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Derepression of β-1,3-Glucanases in *Penicillium italicum*: Localization of the Various Enzymes and Correlation with Cell Wall Glucan Mobilization and Autolysis

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The localization of the derepressible β-1,3-glucanases of *Penicillium italicum* and the cell wall autolysis under conditions of β-1,3-glucanase derepression (24 h in a low-glucose medium) were studied. About 15% of the total activity was secreted into the culture medium during the 24-h period and consisted of similar amounts of each of the three β-1,3-glucanases (I, II, III) produced by this species. Treatment of derepressed mycelia with periplasmic enzyme-inactivating agents resulted in a loss of 45% of the mycelium-bound β-1,3-glucanase. Analysis of periplasmic enzymes solubilized by 2 M NaCl or by autolysis of isolated cell walls revealed that only β-1,3-glucanases II and III were bound to the cell wall. These two enzymes were capable of releasing in vitro reducing sugars from cell walls, whereas β-1,3-glucanase I was not. In addition, the autolytic activity of cell walls isolated from derepressed mycelium was greater than that of cell walls isolated from repressed mycelium. The incubation of the fungus in the low-glucose medium also resulted in the in vivo mobilization of 34% of the cell wall β-1,3-glucan, and this mobilization was fully prevented by cycloheximide, which also blocked derepression of β-1,3-glucanases. Derepression of β-1,3-glucanase seems to be coupled to the mobilization of cell wall glucan.

It is generally accepted that some of the fungal autolytic enzymes must be bound to the cell wall because preparations of isolated cell walls undergo autolysis, thus causing the solubilization of some of their structural components (14). Among these lytic enzymes are β-1,3-glucanases which not only have been isolated from cell walls (5) but also have been shown to contribute to the lysis of these structures (1).

Many details of the β-1,3-glucanase system of *Penicillium italicum* and its regulation have been studied in our laboratory. The system is subject to catabolite repression (19) and consists of three enzymes, which we refer to as β-1,3-glucanases I, II, and III. During active growth, when an excess of glucose is present, only small amounts of β-1,3-glucanases II and III are produced, whereas the absence of glucose not only results in a greater rate of synthesis of these two enzymes, but also triggers the production of β-1,3-glucanase I, which is undetectable in actively growing mycelium (18). The derepression of the β-1,3-glucanase system under conditions of carbon source starvation might be directed toward the mobilization of some of the cell wall or extracellular glucan. However, there are no references about the localization of β-1,3-glucanases in *P. italicum* to support this suggestion. In this communication we show that, after 24 h of derepression, only β-1,3-glucanases II and III were bound to the cell wall, although all three of the β-1,3-glucanases might be found in the cytoplasm and in the culture fluid. Incubation for 24 h in the derepression medium also led to the mobilization of 34% of the cell wall structural glucan, and this was prevented by cycloheximide, which blocked derepression of β-1,3-glucanases.

MATERIALS AND METHODS

Chemicals. Laminarin was from Koch-Light Laboratories, Colnbrook, Bucks, England; cycloheximide and *p*-nitrophenylphosphate were from Sigma Chemical Co., St. Louis, Mo., and [*U*-^{14}C]glucose (3 mCi/mmol) was from the Radiochemical Centre, Amersham, England. The latter was diluted before use with cold glucose to a specific activity of 1.8 μCi/mmol.

Organism, growth conditions, and preparation of derepressed mycelium. *P. italicum* CECT 2294 was used throughout this study and was maintained on slants of potato extract medium (3). Liquid cultures were grown in modified Czapek-Dox medium containing 150 mM glucose, 0.2% $NaNO_3$, 0.2% K_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4$ (Czapek-glucose medium). Log-phase mycelia were

prepared by inoculating conidia at a concentration of 4×10^6/ml into 1,000-ml flasks with 250 ml of the medium and incubating at 28°C in a Gallenkamp orbital incubator for 48 h.

Incubation of log-phase mycelia in a medium low in glucose content led to derepression of β-1,3-glucanase. Derepressed *P. italicum* mycelia were prepared by transferring actively growing cells (5 mg of dry weight per ml) to 1-liter flasks containing 250 ml of Czapek-glucose medium with a glucose concentration of 2 to 5 mM instead of 150 mM and incubating for 24 h in an orbital incubator.

