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Distribution of Autolysins in Hyphae of *Aspergillus nidulans*: Evidence for a Lipid-Mediated Attachment to Hyphal Walls

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Preparations of broken *Aspergillus nidulans* hyphae contained both free and wall-bound autolysins. The bound enzymes were not solubilized by 8 M LiCl or neutral or anionic detergents; they were readily detached from walls by a cationic detergent or by autodigestion. Once detached, the enzymes did not reassociate with wall to give salt-resistant complexes. Six enzymes hydrolyzing wall polymers were bound to the envelope, and the same activities were also detected among soluble proteins in the cytoplasmic fraction. It is suggested that cytoplasmic vesicles, containing autolysins, are inserted into or trapped by newly formed wall in the growing hypha; these constitute the wall-bound autolysin fraction. Starvation for a carbon source derepressed the synthesis of five out of the six autolysins, and the amounts of both soluble and wall-bound activities increased by one to two orders of magnitude.

Ultrastructural studies have demonstrated that cytoplasmic vesicles aggregate at sites of fungal wall synthesis and then apparently fuse with the cell membrane (13, 14). Furthermore, at least some of these vesicles contain autolytic enzymes (9, 16, 17; I. Polacheck, manuscript in preparation). The packaging of wall-hydrolyzing enzymes within vesicles and their liberation at specific cell sites could thus be part of the mechanisms which regulate and limit the activity of these potentially lethal proteins.

In addition, the interaction between autolysins, once these have reached the outer envelope, and the fungal wall may be complex. This is indicated by the distribution of autolysins in preparations of broken hyphae and yeasts. After cell rupture, wall-hydrolyzing enzymes are found both free in the soluble fraction and tightly bound to the wall (1, 9, 10, 15, 18). Among the factors which could explain such a distribution would be a limited number of enzyme-binding sites on the wall, heterogeneity in the enzyme population regarding affinity for wall polymers, or the attachment of a fraction of the autolysins by processes quite distinct from enzyme-substrate interactions.

So far, there is little information on the nature of the binding of autolysins to wall. We have thus investigated the types of autolysins present in vegetative hyphae of *Aspergillus nidulans* and have attempted to clarify the wall-enzyme association. We find that *A. nidulans* contains at least six different enzymes which can hydrolyze bonds in major wall polymers. A fraction of each of these activities is firmly attached to the wall, and this attachment is mediated by lipids. Furthermore, the levels of both soluble and bound autolysins are markedly influenced by the availability of a carbon source.

MATERIALS AND METHODS

Strains, media, and growth conditions. *A. nidulans* strains R21 (*yel pab1*; see reference 18 for gene symbols), SM16 (*bio1 pab1 mel B2*), 46 NXW (*whi pur15 bio1 cnx*), and BWB 152 (*pab1 bio1 vel*) were grown in mineral salts-glucose medium (18) supplemented with the appopriate growth factors. Conditions of inoculation and incubation were as described previously (18).

Continuous radioactive labeling of mycelium with [*U*-^{14}C]glucose was carried out as previously described (18). To label hyphae with [1-^{3}H]glucose, 2% (wt/vol) glucose and 0.1 μCi of [1-^{3}H]glucose per ml (specific activity, 5.3 Ci/mmol; The Radiochemical Centre, Amersham, England) were added to the medium. Differential labeling of lateral and apical wall was carried out according to the following schedule. Hyphae were grown in 2% (wt/vol) glucose and 2 μCi of [6-^{3}H]-glucose per ml (specific activity, 500 mCi/mmol), filtered, and washed on the filter. They were resuspended in medium containing 0.5% (wt/vol) glucose and 1 or 3 μCi of [*U*-^{14}C]glucose per ml to label the apical wall.

To investigate the effect of carbon starvation, cultures were grown for 15 h in medium containing 2%

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glucose. Part of the cultures was then harvested (exponentially growing mycelium), and part was collected on filters, washed, resuspended in medium lacking glucose, and shaken at 37°C for 5 h (carbon-starved mycelium).

