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Role of Enzymes in Growth and Morphology of *Neurospora crassa*: Cell-Wall-Bound Enzymes and Their Possible Role in Branching

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The cell wall of *Neurospora crassa* contains bound enzymes that can digest its structural polymers. These enzymes are not present at the same levels at all stages of growth. The levels of these autolytic enzymes vary and generally show some relationship to the process of branching. These enzymes were removed from the cell wall by β-mercaptoethanol extraction and were tested for activity against isolated cell wall fractions. Such studies, as well as autolytic studies, showed that enzymes acting on the protein portion of the cell wall (proteases) are more prominent than enzymes that act on the glucan portion (glucanases) of the cell wall. Comparative studies between the wild type and a spreading colonial mutant *spco-1* showed that earlier and higher frequency of branching in *spco-1* was correlated with a greater amount of these enzymes bound to the cell walls. It is concluded from these observations that autolytic enzymes acting on the protein and glucan portion of the cell walls occur as wall-bound and participate in the process of branching in *Neurospora*.

The important role of the cell wall and the relative ratios of cell wall polymers in the control of morphology in *Neurospora crassa* have been clearly demonstrated (3, 6). This relationship was established by studying the cell wall polymers of the wild type and several single-gene-controlled morphological mutants of the fungus. Among the morphological mutants, those that grow compactly and tightly, referred to as "colonial," have higher frequencies of branches per unit length of mycelia than the wild-type strain (3). This might lead to the compactness in growth that appears to distinguish it from the wild type. Thus the process of branching becomes very important and biochemical processes behind the morphological variation due to branching are of interest. It was suggested that changes in the relative ratios of cell wall polymers (6), especially those that clearly add to the structural integrity of the cell wall (7), can result in alteration of branching leading to morphological variation. Studies carried out to determine the presence of enzymes bound to the wall and the activity of these enzymes during the growth of the fungus are reported in this paper. Such enzymes might play a part in the process of branching in the fungus. This study was made after observing the presence of an enzyme in the extract of *Neurospora* with activity toward β-1,3 glucan of the cell wall.

MATERIALS AND METHODS

The strains of *N. crassa* used were Perkins wild type (PA) and a spreading colonial mutant, *spco-1*. Conidia were obtained from cultures of the above strains, grown regularly in 2% agar medium containing 2% sucrose and minimal salt solution. The conidial suspension in water was filtered through glass wool to remove mycelial debris and agar pieces. The conidia in the suspension were counted, and up to 3×10^{10} conidia were inoculated in 150 ml of 2% sucrose-minimal salt medium and grown on a rotary shaker for various periods. After each period, the culture was filtered through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.), and the mycelial mass was suspended in distilled water. These suspensions were treated in an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London) at 20 kc/sec until clean cell walls were obtained. The sonically disrupted sample was centrifuged at $4,000 \times g$ for 15 min, and the supernatant solution was discarded. The residue was washed three times with distilled water and then with 0.1 M potassium phosphate buffer, *p*H 7.0. The breakage of mycelia and purity of cell wall preparations were judged by microscopic examination. The cell wall preparations thus obtained were kept frozen until used for further experiments. Quantitation of the cell wall suspension was done by determining the turbidity of a homogeneous suspension in a Klett-Summerson colorimeter.

Radioactive cell wall was prepared under the same conditions as above except that the cultures were grown in medium containing additions of uniformly

labeled ^{14}C-glucose, leucine-*1*-^{14}C, or uniformly labeled ^{14}C-glucosamine to an extent of 2.5 to 5 μc per 150-ml flask. Uniformly labeled ^{14}C-glucose and leucine-*1*-^{14}C were obtained from the Isotope Division of Bhabha Atomic Research Centre, Trombay, India. Uniformly labeled ^{14}C-glucosamine was purchased from New England Nuclear Corp., Boston, Mass.