Preparation of cell-free extracts and cell walls. Cell-free extracts were obtained by mechanical breakage of the mycelium (suspended in 50 mM acetate buffer, pH 5.1) in a Braun homogenizer, as has been described earlier (19). When used as a source of enzyme, *P. italicum* cell walls were purified from cell-free extracts by low-speed centrifugation. They were washed twice with 50 mM acetate buffer (pH 5.5) containing 0.75 M NaCl and then three times with plain 50 mM acetate (pH 5.5). Each wash lasted for about 20 min, and 200-ml portions of the washing solution were used each time to wash the walls from 200 mg (dry weight) of mycelium. *P. italicum* cell walls, when used as substrates, were obtained by the method of Mahadevan and Tatum (10).

Mobilization of glucan. Mobilization of β-1,3-glucan under conditions of derepression was measured as follows. *P. italicum* was grown for 48 h at 28°C in 100-ml flasks with 25 ml of Czapek-glucose medium containing 150 mM [*U*-^{14}C]glucose. The mycelium was washed, transferred to the derepression medium (with 2 mM [*U*-^{14}C]glucose), and incubated as described above. Samples were taken after 0 and 24 h of derepression, followed by the extraction of β-1,3-glucan by the method of Mahadevan and Tatum (10), and the radioactivity present in the β-1,3-glucan fraction was determined in a Packard Tri-Carb scintillation counter. Vials (3 ml) with 2 ml of scintillation solution [0.35% 2,5-diphenyloxazole and 5% 1,4-bis-(5-phenyloxazolyl)-benzene in toluene] were used, and counting efficiency for ^{14}C was 87%.

Column chromatography and enzyme assays. Diethylaminoethyl (DEAE)-Sephadex A50 column chromatography was performed by applying the enzyme solutions to a column (18 by 2 cm) equilibrated with 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2. The column was washed until no UV-absorbing material eluted and developed with a linear salt gradient (0 to 1 M NaCl) in the same buffer. Fractions (5 ml) were collected in an Ultrorak (LKB Instruments Inc., Rockville, Md.) fraction collector at a flow rate of 6 to 18 ml/h.

Laminarin and pustulan were the substrates used in the assays of β-1,3-glucanase and β-1,6-glucanase, respectively. The assays were performed as previously described (19) and were based on the release of reducing sugar groups from these substrates by the action of the enzymes. One unit of activity was the amount of enzyme catalyzing the release of an amount of reducing sugars equivalent to 1 nmol of glucose per min under the conditions of the reaction.

Alkaline phosphatase was assayed with *p*-nitrophenylphosphate as substrate. Final concentrations of buffer (Tris-hydrochloride, pH 8.7) and substrate were 50 mM and 0.25% respectively; the mixtures were incubated at 37°C for 30 min, and the amount of *p*-nitrophenol was determined spectrophotometrically by measuring optical density at 410 nm. One unit of enzyme catalyzed the release of 1 nmol of *p*-nitrophenol per min under the conditions of the reaction. Protein was measured by the method of Lowry et al. (9). Reducing sugar groups were determined by the method of Somogyi (21) and Nelson (12).

RESULTS

Localization of β-1,3-glucanases in derepressed *P. italicum* mycelium. We have shown previously that the activation of β-1,3-glucanase synthesis which takes place during cell incubation in a low-glucose medium is also accompanied by the secretion of a certain amount of enzyme, which can be detected in the culture fluid (19). Figure 1 shows an analysis by DEAE-Sephadex column chromatography of extracellular β-1,3-glucanases secreted after 24 h of incubation in that medium; these represent approximately 15% of the total activity. We can conclude from these results that portions of all three enzymes (β-1,3-glucanases I, II, and III) found to be present in the cytoplasm (18) are secreted into the culture medium. In this case the concentrations of the three enzymes, in terms of units, are very similar; this contrasts with their relative proportions in supernatant fluids of cell-free extracts in which the peak of β-1,3-glucanase II is much more intense, compared with those of β-1,3-glucanase I and III. This suggests differences in their rate of secretion into the culture medium.