Breakage and fractionation of hyphae. Hyphae were broken, and the wall fraction was separated and purified as previously described (18), except that 0.02 M phosphate buffer, pH 7, was used during sonic treatment and washing. Experiments with strain 46 NXW, whose RNA can be labeled specifically with adenine, showed that cytoplasmic contamination of the wall fraction did not exceed 0.5%. A membrane preparation was obtained from the soluble fraction by centrifugation at 105,000 × *g* for 60 min. When necessary, the soluble fraction was dialyzed against water and concentrated by lyophilization.

Solubilization of wall-bound autolysins by LiCl, reducing agents, and detergents. Various concentrations of the extracting agents were added to purified hyphal walls in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7. After the suspensions were stirred for 30 min at 4°C (when sodium dodecyl sulfate [SDS] was used, 20°C), they were filtered through membrane filters (all membrane filters used in this work had an average pore size of 0.45 μm). Wall suspensions with no additions, stirred for 30 min at 4 or 20°C, served as controls. Prior to assaying, the filtrate was dialyzed against 0.02 M phosphate buffer, pH 7 (0.02 M Tris buffer, pH 7, for SDS), and the same buffer was used to wash the walls remaining on the filter.

To release bound enzymes by autodigestion of the supporting walls, the walls (concentration, 5 mg/ml) were shaken at 37°C in 0.05 M phosphate buffer, pH 7.

Assay of autolysins with wall as substrate. The reaction mixture contained enzyme, 5 mg of purified walls per ml (heated for 5 min to 100°C in 4% SDS to inactivate endogenous autolysin), and 0.05 M phosphate buffer, pH 7. Samples were filtered through membrane filters, and soluble wall products were estimated chemically or, when uniformly labeled walls with known specific activity were used, by liquid scintillation counting. Release of soluble products was linear with time for at least 5 h and proportional to enzyme concentrations over a 50-fold range.

To measure bound autolysins, the supporting walls were incubated as described above to release the attached enzymes, and assays were conducted on the filtrate. When the supporting walls themselves were used as substrate, linear rates of product release were obtained, and these corresponded closely to the rates obtained with the detached enzymes in the standard assay.

Assays of individual enzymes. The pH optimum, cofactor requirements, and range of activities which give linearity with time and enzyme concentration were determined for each enzyme activity. The composition of assay mixtures was based on this data, and all incubations were carried out at 37°C.

Proteases were measured with hide powder azure (B grade, Calbiochem, Lucerne, Switzerland) as substrate in 0.05 M phosphate buffer, pH 7, containing 0.01 M $MgCl_2$. After the addition of trichloroacetic acid (final concentration, 10%), the undigested proteins were removed by filtration, and the concentration of soluble products was determined by the optical density change at 595 nm.

β-Glucosidase (EC 3.2.1.21), β-*N*-acetylglucosaminidase (EC 3.2.1.30), and α-glucosidase (EC 3.2.1.20) were measured with *p*-nitrophenyl (PNP)-β-D-glucoside, PNP-*N*-acetyl-β-D-glucosaminide, and PNP-α-D-glucoside, respectively, as substrates. The assay buffers were 0.05 M citrate (pH 5), 0.05 M citrate (pH 4.5) containing 0.01 M $CaCl_2$, and 0.05 M citrate (pH 5), respectively. The reactions were stopped after 30 min of incubation by adding 0.8 M glycine-NaOH buffer, pH 10.5, at a final concentration of 0.27 M, and the changes in optical density were read at 405 nm.

Chitinase (EC 3.2.1.14) was assayed in a reaction mixture containing 5 mg of colloidal chitin per ml (2) in 0.05 M citrate buffer, pH 4.5. After 4 h of incubation, the reaction was stopped by heating at 100°C for 5 min, and the increase in reducing sugar was measured with neocuproine (8), using an *N*-acetylglucosamine standard.

Laminarinase (EC 3.2.1.6) was measured in 0.05 M citrate buffer, pH 4.5, containing 4 mg of laminarin per ml. After 2 h of incubation, the reaction was stopped by heating to 100°C for 5 min, and the increase in reducing sugar was measured with neocuproine (8), using D-glucose as a standard.

