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Cell Wall Alterations Associated with the Hyperproduction of Extracellular Enzymes in *Neurospora crassa*

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A pleiotropic mutation in *Neurospora* (*exo-1*), which confers derepression of α-amylase, glucoamylase, β-fructofuranosidase, and trehalase, appears to also affect the composition of the cell wall. Segregants resulting from the backcross of *exo-1* to the wild-type strain from which it derived are altered in the ratio of galactosamine to glucosamine in hydrolysates of isolated cell walls. Conidial cell walls exhibit a marked decrease in the amount of galactosamine in both *exo-1* and *exo-1*$^+$ strains. Increased levels (approximately sevenfold) of amylase are found in conidia of *exo-1*, as compared with those of *exo-1*$^+$.

A single gene mutation mapping near the mating-type locus on linkage group I in *Neurospora* was shown to have pleiotropic effects upon the de novo production of the extracellular enzymes α-amylase (3.2.1.1; 1,4-glucan-4-glucanohydrolase), glucoamylase (EC 3.2.0.3; 1,4-glucan glucohydrolase), trehalase (EC 3.2.1.28; trehalose 1-glucohydrolase), and β-fructofuranosidase (EC 3.2.1.26; β-D-fructofuranoside fructohydrolase). In strains carrying mutations of these particular loci, designated *exo-1* and *exo-2*, the aforementioned enzymes are hyperproduced under conditions of carbon source deprivation resulting in elevated levels in mycelia and in the culture medium. Strains containing the wild-type allele have little or no detectable carbohydrase activity under identical conditions (3). In this communication, we report that the *exo-1* mutation causes alterations in the amino sugar concentration of the cell wall in addition to loss of normal regulation of extracellular enzyme activity.

MATERIALS AND METHODS

Strains. The wild-type strain used in this study, 74-OR-23-1A, was obtained from the Fungal Genetics Stock Center. Strain SF26, carrying the *exo-1* mutation, was derived from 74-OR-23-1A as previously described (3).

Stock cultures of the mutant and wild-type strains have been consistently maintained on silica gel (Ogata, Neurospora Newslett. 1:13), and new cultures were initiated with conidia from the silica gel stocks.

The strains used in the experiments described in this investigation were random ascospore segregants of a backcross of *exo-1* to 74-OR-23-1A.

Cultural conditions. Stock cultures for production of conidia were grown in 125-ml flasks of Vogel's minimal medium supplemented with 1% sucrose, 1% glycerol, and 1½% agar. The conidia from 7-day-old cultures were harvested by suspension in distilled water and filtered through Dacron wool to remove mycelial debris. Suspensions were then adjusted to about 10^7 cells/ml by turbidimetry. Nine milliliters of the suspension was inoculated into 1 liter of Vogel's minimal medium containing either 0.5% or 2% sucrose in Fernbach flasks. The flasks were incubated with shaking (120 rev/min) at a temperature of 30 C in a New Brunswick Psychrotherm Shaker/Incubator.

Cell wall preparation. Mycelial cell walls were prepared essentially as described by Mahadevan and Tatum (8). At the desired time, the mycelia were filtered from the flasks with suction and washed with ice-cold water, frozen on dry ice, and lyophilized. Subsequent to grinding in a Wiley mill, the powdered mycelia were shaken overnight in 1% sodium dodecyl sulfate, washed with water, and dehydrated in ethanol. Conidial walls were isolated by the procedure of Mahadevan and Mahadkar (7).

Glucoamylase was assayed on the culture filtrate as previously described (3). The conidia were disrupted by sonic treatment with a Branson sonifier. Fifteen minutes of sonic treatment at the highest setting, in short bursts of 3 min, was found necessary for complete disruption of conidia. Cooling was by ethylene glycol and dry ice (2).

Assays of amino sugars. Dried cell wall powder was hydrolyzed in 6 N HCl in sealed, evacuated tubes at 110 C for 24 hr (6). Galactosamine and glu-

cosamine were analyzed by the automated ion-exchange chromatography method of Lee, Scocca, and Muir (5).

Glucamylase purification. Glucamylase was purified by coprecipitation from culture media with glycogen (3) and rendered electrophoretically pure by ion-exchange chromatography on CG-50 by the method of Fass (Doctoral thesis, Florida State University, 1969).