Part of the mycelium-bound β-1,3-glucanase

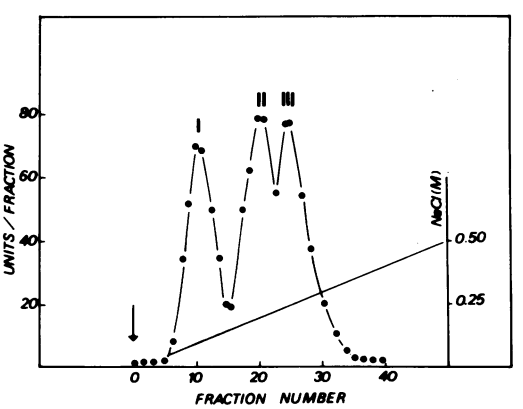

FIG. 1. *Analysis by DEAE-Sephadex column chromatography of β-1,3-glucanases secreted by P. italicum after 24 h of incubation in low-glucose medium. Culture fluid (750 ml) was dialyzed against 5 mM acetate buffer (pH 5.5) and then against 50 mM Tris-hydrochloride (pH 7.25) and finally chromatographed in DEAE-Sephadex A50.*

must be located in the periplasmic space because the sedimentation of cell walls from cell-free extracts by low-speed centrifugation caused a loss of 30% of the activity present in those extracts (data not shown). In fact, when β-1,3-glucanase was assayed in cell wall preparations, a value of 0.66 U per mg (dry weight) of cell wall was found. This is much higher than the value obtained for β-1,6-glucanase (0.09 U), an essentially extracellular enzyme. Periplasmic β-1,3-glucanase should be destroyed by treatment of the mycelium with 0.1 M HCl (4) or trypsin (6), which have been used for inactivating cell wall-bound enzymes without affecting those located within the cytoplasm. As shown in Fig. 2 and 3, this kind of treatment destroys approximately 45% of the mycelium-bound β-1,3-glucanase. As a control, we also followed the effect of these treatments on alkaline phosphatase. Cell-free extracts of *P. italicum* derepressed mycelium had 10.6 U of this enzyme per mg of mycelium, and only 0.48 U (4.5% of the total) were detected when it was assayed in whole cells. Alkaline phosphatase is therefore an essentially intracytoplasmic enzyme in *P. italicum*, as it is in *Neurospora crassa* (11), which remains unaffected by the treatment with HCl or trypsin (Fig. 2 and 3).

Bearing in mind the periplasmic localization of part of the β-1,3-glucanase activity, it was of interest to determine which one(s) of the three enzymes was present in the periplasmic space. Several of the treatments (8) which have been devised for the extraction of periplasmic enzymes in fungi were used in our experiments involving extraction of β-1,3-glucanase from derepressed *P. italicum* mycelium; their efficiency is indicated in Table 1. Treatment with 2 M

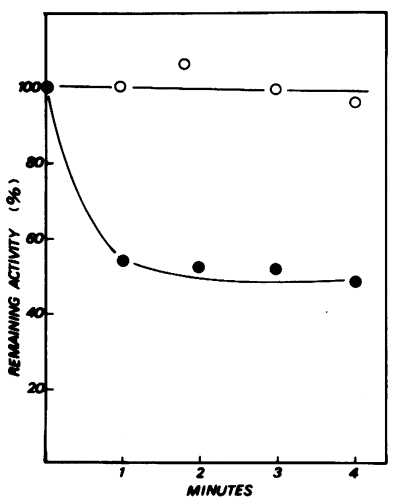

FIG. 2. *Effect of treatment with 0.1 M HCl on mycelium-bound alkaline phosphatase* (○) *and β-1,3-glucanase* (●). *Portions of 37 mg (dry weight) of derepressed P. italicum mycelium were suspended in 25 ml of 0.1 M HCl; after different periods of treatment, the pH was brought to 5.5 by the addition of 0.2 M sodium acetate, and the enzymes were assayed in cell-free extracts. Values express percentage of specific activity of each enzyme remaining after the treatment.*