Enzyme units. Enzyme activities were expressed in arbitrary units, 1 unit being the amount of enzyme which (i) liberates 1 μg of soluble components from hyphal walls per h (autolytic activity) or (ii) produces an increase of 1.0 optical density unit per h under the assay conditions (protease, chitinase, laminarinase, α-glucosidase, β-glucosidase, β-acetylglucosaminidase).

Polyacrylamide disc gel electrophoresis. For separation under nondenaturing conditions, cylindrical gels were prepared as described by Davies (7). Stacking gels contained 2.5% (wt/vol) acrylamide in Tris-phosphate buffer, pH 6.6, and separating gels contained 6.4% (wt/vol) acrylamide in Tris-hydrochloride buffer, pH 8.8. The electrode buffer was glycine-Tris, pH 8.3. Samples containing 50 μg of protein, 50% (wt/vol) glycerol, and bromophenol blue as indicator were loaded onto each gel. Electrophoresis was carried out at 4°C and 5 mA/gel. Protein bands were stained with Coomassie brilliant blue (5).

SDS-polyacrylamide gel electrophoresis was carried out as described in reference 11.

Enzyme activity was located in the gels by in situ staining or by cutting the gel into segments and assaying activity in the segments. For in situ staining, the gels were first rinsed with water and incubated for 30 min at room temperature in 0.2 M acetate buffer, pH 4.9. To detect α- and β-glucosidase and β-acetylglucosaminidase, the gels were immersed in the appropriate buffer (see above) containing the pertinent PNP-glycoside. After incubation at 37°C, the gels were washed, and the appearance of color at alkaline pH was scored. Laminarinase activity was located by incubating gels with 0.05 M phosphate buffer, pH 7, containing 5 mg of laminarin (Sigma Chemical Co., St. Louis, Mo.) per ml. Free reducing groups resulting from laminarinase activity were detected with triphenyl tetrazolium chloride, as described in reference

12. Chitinase activity was localized by a similar method, the gels being incubated in colloidal chitin (2) in 0.05 M phosphate buffer, pH 7.

To prepare gel segments, the gels were frozen at −60°C and mechanically cut into consecutive 2-mm segments. Each segment was macerated in the assay mixture appropriate to the activity being tested. After incubation at 37°C, the suspension was filtered through a membrane filter (average pore size, 0.45 μm), and the products in the filtrate were determined as described above.

Polysaccharide hydrolysis, chromatography, chemical assays, and radioisotope determintions. Polysaccharides and oligosaccharides were hydrolyzed with 3 N HCl (18). Sugars were separated by thin-layer chromatography as described previously (18). Determinations of sugars, amino sugars, amino acids, and proteins were carried out as previously described (18), with the exception of free reducing groups, which were assayed with neocuproine (8). Radioisotope contents were determined by liquid scintillation counting in a Tri-Carb spectrometer (Packard Instruments Co., Downers Grove, Ill.).

RESULTS

Solubilization of wall-bound autolysins. In preparations of broken hyphae, LiCl (in concentrations of 1 to 8 M), Triton X-100 (0.01, 0.1, and 1%), dithiothreitol (0.005 M), and SDS detached only 10% or less of the bound enzymes (Table 1). The autolysins were remarkably resistant to inactivation by these reagents; at the highest concentrations, only SDS and cetyltrimethyl ammonium bromide inhibited soluble autolysins (Table 1). The cationic detergent cetyltrimethyl ammonium bromide was the only one of the compounds tested which proved effective and released bound enzyme.

Autodigestion of the supporting wall also led to enzyme release (Table 2). During autodigestion, extensive enzyme detachment had occurred when only a small proportion of the wall had been converted to soluble fragment (Table 2). The presence of insoluble wall was therefore not sufficient to remove free enzymes from solution. This was confirmed by mixing solubilized enzymes with purified, SDS-treated walls (final wall concentration, 5 mg/ml). After the wall was removed by filtration and washed with 0.05 M phosphate, 97% of the original autolysin activity was still found to be in the filtrate and washings, and only 3% had reattached to the walls.