RESULTS

A comparison of the amino sugar content of the cell walls of wild-type and *exo-1* segregants is shown in Table 1. It can be seen that there is at least a 30% increase in the amount of galactosamine in *exo-1* cell walls as compared to *exo-1*$^+$ isolated from mycelia grown in 2% sucrose. After 24 hr of growth, reducing sugar was present in the medium, and there was little detectable intracellular or extracellular amylase activity. Thus, under conditions of active vegetative growth, there appears to be an increase in the amounts of galactosamine in cell walls of the mutant strain when compared with the wild-type strain. If cultures of the various segregants are allowed to undergo starvation, a condition (40 hr) in which extracellular enzyme is produced de novo in *exo-1* but not in *exo-1*$^+$ strains (3), marked differences in galactosamine in the cell walls of *exo-1* and *exo-1*$^+$ strains are found (Table 2).

It was of interest to determine whether these increases in galactosamine also were present in conidial cell walls. Cell walls from the mutant and wild-type segregants were isolated by the procedure of Mahadevan and Mahadkar (7).

The results of analysis of amino sugars of conidial cell walls are shown in Table 2. Only trace amounts of galactosamine were found in the hydrolysates of cell walls of either strain under the conditions used.

Total amylase activity, which can be removed from conidia by washing with water, is also shown in Table 2. Since negligible additional enzyme activity could be released from conidia through disruption by sonic treatment and assay of the extracts or of the cell wall, it appears that most of the total amylytic activity is on the surface of the conidium. The high amylase activity found in vegetative, starved *exo-1* cultures is also reflected in the *exo-1* conidia (Table 2).

The amino sugar composition of carbohydrate associated with purified glucoamylase consists of: galactosamine, 138 nmoles/mg, 4 μmoles/μmole of glucamylase (assuming the molecular weight of glucamylase is 2.5×10^3 [Fass, 1969]); and glucosamine, 35 nmoles/mg, 1 μmole/μmole of glucamylase. Galactosamine predominates, comprising 75% of the amino sugar of this carbohydrate.

DISCUSSION

In these investigations, it was found that changes occur in the concentration of amino sugars of the cell wall and that these changes are correlated with the production of extracellular glucoamylase.

The concentration of galactosamine in the cell walls of the *exo-1* strain appears to be elevated during active, exponential growth, at a

TABLE 1. *Glucosamine and galactosamine in mycelial cell walls of wild-type and exo-1 segregants*

Culture	Isolate	Genotype	Total glucamylase[a]	Glucosamine[b]	Galactosamine[b]	Galactosamine/glucosamine
24-Hour cultures, 2% sucrose	20	*exo-1*$^+$	<1	0.365	0.134	
				0.363	0.130	0.36
	12	*exo-1*$^+$	<1	0.316	0.128	
				0.362	0.125	0.39
	13	*exo-1*	<1	0.341	0.183	
				0.345	0.183	0.53
	16	*exo-1*	<1	0.383	0.187	
				0.393	0.201	0.50
40-Hour cultures, 0.5% sucrose	12	*exo-1*$^+$	<1	0.325	0.049	0.150
	7	*exo-1*$^+$	<1	0.256	0.066	0.258
	19	*exo-1*$^+$	<1	0.303	0.058	0.190
	4	*exo-1*	10	0.288	0.186	0.64
	17	*exo-1*	14	0.245	0.267	1.09
	16	*exo-1*	12	0.228	0.233	1.02

[a] Micromoles of glucose liberated per minute per milligram of mycelial protein.
[b] Micromoles per milligram of dry cell wall.

TABLE 2. *Glucosamine and galactosamine in conidial cell walls of wild-type and exo-1 strains of Neurospora*

Isolate	Genotype	Glucosamine (μmoles/mg of dry wall)	Galactosamine (μmoles/mg of dry wall)	Amylase[a]
20	*exo-1*$^+$	0.61	<0.01	2.8
13	*exo-1*	0.78	<0.01	8.0

[a] Includes total (α and γ) amylase activity units/10^6 cells.

period when little enzyme activity is found in either mutant or wild-type strains. Galactosamine then increases threefold relative to the galactosamine of wild-type strain, during starvation and accompanying glucoamylase production.