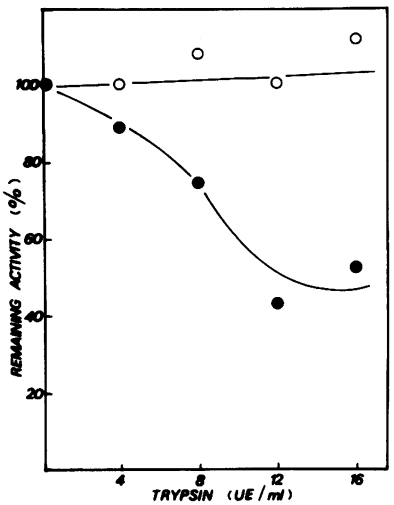

FIG. 3. *Effect of trypsin on mycelium-bound alkaline phosphatase* (○) *and β-1,3-glucanase* (●). *Portions of 37 mg (dry weight) of derepressed mycelium were suspended in 25 ml of Czapek-glucose medium (with 5 mM glucose) having the indicated trypsin concentrations and shaken at 25°C for 75 min, after which cell-free extracts were prepared for assaying the enzymes. UE, Units of enzyme.*

TABLE 1. *Extraction of periplasmic β-1,3-glucanase*[a]

Extracting solution	Amt of enzyme solubilized (U/ml)
Acetate buffer, pH 5.5 (50 mM)	0
Triton X-100 in acetate[b]	—[c]
Tween 80 (0.5% in acetate)	2.33
Tween 20 (0.5% in acetate)	2.33
Brij 58 (0.5% in acetate)	1.10
Deoxycholate (0.5% in 50 mM Tris-hydrochloride, pH 7.5)	2.83
NaCl (2 M in acetate)	3.10
Ethylenediaminetetraacetate (1 to 5 mM)	1.30

[a] For extraction, 15 mg (dry weight) of derepressed mycelium was suspended in 5 ml of the extracting solution. The suspension was shaken for 20 min and filtered through Whatman no. 3 filter paper, and β-1,3-glucanase was assayed in the filtrates.

[b] Acetate, 50 mM acetate buffer (pH 5.5).

[c] —, Strong interference with reducing sugar determination.

NaCl would appear to be the most efficient, although it does not yield a quantitative extraction. DEAE-Sephadex column chromatography of the β-1,3-glucanases extracted with 2 M NaCl revealed two peaks of activity, with elution patterns which corresponded to those of β-1,3-glucanases II and III (Fig. 4); the latter was more intense. Similar results were obtained when β-1,3-glucanases solubilized by autolysis of purified cell wall preparations (55% of the activity bound to the cell wall was solubilized by this procedure) were chromatographed (Fig. 5). This indicates that only β-1,3-glucanases II and III are bound to the cell wall and that the latter is present in higher proportions. The data given in Table 2 also favor this interpretation; whereas β-1,3-glucanase I has no effect on isolated *P. italicum* cell walls, the other two are able to release reducing sugar groups from them, and the activity of β-1,3-glucanase III is much more intense in this reaction than is that of β-1,3-glucanase II.

Cell wall autolysis in derepressed *P. italicum* mycelium. Isolated *P. italicum* cell walls are capable of undergoing autolysis and, hence, the release of reducing sugar groups. As shown in Fig. 6, cell walls isolated from mycelium which was incubated for 24 h in a medium with 2 mM glucose autolyzed at a faster rate and to a greater extent than did those isolated from mycelium incubated in 150 mM glucose. It is also clear that, when cycloheximide, which prevented derepression of β-1,3-glucanase (Fig. 7), was added to the derepression medium, the release of reducing sugar groups by cell wall autolysis was almost negligible.