Nature of enzymes bound to hyphal walls. Enzymes free from walls hydrolyzed colloidal chitin, insoluble β-(1,3)-glucan (laminarin), and protein. The presence of β-glucosidase, α-glucosidase, and β-acetylglucosaminidase was shown by the hydrolysis of PNP-glycosides. Polyacrylamide gel electrophoresis gave 12 bands which stained with Coomassie brilliant blue (Fig. 1). One band hydrolyzed chitin but had no β-acetylglucosaminidase activity; another hydrolyzed laminarin but not PNP-β-D-glucoside. Two distinct β-glucosidases and two β-acetylglucosaminidases could be identified. Protease and α-glucosidase were not detected on the gels, possibly because of their relatively low activities in the starting material.

Thin-layer chromatography and reaction with glucose oxidase showed that autolytic wall digests contained free glucose and *N*-acetylglucosamine. This confirmed the presence of glucosidases and of β-acetylglucosaminidase. Autolytic wall digests contained considerable amounts of oligosaccharides, as shown by a ratio of total to reducing sugar of 2.2:1. Column chromatography demonstrated that the oligosaccharides were very heterogeneous in size (Fig. 2); the largest

TABLE 2. *Release of bound autolysins by autodigestion of supporting walls of strain R21*

Time of incubation	Bound enzyme[a]	Detached enzyme[a]	% of wall solubilized
0	720	0	
15 min	595	120	
30 min	550	160	
60 min	435	276	0.8
120 min	350	320	1.5
240 min	85	524	2.2
22 h	60	628	

[a] Numbers are autolysin units (wall as substrate) per 70 mg of wall dry weight.

TABLE 1. *Detachment of bound autolysins from purified walls of strain R21*

Reagent[a]	Concn	Inhibition of soluble enzyme (%)	Enzymes remaining bound[b]	Enzymes detached[b]	% Extraction
None			282	0	
CTAB[c]	0.1%	9	28	254	90.1
	5%	91	27	6	
SDS	4%	40	258	17	6.2
Triton X-100	1%	2	261	14	5.1
LiCl	8 M	7	248	4	1.6

[a] Extraction with SDS was carried out at 20°C; all others were at 4°C.
[b] Numbers are autolysin units (wall as substrate) per 20 mg of wall dry weight.
[c] CTAB, Cetyltrimethyl ammonium bromide.

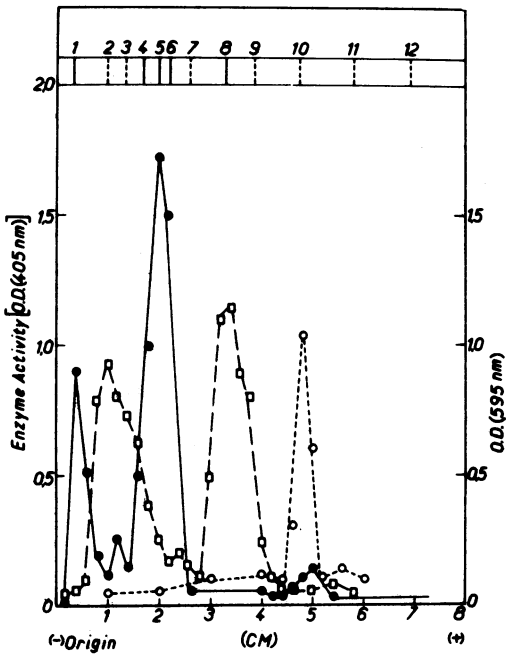

FIG. 1. *Location of proteins and enzyme activities after electrophoresis of wall-bound enzymes in polyacrylamide gels. Gels were cut into 2-mm slices, and hydrolysis of substrates by individual slices was determined as described in the text. Substrates were: (●) PNP-β-glucoside; (□) PNP-N-acetyl-β-glucosaminide; (○) colloidal chitin. Location of bands staining with Coomassie brilliant blue are shown and numbered at the top (solid line, deeply stained; broken line, faint band). O.D., Optical density.*

fragments had molecular weights in excess of 10^5. The oligosaccharides contained all of the mannose solubilized from the wall, as well as glucose and galactose residues. Their more detailed structure is being investigated.