There is also a decrease in glucosamine in 40-hr cultures of *exo-1*, as well as a drop in the levels of glucosamine and galactosamine in wild type during starvation. At this stage of investigation, it is not possible to adequately explain these observations, particularly without data on the levels of intracellular pools of intermediates in the biosynthesis of the amino sugars and polymers in question.

It is conceivable that autolytic enzymes, active in the later stages of growth of the culture, are responsible for decreases in these amino sugars observed in both strains during starvation. In this regard, perhaps the genetic lesion in *exo-1* is one that causes an alteration in activity of a specific hydrolase which normally cleaves galactosamine from the polymers of the cell wall of wild type during the stationary phase of the culture.

If it can be shown that the galactosamine-containing carbohydrate, which copurifies with glucoamylase, is covalently bound to the protein, then an interesting relationship may exist between the galactosamine of the glucoamylase and that contained in the cell wall.

A comparison, by isoelectric focusing, of the glucoamylases of *exo-1* and the wild-type enzyme by Fass (Doctoral thesis, Florida State Univ., 1969) has shown that differences exist in the isoelectric points of the enzymes produced by the two strains: the wild type IEP = 5.2, *exo-1* IEP = 5.9. Studies of other parameters, such as K_m, *p*H optima, and molecular weight, uncovered no other differences between the glucoamylases from mutant and wild-type strains. Thus far, it has not been possible to purify sufficient glucoamylase from the wild-type strain, 74A-OR-23-1A, to determine amino acid and amino sugar concentrations, as has been done for *exo-1*.

It has been shown (1) that the cause of cell wall alterations can be complex and indirect. But, in the studies reported here, the same or closely linked genes would appear to affect both the control of synthesis of several extracellular glycoprotein enzymes and also cell surface structure. Although the cause and effect relationship between the two kinds of phenomena have not been established, it is of interest that the cell wall change in *exo-1* is manifested during exponential growth, a period when negligible glucoamylase activity is found in the culture.

Mahadevan and Tatum showed that a galactosamine polymer is found in the alkali-soluble outer layer of the cell wall (8).

In this regard, it is significant that glucoamylase probably contains galactosamine, and both the cell wall galactosamine and the galactosamine-containing enzyme are elevated in *exo-1*. It has been found that greater than 90% of glucoamylase is in the medium, whereas about 30% of the remainder of the glucoamylase, which is found in cell-free extracts, is tightly bound to the mycelial cell wall and cannot be removed by homogenization of the wall fraction in 1% Triton X-100 (*unpublished data*). It is conceivable that glucamylase is bound in some manner to the outer layer of the cell wall through the galactosamine of the wall and of the enzyme.

It has been shown that binding sites exist for alpha-amylase in *Aspergillus oryzae* (9). It is probable that similar binding sites exist for the amylases of *Neurospora*.

There is perhaps a relationship between the attachment to the cell wall and the control of synthesis of such glycoproteins. Lampen (4) proposed a model in which bacterial penicillinase is externalized by growth and formation of a membrane-enzyme complex. Fass expanded upon Lampen's model to include a regulatory function for the *Neurospora* glucoamylase system. He proposed that there are specific binding sites for glucoamylase which, when filled, repress further synthesis of the particular enzyme. If a mutation, such as *exo-1*, causes improper assembly of cell wall or enzyme components, binding would be altered, preventing proper binding of glucoamylase, thus affecting its regulation. The loss of regulation would be amplified during starvation. The preliminary evidence described in this communication, alteration of the cell wall manifested in *exo-1*, could support this model of cell surface regulation, but further experimentation

will be necessary to rule out other models.

The major constituents of conidial walls were identified by Mahadevan and Mahadkar (7). They reported chromatographic evidence for galactosamine in the alkali-soluble fraction (their fraction I) but did not quantitate this observation. However, they reported that the level of fraction I doubled in conidial walls in comparison to that found in mycelial walls. If the amount of this wall fraction increases, it may be due to increases in the glucan or polypeptide moiety of the complex. It may well be that fraction I was lost during sonic treatment in our procedure. The method of wall isolation was identical to that used by Mahadevan and Mahadkar, although amino sugar analysis was accomplished on total conidial cell wall, not on isolated fraction I. Further studies are obviously necessary on the isolated conidial wall fraction to substantiate that such a drastic developmental change in cell wall composition occurs during conidial differentiation.

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