Cell-wall associated enzyme activity towards laminarin (β-1,3 glucan) was determined by incubating the wall with laminarin [obtained from International Seaweed Research Station and purified by the method of Tanaka (*personal communication*)] in a concentration of 1 mg/ml in 0.1 M citrate buffer (citric acid-sodium citrate), *p*H 5.0. After incubation for 18 hr, the sample was centrifuged, and the glucose present in the supernatant fluid was estimated with the glucostat reagent (8) obtained from Worthington Biochemical Corp., Freehold, N.J. Laminarinase activity has been expressed as units per milligram of glucose released during the incubation period.

The presence of cell-wall-bound enzymes that hydrolyze cell wall polymers was demonstrated as follows.

Radioactively labeled cell walls were washed twice with distilled water and buffer and then with 0.1 M sodium chloride followed by buffer (0.1 M potassium phosphate, *p*H 7.0). They were incubated in 0.1 M buffer, *p*H 5.0 (citrate) or *p*H 7.0 (phosphate buffer) for 18 hr at 37 C and centrifuged. The clear supernatant fluid was separated from the cell walls, and the radioactivity in the supernatant fluid was determined in a Tri-Carb scintillation spectrometer (Packard Instrument Corp., Downers Groves, Ill.). The amount of radioactivity released was taken as an indication of the degree of digestion of the cell wall polymers by the wall-bound enzymes. This is referred to as autodigestion or autolytic capacity. By growing the culture in ^{14}C-glucose- or ^{14}C-leucine-containing medium, the polymers in the cell wall of the mycelia were already labeled and acted as the substrates for the wall-bound enzymes, whose degradation could be followed by the estimation of the amount of radioactivity released in the supernatant fluid. This autodigestibility or autolysis is proportional to the amount of cell wall used within a limited time (Fig. 1).

Before incubation for autodigestion, the amount of cell wall used was determined by measuring the density of the suspension in a Klett-Summerson colorimeter. A homogenous suspension of sonically disrupted sample of cell wall showed a linear relationship between amount of cell wall (obtained by serial dilution) and turbidity determined by using the colorimeter with a red filter (620 nm; Fig. 1). In a suspension of cell wall showing a Klett reading of 100 (which is calculated as an optical density of 0.2), the amount of cell wall was 30 mg, dry weight. In this way, the autolytic capacity for a known amount of cell wall was determined by measuring the radioactivity released during 18 hr of incubation in buffer at 37 C. From these data, autolytic capacity per unit amount of cell wall (per 100 Klett units = 30 mg, dry weight) or of the total cell wall obtained from any growth period (counts per minute released per unit amount of cell wall times total cell wall obtained from a growth period) was also recorded. In the data presented in all experiments, comparison of the total autolytic capacity associated with the total wall of culture grown for various periods of time was made. Such comparison was made for culture grown in ^{14}C-glucose- and ^{14}C-leucine-containing medium. It is expected that ^{14}C-glucose will go into at least two of the major fractions of the cell wall identified and characterized earlier (6), i.e., protein-polysaccharide complex and β-1,3 glucan. ^{14}C-leucine will go into proteins of the cell wall.

Fractionation of the cell wall of *N. crassa* was done by the method of Mahadevan and Tatum (6). In this method, the first 2 N sodium hydroxide-soluble portion of the cell wall was determined as protein-polysaccharide complex (fraction I), fraction II contained easily acid-soluble glucan portion, and fraction III contained alkali-soluble-1,3 glucan. The residue after removal of the above fractions was characterized as chitin (fraction IV).

RESULTS

Preliminary tests showed that finely homogenized cell wall could be quantitated by measuring the turbidity of the suspension in a Klett-Summerson colorimeter with a red filter (620 nm). The linear relationship of density (calculated from Klett readings) to different amounts of cell wall (obtained by serial dilution of a suspension) is shown in Fig. 1. Tests of bound enzyme activity against laminarin or autolytic capacity of cell wall showed that there is a direct relationship between concentration (amount) of cell wall in a suspension and degree of autodigestion (Fig. 1). It was observed that heating the cell walls for 10 min in boiling water destroyed the autolytic nature of the cell walls. In all experiments, cell walls used for autolytic tests were repeatedly washed with buffer, and then with 0.1 M sodium chloride followed by buffer, until there was no release of radioactivity in the wash supernatant fluids.

The log-phase growth period of *N. crassa* was found to be between 12 and 20 hr after incubation. In vigorously aerated bubbler-type or shake cultures, germination does not start before 90 to 120 min after inoculation. The total time involved for complete germination may extend to 10 to 12 hr. The germ tubes start branching only after 10 hr. Branches are then produced along with growth and elongation of the mycelia. In the mutant *spco-1*, germination of conidia started by 90 min, and by 6 hr most of the conidia had germinated. One or two branches on the germ tubes were produced by 6 to 8 hr of growth. This is a significant departure from what is seen in the wild type. We took advantage of the earlier germination and increased frequency of branching in an attempt to correlate branching and the role of cell-

wall-bound hydrolytic enzymes in relation to branching.

Cell-wall-bound enzyme activity toward laminarin (β-1,3 glucan). The cell-wall-bound enzyme activity (laminarinase) toward laminarin (β-1,3 glucan) in the wild type is shown in Fig. 2. The activity is based on the amount of glucose released by the action of a known amount of cell wall (30 mg, dry weight) on laminarin. Laminarin was used as a substrate for this test because of the demonstration of the presence of β-1,3 glucan in the mycelial wall of *Neurospora* (6). Figure 2 shows this laminarinase activity associated with the cell wall obtained from various growth periods. It can be seen that the cell-wall-bound enzyme activity fluctuated, indicating that the walls from the various growth periods contain different amounts of this enzyme.

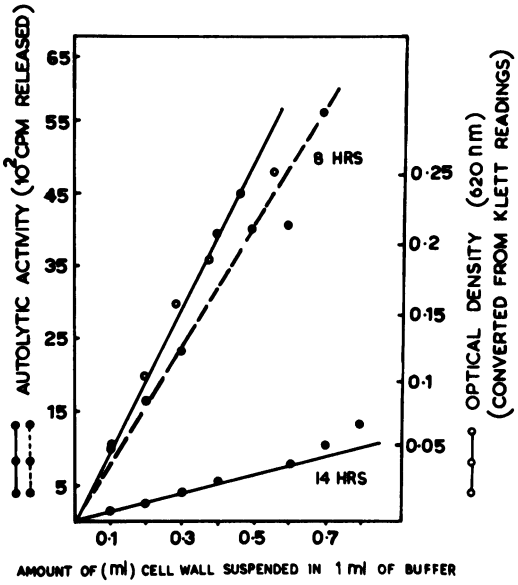

FIG. 1. *Linear relationships of amount of homogenized cell wall suspension to its optical density and autolytic capacity. Autolytic capacity was determined for cell walls at 8 and 14 hr of growth.*

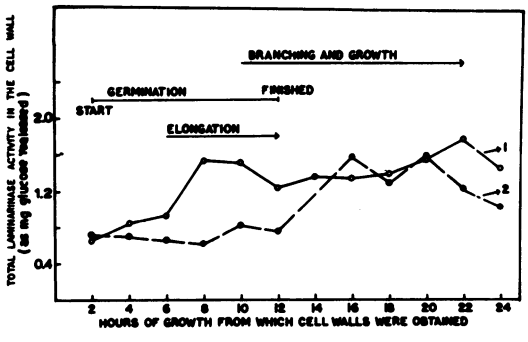

FIG. 2. *Wall-bound laminarinase activity of Neurospora crassa (PA) in the cell walls of various growth periods from two experiments (1 and 2).*

The above observation indicated that there are enzymes associated with the cell wall that could attack the cell wall β-1,3 glucan, and perhaps also other cell wall polymers. This was tested by autodigestion experiments with the cell wall.

Autodigestion of cell walls: ^{14}C glucose-labeled wall. As mentioned earlier, the radioactivity released on incubation of ^{14}C-glucose-labeled cell wall preparation in buffer at 37 C for 18 hr was used as an indication for autolytic capacity of a known amount of cell wall. Figure 3 shows autolytic capacity associated with the entire (total) cell wall obtained from cultures of PA wild type that had been grown for various periods. The cell walls from 8- to 12-hr growth periods have greater activity, followed by increased activity in the wall of 12- to 22-hr growth periods. This autolytic degradation involved primarily ^{14}C-glucose-labeled polymers of the cell wall. This has also been determined.

It was consistently observed that the autolytic capacity of the cell wall does not show uniform rise or decrease but fluctuates as with the wall-bound laminarinase activity. The period of incubation of the cell wall (18 hr) is one that allows expression of maximum autolytic capacity, so that the comparison of this property from cell walls of various growth periods does have significance.

Cell wall polymers degraded by autodigestion. To determine which polymers of the cell wall were degraded by autodigestion, cell walls were frac-

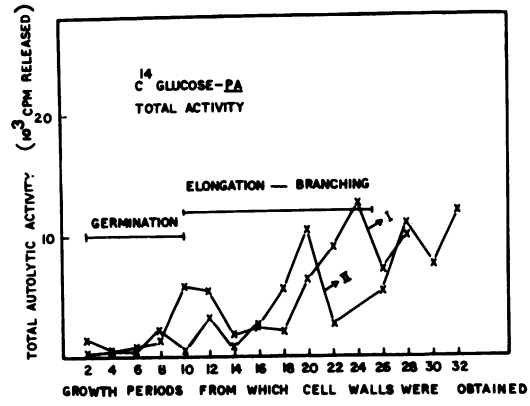

FIG. 3. *Total autolytic activity associated with cell walls from various growth periods of wild type (PA) from two experiments (I and II). Cell walls were labeled with ^{14}C-glucose.*

tionated before and after autodigestion. The fractionation method was the same as that used earlier, which had characterized four fractions (6). In the present experiment, a sample of cell wall was repeatedly autodigested by replacing the buffer every 24 hr. The autodigestion was done until very little radioactivity appeared in the supernatant fluid. Both the samples of cell wall, one subjected to autodigestion and then fractionated and the other fractionated without autodigestion, had the same initial amount of radioactivity. In two experiments (Table 1) with cell wall from the wild-type culture grown for 16 hr, the glucose polymer of fraction I (protein-polysaccharide complex) was degraded more than fractions II and III. Chitin (fraction IV) was degraded to a much lesser extent than all the other polymers.

Autodigestion associated with the cell wall of spreading colonial mutant (spco-1). The autolytic capacity of ^{14}C-glucose-labeled cell wall of the various growth periods from the *spco-1* mutant is shown in Fig. 4. This shows that the total activity associated with total wall from each growth period is much greater than that seen in the wild type. The fluctuation in activity associated with cell wall samples is also seen in this mutant strain, like the wild type.

Autodigestion in specifically labeled cell walls. Cell walls of *N. crassa* (PA) grown for 16 hr were labeled with ^{14}C-leucine and ^{14}C-glucosamine by growing a culture in supplemented 2% sucrose medium. Cell wall containing the labeled compounds, on fractionation, showed that leucine was preferentially incorporated into fraction I of the cell wall and ^{14}C-glucosamine into fraction IV (chitin; Table 2). In both cases, cell walls were incubated for autodigestion, and autolytic capacity was determined.

Table 3 shows the autolytic capacity of the 16-hr cell wall represented as radioactivity released during the autolysis of the wall sample. The amount of degradation (as percentage) of the cell wall for the ^{14}C-leucine-labeled cell wall was 28%, as compared to 6% degradation of ^{14}C-glucosamine-labeled cell wall. This indicates that the protein portion of fraction I (protein-polysaccharide complex) is degraded much more than the chitin portion (fraction IV) of the cell wall by the cell-wall-bound enzymes. This can be due to the presence of larger amounts of proteolytic enzymes and less chitinolytic enzymes.

^{14}C-leucine-labeled cell wall of wild type and spco-1. The results of autodigestion experiments with cell walls from various growth periods after labeling them with ^{14}C-leucine are shown in Fig. 5. It is clear that the autolytic capacity toward ^{14}C-leucine-labeled polymers (protein-polysaccharide in fraction I) of the cell wall is greater than the autolytic activity toward glucose polymers. This

TABLE 1. *Autolytic degradation of ^{14}C-glucose-labeled cell walls*[a]

Determination	Radioactivity before autodigestion	Radioactivity after autodigestion	Loss[b]
Expt no. 1			
Fraction I	20,000	15,900	4,100 (20)
Fraction II	23,500	19,400	4,100 (17)
Fraction III	6,300	5,500	800 (12)
Fraction IV	4,480	6,750	
Counts/min in the total cell wall used for fractionation	58,000	47,000	
Expected loss	11,000		
Counts/min in the pooled supernatant period after autodigestion	9,000		
Expt no. 2			
Fraction I	75,075	56,280	18,975 (25)
Fraction II	44,650	36,480	8,170 (18.1)
Fraction III	22,980	20,160	2,820 (12.3)
Fraction IV	9,675	9,200	475 (4.9)
Counts/min in the total cell wall used for fractionation	167,000	130,000	
Expected loss	37,000		
Counts/min in the pooled supernatant fluid after autodigestion	31,000		

[a] Cell wall from wild-type strain PA grown for 16 hr was used for autodigestion.
[b] Numbers in parentheses indicate percentage of loss.

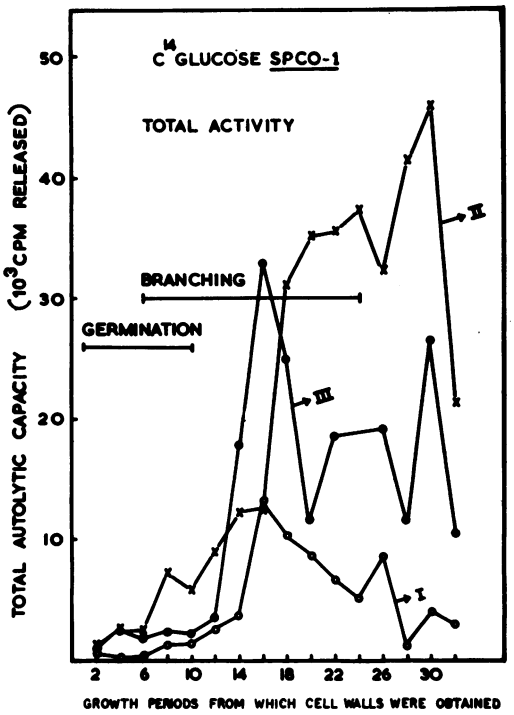

FIG. 4. *Total autolytic activity associated with cell walls from various growth periods of spco-1 from three experiments (I, II, III). Cell walls were labeled with ^{14}C-glucose.*

TABLE 2. *Distribution of radioactivity in the total cell wall obtained from ^{14}C-leucine and ^{14}C-glucosamine-labeled Neurospora (PA)*[a]

Fraction	Total counts/min in the fraction	
	^{14}C-leucine-labeled wall	^{14}C-glucosamine-labeled wall
I (Protein-polysaccharide complex)	21,500	3,400
II (Soluble β-1,3 glucan)	2,250	9,600
III (β-1,3 Glucan)	275	5,610
IV (Chitin)	1,450	25,100

[a] Cell walls prepared from cultures labeled appropriately were fractionated by the method of Mahadevan and Tatum (6). Radioactivity was determined in the soluble phase of each fraction (i.e., before precipitation of fraction I and fraction III) except in fraction IV, which was counted as a homogenous suspension. Amount of cell wall used for fractionation in both cases was the same.

is true for both the wild type and the mutant. Furthermore, the mutant *spco-1* has more enzyme activity toward proteins as compared to the wild type. The mutant and the wild-type cultures were

TABLE 3. *Autodigestibility of ^{14}C-leucine- and ^{14}C-glucosamine-labeled cell wall of wild-type (PA) Neurospora at 16 hr of growth*

Determination	^{14}C-leucine-labeled culture	^{14}C-glucosamine-labeled culture
Total counts/min in the cell wall used for autodigestion[a]	2,840	3,592
Counts/min released during autodigestion	800	200
Percentage of degradation	28.2	6
Total amount of wall isolated from the culture (OD units)[b] at 16 hr of growth	4.0	4.4
Total autodigestibility[c] of the cell wall (as counts/min released)	16,000	4,520

[a] Labelled cell wall containing a known amount of radioactivity was autodigested for 16 hr, and the amount of autodigestion was determined as radioactivity released in the supernatant fluid.

[b] OD, optical density.

[c] Total autodigestibility is equal to autodigestive capacity of unit amount times total cell wall from the culture.

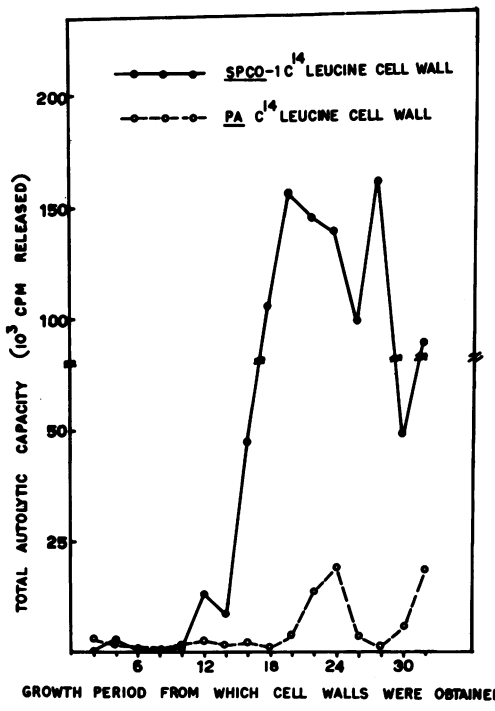

FIG. 5. *Total autolytic activity associated with ^{14}C-leucine labeled cell walls from various growth periods of PA and spco-1.*

started with similar amounts of conidia. The amount of cell wall at each growth period was greater in the mutant. However, this does not contribute completely to the greater amount of autolytic activity. The autolytic activity per unit amount of cell wall (30 mg, dry weight) was also greater in the *spco-1*. This was true for both ^{14}C-glucose- and ^{14}C-leucine-labeled walls.

β-Mercaptoethanol treatment of cell wall. Treatment of clean cell wall preparations repeatedly with β-mercaptoethanol leads to a decrease in the autolytic capacity of the cell wall. This is clearly shown in data presented in Table 4, where 50% of the autodegradative capacity of cell wall was lost due to β-mercaptoethanol washing.

The wash or extract obtained by cell wall wash was freed from β-mercaptoethanol by dialysis. This sample was found to contain 980 μg of proteins. This was obtained from a suspension of cell wall with an optical density of 12 units (1.8 g of cell wall). This wash also showed enzyme activity as indicated by the release of radioactivity in the supernatant fluid when it was incubated with labeled cell wall polymers. The data of two such experiments are presented in Table 5. The protein portion of fraction I appears to be degraded to a greater extent than others. The increased activity of this wash toward the protein portion of fraction I is consistent with the result of greater autolytic capacity seen in ^{14}C-leucine-labeled cell wall of both wild type and *spco-1* compared to autolytic capacity toward glucan portion of the wall (fraction III).

DISCUSSION

de Terra and Tatum (4) reported that presence of snail digestive juice in agar medium caused morphological growth of *Neurospora* due to higher frequency of branching. It was suggested by them that the increase in branching might be due to lytic action of the enzyme on the cell walls creating weakened areas which might be pushed out by cytoplasmic flow to initiate a branch.

This possibility could be investigated by studying the enzymes present in *Neurospora* that can act on cell wall polymers. It would be interesting if such enzymes are present as a form bound to the cell wall. The indication that such a feature existed was shown by the hydrolysis of laminarin by cell wall of *N. crassa* (Fig. 2). This activity was associated with the cell wall. Laminarin is β-1,3 glucan, and a similar polymer has been demonstrated in the cell wall of *N. crassa* (6).

Autolytic experiments with cell walls labeled with ^{14}C-glucose or ^{14}C-leucine showed that cell walls have autolytic capacity. This is true for both the wild type and the spreading colonial mutant *spco-1*. The autolysis leads to removal of cell wall polymers due to their hydrolysis by wall-bound enzymes (Table 1). The autolytic capacity of

TABLE 4. *Loss of autolytic capacity of ^{14}C-glucose labeled cell wall of wild-type strain due to β-mercaptoethanol treatment*

Condition	Total radioactivity in the cell wall[a] used for autodigestion	pH of autodigestion	Total autolytic capacity (total released)	Percentage of degradation
Control	93,200	5.0	13,195	14
After β-mercaptoethanol wash	64,400		5,100	7.8
Control	74,800	7.0	11,375	15
After β-Mercaptoethanol wash	56,400		4,350	7.15

[a] Total cell wall used for autodigestion before and after β-mercaptoethanol wash had an optical density of 7.3 (1.06 g, dry weight). The β-mercaptoethanol wash was tested for enzyme activity toward cell wall polymers. Total protein in the wash was 980 μg.

TABLE 5. *Enzyme activity present in β-mercaptoethanol wash of cell wall from wild type*

Determination	Expt no.	^{14}C-leucine-labeled fraction I	^{14}C-glucose-labeled fraction I	^{14}C-glucose-labeled fraction III
Total radioactivity in the fraction used as substrate	1	880	14,000	18,200
	2	1,780	6,000	6,430
Counts/min released in the reaction mixture	1	250	855	950
	2	750	335	1,020
Percentage of degradation	1	28.5	4.5	5.2
	2	42	5.6	15.8

cell walls from various growth periods is not uniform but fluctuates. Generally, cell walls of growth periods 8 to 12, 14 to 18, and 22 to 26 hr have greater autolytic capacity. From this it may be concluded that the enzymes present at any time of growth and participating in the autolysis do get distributed in the cell wall as its surface increases, until more enzymes are added to the wall. This addition or accumulation perhaps occurs at intervals. Cell-wall-bound enzymes are removable with β-mercaptoethanol. Such β-mercaptoethanol washes of cell walls do have proteins that could degrade cell wall polymers (Table 5).

Lastly, the autolysis of the protein portion of fraction I (protein-polysaccharide complex) is greater than the polysaccharide portion of fraction I or β-1,3 glucan (fraction III) or chitin (fraction IV). The spreading colonial *spco-1*, which has earlier and higher frequency of branching, has a greater amount of autolytic ability associated with its wall. This may have some significance for the larger amount of branching.

The action of hydrolytic enzymes on cell walls at various morphological states of an organism has been reported in several instances. Brock (1) showed that in the yeast *Hansenula wingei*, during the process of conjugation and budding, there is softening of cell wall by enzymes whose synthesis was induced. Electron-microscopic studies (2) of conjugating cells also showed cell wall changes including dissolution of wall before conjugation. An enzyme extracted from *Bacillus cereus* caused germination of spores, and it was suggested by Gould, Hutchins, and King (5) that the enzyme substrate was probably the structural material of the spore coat. Autolysis was localized in the cell walls of *Streptococcus faecalis* by similar autolytic studies of ^{14}C-lysine-labeled cell walls of the organism (9).

Thus, in *Neurospora*, our data tend to show the presence of cell-wall-bound enzymes that are capable of hydrolyzing cell wall polymers. The presence of such enzymes may have some significance to the branching process in the mycelium. The role such enzymes can play was indicated by the observation of de Terra and Tatum (4) mentioned at the beginning of the discussion.

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