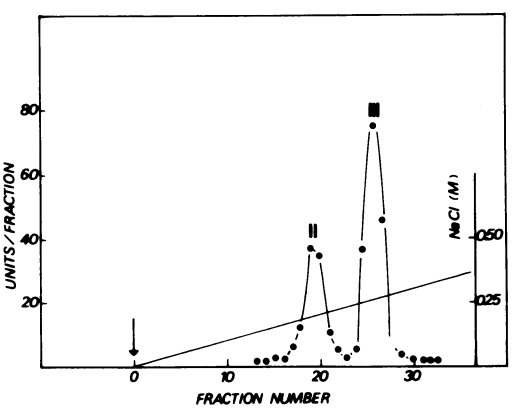

FIG. 4. *Analysis by DEAE-Sephadex column chromatography of β-1,3-glucanases extracted from derepressed mycelium with 2 M NaCl. A 9-g (dry weight) amount of derepressed P. italicum mycelium was suspended in 500 ml of 50 mM acetate buffer (pH 5.5) containing 2 M NaCl and shaken for 30 min at 28°C. The cells were centrifuged, and the supernatant fluid was dialyzed against 5 mM acetate (pH 5.5) and then against Tris-hydrochloride (pH 7.25) to be finally chromatographed in DEAE-Sephadex A50.*

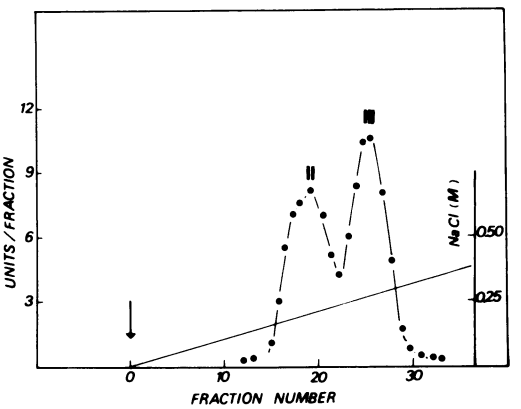

FIG. 5. *Analysis by DEAE-Sephadex A50 column chromatography of cell wall-bound β-1,3-glucanases solubilized by autolysis of isolated cell walls. Cell walls isolated from 750 mg (dry weight) of P. italicum derepressed mycelium were suspended in 50 ml of 50 mM acetate buffer (pH 5.5), subjected to sonic treatment, and dialyzed for 65 h against the same buffer. The cell walls were centrifuged, and the supernatant was dialyzed against 50 mM Tris-hydrochloride (pH 7.25) and applied to a DEAE-Sephadex column.*

TABLE 2. *Lytic action of β-1,3-glucanases on isolated P. italicum cell walls*[a]

Enzyme	Amt of reducing sugar released (nmol/ml per enzyme unit)
β-1,3-Glucanase I	0
β-1,3-Glucanase II	5
β-1,3-Glucanase III	140
β-1,3-Glucanases I, II, and III	58

[a] The enzymes were separated by DEAE-Sephadex column chromatography of cell extracts as described previously (18). Assay mixtures contained 10 U of enzyme or the mixture and 10 mg (dry weight) of cell walls in 2 ml of 50 mM acetate buffer, pH 5.5. After incubation for 8 h at 30°C in screw-capped tubes, the amount of reducing sugar released was determined.

Derepression of β-1,3-glucanase not only is paralleled by a more intense autolysis of isolated cell walls in vitro, but is also accompanied by an effect with regard to the mobilization of β-1,3-glucan in vivo. Table 3 shows that, upon incubation of mycelium with radioactively labeled glucan for 24 h in a medium with 2 mM [^{14}C]-glucose, approximately 34% of the glucan was mobilized and this mobilization was fully prevented when the incubation was performed in the presence of cycloheximide.

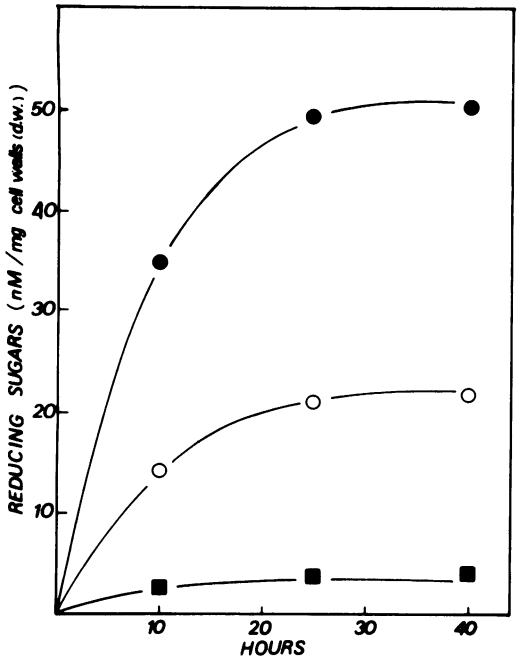

FIG. 6. *Autolysis of cell walls isolated from P. italicum mycelium. A 20-mg (dry weight) amount of cell walls was suspended in 5 ml of 50 mM acetate buffer (pH 5.5) and incubated at 30°C with occasional shaking. At the indicated times samples were taken and centrifuged, and the reducing sugar groups in the supernatants were determined. The cell walls had been isolated from mycelium incubated for 24 h in Czapek-glucose medium containing 150 (○) or 5 mM glucose (●) or 5 mM glucose plus 50 μg of cycloheximide per ml (■).*

DISCUSSION

Many of the enzymes produced by microorganisms are subject to glucose repression (13). Among them there are not only enzymes involved in sugar catabolism but also many others, such as those which participate in morphogenetic events (for example, the enzymes needed for bacterial sporulation or for development in the slime mold *Dictyostelium discoideum* [15]). The β-1,3-glucanase system of *P. italicum* also belongs to this class; synthesis of the enzymes is subject to glucose repression (19) exerted at a pretranslational level (20). The system consists of three enzymes, β-1,3-glucanase I, which degrades glucan at intermediate points of the polymer chain, β-1,3-glucanase II, which acts at the nonreducing end, and β-1,3-glucanase III, which has both mechanisms of action (18). The incubation of log-phase *P. italicum* in a low-glucose medium not only leads to a greater activity in the synthesis of β-1,3-glucanases II and III, but at the same time determines the appearance of

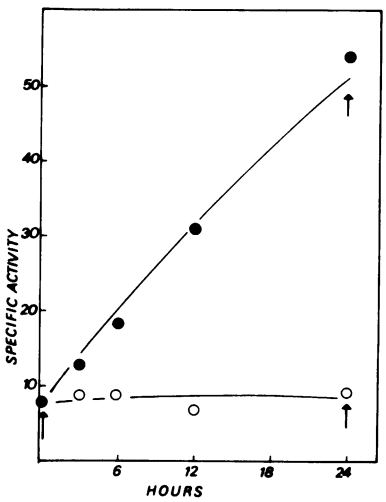

FIG. 7. *Effect of cycloheximide on derepression of β-1,3-glucanase. Log-phase P. italicum cells were incubated in Czapek-glucose medium with 5 mM glucose in the presence (○) or absence (●) of 50 μg of cycloheximide per ml. At the indicated times the specific activity of β-1,3-glucanase was determined in cell-free extracts of samples taken from the culture.*

TABLE 3. *Mobilization of β-1,3-glucan under conditions of β-1,3-glucanase derepression*

Length of depression (h)	Radioactivity (cpm of ^{14}C) in β-1,3-glucan extracted from 1 mg (dry weight) of mycelium		
	Expt 1	Expt 2	Control (+cycloheximide)
0	613	478	504
24	401 (34.6)[a]	317 (33.7)	603 (0)

[a] Values in parentheses represent the percentage of radioactivity lost after 24 h of derepression.

β-1,3-glucanase I, which is undetectable in actively growing cells. We believe that the study of the localization of these enzymes in derepressed mycelium should contribute to the interpretation of the physiological significance of β-1,3-glucanase derepression. A scheme of the process of secretion of β-1,3-glucanases by *P. italicum* after 24 h of derepression, based on the evidence in this paper and in a previous one (18), is depicted in Fig. 8; 45% of the total activity is located in the cytoplasm, in which the most abundant of the three enzymes is β-1,3-glucanase II. Another 40% is located in the periplasmic space, where only β-1,3-glucanases II and III seem to be present; and the remaining 15% is extracellular and is made up of similar concentrations of each of the three enzymes. These results point to the existence of important differences among these three enzymes in their

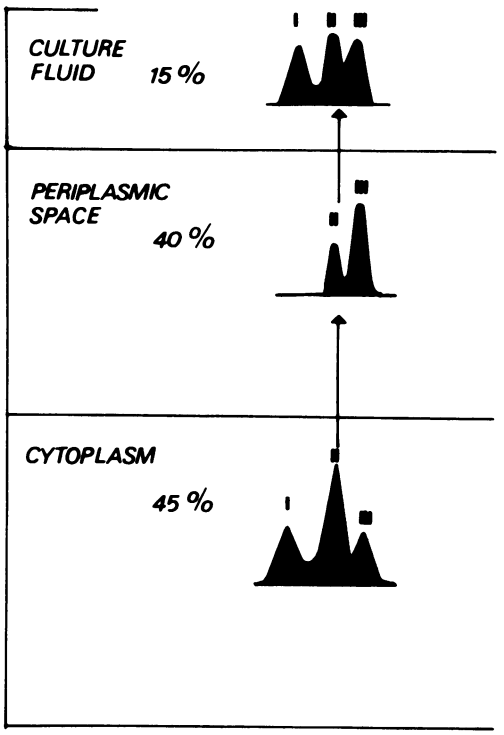

FIG. 8. *Spatial distribution of β-1,3-glucanases produced by P. italicum after 24 h of incubation in a low-glucose medium.*

affinity for the homologous cell walls. β-1,3-Glucanase I seemed to have a very low affinity because it neither bound to the cell walls nor was capable of releasing reducing sugar groups from them in vitro. The production of β-1,3-glucanase I therefore must be directed to the degradation of extracellular glucan.

On the other hand, the fact that β-1,3-glucanase II and in particular β-1,3-glucanase III were capable of binding to the cell walls and hydrolyzing some of their components in vitro is indicative of a higher affinity for this structure. The existence among β-1,3-glucanases of differences in affinity for natural substrates has been detected in some cases; some of the β-1,3-glucanases produced by *Bacillus circulans* WL-12 (16, 17) are lytic for yeast cell walls, whereas others are not. The interpretation of these differences is problematical because cell wall binding of lytic enzymes in vitro as well as in vivo might be affected not only by the different molecular arrangements of the substrates but also by other cell wall components; the teichoic acid content of the cell walls of gram-positive bacteria, for example, seems to affect the binding of peptidoglycan-hydrolyzing enzymes (7).

In any case, it seems clear from the evidence reported above that derepression of β-1,3-glucanases in *P. italicum* was associated with an intense degree of autolysis of isolated cell walls together with the in vivo mobilization of a significant proportion of the cell wall β-1,3-glucan. This indicated that a correlation existed between the two phenomena and suggested that, when the carbon source concentration was very low or exhausted, part of the cell wall glucan could be mobilized for the purpose of supplying energy. A similar event occurs with α-1,3-glucan in *Aspergillus nidulans*; activation of α-1,3-glucanase in glucose-starved mycelium is probably directed to the mobilization of α-1,3-glucan to provide the energy needed for the morphogenetic events involved in cleistothecium formation (22). Although no formation of sexual structures has been reported to occur in an imperfect fungus such as *P. italicum*, important biochemical events also take place in this species when glucose repression ceases, and the formation of conidia is one of its consequences. We have repeatedly pointed out that no role can be unequivocally attributed to β-1,3-glucanases. The mobilization of cell wall glucan for the purpose of supplying energy could be one role, in addition to their participation in wall growth and extension, as it has been hypothesized previously (2).

The diversity of the mechanisms of action and the properties of β-1,3-glucanases increases the problem of interpreting their exact role. This diversity may even be extended to their regulation by glucose. Recently, we have found that the glucose repression of β-1,3-glucanases which occurs in *P. italicum* and *N. crassa* does not hold for *Trichoderma viride* and *Saccharomyces cerevisiae*, in which synthesis of β-1,3-glucanase only takes place under conditions of active growth in the presence of excess of glucose (F. del Rey, I. García Acha, and C. Nombela, J. Gen. