7	49.5
100	34.0	51.9	55.4
150	41.8	62.6	63.2
200	48.2	89.9	68.1
250	57.6	100	69.3
Melanin preincubated with substrate			
50	5.1	6.0	24.7
100	11.0	13.1	30.3
150	18.3	19.7	34.0
200	22.9	26.7	37.2
250	28.8	35.6	40.0

several substrates from enzymatic hydrolysis in the course of plant infection by pathogenic microorganisms (4), the present data do not indicate whether the formation of a resistant melanin-substrate complex is the reason for the retardation in hydrolysis rates, inasmuch as the melanin may have affected the enzyme during the second incubation period rather than complexing with the substrate in the preincubation phase.

Data supporting the hypothesis that melanin may complex with and protect at least certain substrates were obtained by preincubating casein for varying periods with the melanin in 0.1 M acetate buffer (*p*H 5.0) prior to addition of the *B. subtilis* protease. The final reaction mixture contained 100 μg of melanin, 150 μg of casein, and 10 μg of the protease preparation per ml. After the preincubation period, the protease contained in 0.067 M phosphate buffer (*p*H 7.7) was added, and the mixture was incubated for 20 min at 37 C prior to determining the extent of casein digestion. The results in Table 3 show that the inhibition of protein hydrolysis increases with progressively greater periods of preincubation of casein with melanin. The most plausible explanation for these observations is that melanin binds with the protein to make it less available to attack and that the extent of protection increases with greater contact time of the polyaromatic with the substrate. On the other hand, if there had been a significant removal of the

enzyme inhibitor in these experiments because of a complexing of the melanin with the casein, a diminution rather than an increase in inhibition with greater periods of preincubation might have been expected.

It has been suggested that quinones are generated during the formation of melanins from either tyrosine or DOPA (11), and that these serve to retard the hydrolysis of cell wall constituents which are susceptible to lytic enzymes. To determine whether substances generated during melanin formation inhibited the lytic enzymes, 20 ml of a DOPA-melanin solution, originally containing 98.6 mg of DOPA before autooxidation, was dialyzed against 5 liters of distilled water, and the solution containing the diffusible materials was concentrated to a point that it contained a quantity of low molecular weight components equivalent to that which would be found in the standard melanin solution prepared by Akino's (1) method. These low molecular weight compounds did have a slight toxic effect on the glucanase and chitinase, the activity catalyzed by the latter being more susceptible to inhibition (Table 4). It is thus possible that low molecular weight aromatic products appearing during melanin formation also prevent enzymatic lysis of fungi containing glucans and chitin in their hyphal or spore walls. The inhibition by the diffusible fraction became more pronounced as the time of dialysis employed to prepare this fraction was increased.

If melanins do indeed have an ecological significance by protecting fungal hyphae or spores from microbiological lysis in natural ecosystems, the polyaromatic probably must either be resistant to degradation so that its protective influence would be prolonged, or it must be generated continually by the fungus. The latter possibility seems unlikely for a melanin-containing resting structure like the sclerotium (2), which often persists in a viable state in nature for many years without appreciable further formation of the melanin-containing sclerotial rinds. To determine whether melanin is resistant to decomposition by a highly heterogeneous, natural microbial community, 35.4 μc of ^{14}C-labeled synthetic melanin was added to 100 g of potting soil contained in a 500-ml Erlenmeyer flask. To another sample of the soil was added 50 μc of ^{14}C-tyrosine, the monomer from which the melanin was synthesized. The soil samples were moistened, and air freed of CO_2 by passage through soda lime was circulated over the soils, which were incubated at room temperature.

The results summarized in Fig. 2 show the marked resistance of melanin to decomposition. Even after 8 weeks, only 8.6% of the ^{14}C was recovered as CO_2, and more than half of this carbon was volatilized in the first 4 days, possibly as a result of the degradation of the low molecular weight portion of the melanin complex. Tyrosine, by contrast, was rapidly decomposed, more than half of the labeled carbon

TABLE 3. *Effect of preincubation time of melanin with casein on rate of casein hydrolysis by protease*

Preincubation time	Decrease in rate of casein hydrolysis[a]
min	%
0	8.1
15	12.8
30	30.2
45	35.4
60	42.0
120	44.3

[a] Compared to the rate of casein hydrolysis in the absence of melanin.

TABLE 4. *Inhibitory effects of diffusible intermediates of DOPA melanins on β-(1 → 3) glucanase and chitinase*

Diffusible fraction	Inhibition (%)	
	β-(1 → 3) Glucanase	Chitinase
ml		
0.2	1.8	3.0
0.4	3.2	5.3
0.6	5.2	8.0
0.8	7.8	11.1
1.0	9.7	13.2

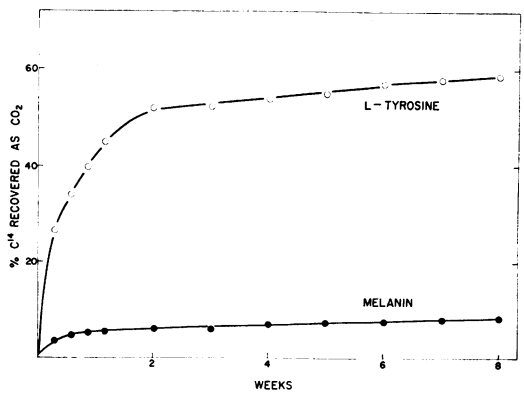

FIG. 2. *Persistence of melanin and L-tyrosine in natural soil.*

applied being converted to CO_2 in the first 2 weeks. The slow release of the remaining ^{14}C added in the form of tyrosine undoubtedly results, at least in part, from the incorporation of the carbon into microbial cell constituents, but some of the tyrosine may have been converted to refractory polyaromatic substances during the incubation period. The resistance of melanin to microbial destruction is also suggested by the inability to isolate a microorganism capable of utilizing melanin as the sole carbon source. Earlier attempts to obtain microorganisms capable of lysing *Rhizoctonia solani* and *Cladosporium* sp., fungi producing hyphae rich in melanin, were also unsuccessful (2).

DISCUSSION

The suggestion that melanins account for the resistance of certain fungi or fungal structures to enzymatic lysis was advanced in view of the correlation between resistance of these organisms or structures to lysis and their possession of melanin pigments. Thus, *R. solani* and *Cladosporium* sp. hyphae, *S. rolfsii* sclerotia, and *A. phoenicis* conidia contain melanin and are not attacked by lytic microorganisms or by a β-(1→3) glucanase-chitinase mixture that digests melanin-free *Fusarium solani*, *A. phoenicis*, or *S. rolfsii* hyphae or the *A. phoenicis* conidia from which essentially all of the melanin-containing spicules are removed (2, 10).

The present findings with *A. nidulans* provide a more direct line of evidence for the causal relationship between the presence of the dark pigment in fungal structures and their resistance to enzymatic digestion. Walls of the melanin-forming wild type were not appreciably solubilized except in very young cultures, in which little pigment appeared, whereas the hyaline filaments of the essentially melanin-less mutant were exhaustively degraded by the two enzymes employed. Moreover, as the content of melanin-like materials in the mycelium increased with age, its suitability as a substrate for the glucanase and chitinase declined. It is apparent that the relation between melanin content and the extent of digestion of *A. nidulans* walls is not linear; however, it would be most surprising to have a linear relationship with a substance that is both water-insoluble and is impregnated in the surface structure of the fungus.

Two hypotheses have been advanced to account for the protection afforded by the melanin: the polyaromatic may shield the more biodegradable polysaccharides of fungal cell walls by overlaying or combining with them, or the melanin may itself inhibit one or more of the enzymes participating in lysis of fungi (2). The proposal that the polyaromatic substance serves as a barrier to enzymatic hydrolysis of surface-localized polymers is made more plausible by the finding that the rate of casein hydrolysis by a protease is diminished when the protein is complexed with melanin. On the other hand, the pigment or low molecular weight compounds formed from melanin precursors inhibit chitinase, the β-(1→3) glucanase, and protease to varying extents, and such toxic effects could account for fungal resistance to lysis. Enzyme inhibition by simple aromatic compounds has been reported by several investigators (4, 7).

In order for any substance to be effective in nature in protecting a fungus or a fungal structure from attack by parasitic coinhabitants of the same ecosystem, the protective agent must itself be long-lived or it must be regenerated as rapidly as it is destroyed. The fact that a synthetic melanin, but not the amino acid monomer from which it was made, is extremely resistant, even when exposed to the activities of a biochemically versatile natural community of microorganisms, appears to satisfy the requirement for durability.

Further study is required to determine whether shielding the substrate of the lytic enzymes, whether complexing or inhibiting the enzymes themselves, or whether both are responsible for the resistance of these microbial structures to digestion by lytic microorganisms. The prolonged persistence of melanin and melanin-containing microbial structures in soil, a habitat containing species capable of metabolizing a wide range of natural products, lends credence to the hypothesis that an underlying substrate or a polysaccharide complexed in some way with the resistant polyaromatic is protected from the enzymes of fungal parasites. Nevertheless, the inhibition of hyaluronidase (1), polygalacturonase (3), and of the lytic enzymes by melanin or DOPA oxidation products may be of significance. It would be of interest to know whether other polymeric materials in addition to melanin serve a similar protective role in nature.

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