Distribution of autolysins in preparations of broken hyphae. After the breakage of exponentially growing hyphae, a major part of the enzymes acting on whole walls or on specific wall components was found in the soluble fraction (Tables 3 and 4). Enzymes with the same activities were, however, also tightly attached to the wall fraction. The ratio of bound to total enzyme varied considerably among the different activities, and the bound autolysins had, with the exception of α-glucosidase and β-acetylglucosaminidase, a markedly higher specific activity. We detected autolysins in the culture medium, but a purified membrane fraction showed only low activity (Table 3). All three strains examined in this work gave comparable results.

To determine if autolysin attachment is an artefact of hyphal breakage, we radiolabeled hyphae by growth on [^{3}H]glucose. The mycelium was then sonically treated in the presence of purified walls labeled by growth on [^{14}C]glucose and heated with SDS to inactivate autolysins. After breakage, purification of the wall fraction, and incubation, the release of isotopes showed that the walls from the originally intact hyphae carried almost all the attached enzyme (Table 5). The added walls were only digested at significant rates when enzyme started to detach from the ^{3}H-labeled walls (see Table 2).

Incubation of exponentially growing hyphae in media lacking a carbon source led to a striking increase in total autolytic activity and in that of individual enzymes (Tables 3 and 4). Although the wall-bound enzymes increased by an order of magnitude, the biggest differences were found in the amounts of soluble enzyme. Cyclohexi-

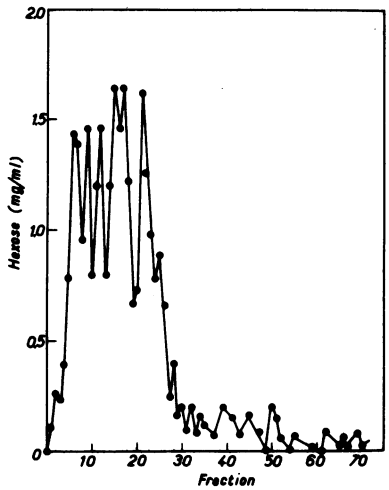

FIG. 2. *Column chromatography on Sephadex G200 of autolytic wall digest. Walls of strain R21 were allowed to autolyze until the plateau level was reached; 176 mg of soluble wall products was loaded onto the column. Fractions of 3 ml were collected; fraction 1 corresponds to the end of void volume elution.*

TABLE 3. *Autolysin distribution in preparations of broken hyphae of strain R21*

Fraction	Carbon source prior to harvest	Total enzyme[a]	Sp act (units/mg of protein)
Wall	+	2,975	1,050
	−	11,736	7,523
Soluble	+	2,761	27
	−	30,882	722
Culture medium	+	826	45
	−	2,851	361
Membrane	+	22	4

[a] Enzyme units obtained from breakage of 1.1 g (dry weight) of hyphae. Enzyme activity was determined with wall as substrate as described in the text.

TABLE 4. *Autolysin contents of exponentially growing and starved hyphae of strain R21*

Carbon source prior to harvest	Enzyme substrate	Wall fraction		Soluble fraction	
		Total enzyme[a]	Sp act (units/mg of protein)	Total enzyme[a]	Sp act (units/mg of protein)
+	Chitin	22.00	10.50	125.0	1.400
−	Chitin	702.00	467.00	18,643.0	302.000
+	Laminarin	945.00	446.00	6,875.0	55.000
−	Laminarin	3,145.00	1,372.00	31,789.0	515.000
+	β-Glucoside	162.00	57.00	282.0	2.600
−	β-Glucoside	443.00	278.00	2,291.0	54.000
+	Protein	0.86	0.30	1.4	0.013
−	Protein	8.06	5.17	72.0	1.680
+	α-Glucoside	21.00	7.40	206.0	3.900
−	α-Glucoside	30.00	14.50	425.0	4.800
+	β-*N*-acetylglucosaminide	6.10	2.10	206.0	1.900
−	β-*N*-glucosaminide	19.60	12.60	415.0	9.700

[a] Enzyme units in material obtained from 3.6 g (dry weight) of hyphae. Enzyme activity was determined as described in the text.

TABLE 5. *Binding of autolysins during hyphal breakage to exogenously added walls*

Incubation time (min)	Hyphal wall (^3H)[a] (μg of soluble product/ml)	Exogenous wall (^{14}C)[a] (μg of soluble product/ml)	$^3H/^{14}C$
10	545	48	11.3
20	880	93	9.4
30	1,185	135	8.8

[a] The amounts of mycelium and exogenous wall were adjusted before breakage to give roughly equal amounts of dry weight (10 mg/ml) of each wall after purification. Soluble products were calculated from specific radioactivity measurements of walls and are proportional to the amount of enzyme bound.

mide completely prevented the rise in enzyme activity. It may be pointed out that 4 h of carbon starvation failed to induce α-(1,3)-glucan hydrolase (20) in amounts sufficient to allow detection by our method.

SDS-polyacrylamide gel electrophoresis was used to investigate whether the autolytic enzymes formed in the absence of a carbon source are identical to those made in smaller amounts during exponential growth. The wall-bound autolysins from exponentially growing and from starved hyphae gave patterns which differed in the intensity of some of the bands but not in their positions (Fig. 3). Thus, among the bound autolysins, the same proteins appear to be made but in differing amounts.

Hydrolysis of lateral and apical walls by bound and soluble autolysins. Apical walls are better substrates for added, soluble autolysins than lateral walls and are hydrolyzed preferentially (18; Table 6). This, however, was not the case with bound autolysins (Table 6). During

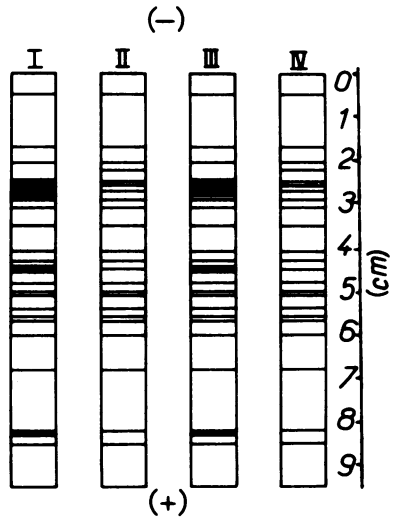

FIG. 3. *SDS-polyacrylamide gel electrophoresis of wall-bound autolysins. A 50-μg amount of protein was loaded onto the gels, and the bands were stained with Coomassie brilliant blue. Gels I (SM16) and III (R21), Proteins from exponentially growing hyphae; gels II (SM16) and IV (R21), proteins from hyphae starved for carbon source.*

the early stages of incubation, bound enzyme digested the supporting lateral wall more rapidly. As incubation proceeded and bound enzyme became detached, the rate of apical wall hydrolysis increased. Since soluble enzymes were tested on SDS-treated walls, a control with untreated walls carrying bound autolysins was included (Table 6). The addition of soluble enzymes to such walls increased the relative rate

TABLE 6. *Digestion of apical and lateral walls by bound and free autolysins*

Incubation time (min)	Autolysin present	Lateral wall digested (^3H) (dpm/ml)	Apical wall digested (^{14}C) (dpm/ml)	$^{14}C/^3H$
15	Bound[a]	2,356	2,425	1.03
	Free[b]	1,432	5,700	3.98
	Bound + free[c]	4,082	9.482	2.32
30	Bound	3,091	4,041	1.31
	Free	2,366	10,894	4.60
	Bound + free	5,565	14,565	2.62
	HCl[d]	50,772	57,888	1.14

[a] Purified walls carrying autolysins served as both substrate and enzyme source.
[b] Bound walls were heated with SDS, and free autolysin was added.
[c] Bound walls with same amount of free added autolysins as in free walls.
[d] Total hydrolysis by heating with HCl.

of apical wall digestion. Thus, the different activities of bound and soluble enzymes appear to be due to either an unequal distribution of bound enzyme or a greater affinity of soluble autolysins for apical wall.

DISCUSSION

Broken hyphae of *A. nidulans,* like those of *Neurospora* (15), *Saprolegnia* (9), and yeasts (10, 16), contained two autolysin populations, soluble enzymes and wall-bound proteins. In *A. nidulans,* bound autolysins remained attached in the presence of 8 M LiCl and, when solubilized by other means, did not readsorb to wall. Thus, the tight association was not due to an exceptionally high affinity of enzyme for substrate. Since the cationic detergent cetyltrimethylammonium bromide, but not the neutral or anionic detergents Triton X-100 or SDS, removed the bound proteins, the attachment appears to be mediated by a noncovalent linkage with negatively charged lipids.

Our method of preparation yields a wall fraction which is largely free from cytoplasmic membranes (18). Furthermore, a membrane preparation contained only small amounts of autolysins and at a low specific activity. The possibility that the enzymes in the wall fraction are present in contaminating fragments of cytoplasmic membranes can thus be ruled out, as can the possibility that attachment is an artefact of cell rupture, since labeled walls added during breakage carried little enzyme. Autolysin attachment to the wall, then, appears to be the result of a cell-directed modification occurring in the growing hyphae.

No firm conclusions concerning the nature of the modification can be drawn from the present data. However, cytoplasmic vesicles from *A. nidulans* hyphae do contain autolysins (Polacheck, in preparation). A simple explanation would therefore be that such vesicles migrate to sites of wall synthesis (13) and that some of them are trapped in newly formed wall. In this view, vesicles inserted into or attached to the wall would be protected during cell breakage; others would be sheared to liberate soluble enzymes.

The suggestion that autolysins are attached to wall during growth raises a problem in control, irrespective of the mechanism of attachment. Wall turnover in growing hyphae is very low (19), even in strain SM16 whose walls lack α-(1,3)-glucan and autolyze very rapidly after isolation and purification. Growing hyphae must therefore possess controls over autolysin activity which are lost on cell breakage. Such controls could consist of soluble inhibitors; it is also possible that breakage and purification partially damage the normally impermeable vesicle membranes. They may then retain enzymes at the low temperatures of purification (solid phase of lipids) but not during incubation at 37°C. It is tempting to speculate that hydrolytic enzymes, in an inactive state, are associated with the wall along the whole length of the growing hypha. Branching would then involve their activation at specific sites, a situation analogous in principle to that described for chitin synthetase in yeast by Cabib and his collaborators (3, 4).

The enzymes attached to hyphal walls were capable of hydrolyzing all the major wall polymers except α-(1,3)-glucan, and among them were two pairs of isoenzymes. It is unlikely that these represent all the autolysins involved in wall metabolism; we did not look for hydrolases acting on mannose- and galactose-containing polymers. The synthesis of both soluble and bound enzymes was strikingly derepressed in the absence of a carbon source. Cohen (6) has described a derepression of proteases in *A. nidulans* starved of carbon; we find that under these conditions five of the autolysins also increased sharply, although noncoordinately, in amount. This derepression may be relevant to the relation between the degree of hyphal branching and wall-bound autolysins (9, 15). Increased branching leads to higher contents of bound autolysins; highly branched hyphae, however, form tighter clumps whose interior may be lacking nutrients. From the SDS-polyacrylamide gel electrophoresis profiles, autolysins formed after carbon starvation appear to be the same proteins made in smaller amounts during exponential growth. It is likely that they function in mobilizing cell polymers during starvation, for exoenzymes acting on exogenous polymers should have been present in the culture medium at a

much higher specific activity.

Somewhat surprisingly, we failed to detect α-1,3-glucan hydrolase in starved hyphae. Zonneveld (20, 21) has shown that synthesis of this enzyme is derepressed in old mycelium growing on agar and that its appearance coincides with the onset of cleistothecia formation. It may be that carbon starvation in shaken cultures is an insufficient trigger.

LITERATURE CITED

1. **Barras, D. R.** 1972. A β-glucan endo-hydrolase from *Schizosaccharomyces pombe* and its role in cell wall growth. Antonie van Leeuwenhoek J. Microbiol. Serol. **38**:65–80.
2. **Berger, L. R., and D. M. Reynolds.** 1958. The chitinase system of a strain of *Streptomyces griseus*. Biochim. Biophys. Acta **29**:522–534.
3. **Cabib, E., and V. Farkas.** 1971. The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. Proc. Natl. Acad. Sci. U.S.A. **68**:2052–2056.
4. **Cabib, E., and R. Ulane.** 1973. Yeast chitin synthetase. J. Biol. Chem. **248**:1451–1458.
5. **Chrambach, A., R. A. Reisfeeld, M. Wyckoff, and J. Zaccari.** 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. **20**:150–154.
6. **Cohen, B. L.** 1973. Regulation of intracellular and extracellular neutral and alkaline proteases in *Aspergillus nidulans*. J. Gen. Microbiol. **79**:311–320.
7. **Davies, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
8. **Dygerts, S., L. H. Li, D. Florida, and J. A. Thoma.** 1965. Determination of reducing sugar with improved precision. Anal. Biochem. **13**:367–374.
9. **Fevre, M.** 1972. Contribution to the study of the determination of mycelium branching of *Saprolegnia monoica* Pringsheim. Z. Pflanzenphysiol. **68**:1–10.
10. **Fleet, G. H., and H. J. Phaff.** 1974. Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall-associated β 1–3 glucanase. J. Biol. Chem. **249**:1717–1728.
11. **Fleming, H., and R. Haselkorn.** 1974. The program of protein synthesis during heterocyst differentiation in nitrogen-fixing blue-green algae. Cell **3**:159–170.
12. **Gabriel, O.** 1971. Locating enzymes in gels. Methods Enzymol. **22**:578–604.
13. **Grove, S. N., and C. E. Bracker.** 1970. Protoplasmic organization of hyphal tips among fungi: vesicles and spitzenkörper. J. Bacteriol. **104**:989–1009.
14. **Keath, J. B., J. L. Gay, and A. D. Greenwood.** 1971. Cell wall formation in the *Saprolegniales*: cytoplasmic vesicles underlying developing walls. J. Gen. Microbiol. **65**:225–232.
15. **Mahadevan, P. R., and V. R. Mahadkar.** 1970. Role of enzymes in growth and morphology of *Neurospora crassa*: cell-wall bound enzymes and their possible role in branching. J. Bacteriol. **101**:941–947.
16. **Matile, P., M. Cortat, A. Wiemken, and A. Frey-Wyssling.** 1971. Isolation of glucanase-containing particles from budding *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **68**:636–640.
17. **Page, W. J., and J. J. Stock.** 1972. Isolation and characterization of *Microsporum gypseum* lysosomes: role of lysosomes in macroconidia germination. J. Bacteriol. **110**:354–362.
18. **Polacheck, Y., and R. F. Rosenberger.** 1975. Autolytic enzymes in hyphae of *Aspergillus nidulans*: their action on old and newly formed walls. J. Bacteriol. **121**:332–337.
19. **Polacheck, I., and R. F. Rosenberger.** 1977. *Aspergillus nidulans* mutant lacking α-(1-3)-glucan, melanin, and cleistothecia. J. Bacteriol. **132**:650–656.
20. **Zonneveld, B. J. M.** 1972. A new type of enzyme, an exosplitting α 1,3 gluean from non-induced cultures of *Aspergillus nidulans*. Biochim. Biophys. Acta **258**:541–547.
21. **Zonneveld, B. J. M.** 1972. Morphogenesis in *Aspergillus nidulans*. The significance of α 1,3 glucan of the cell wall and α 1,3 glucanase for cleitothecium development. Biochim. Biophys. Acta **273**:174–187.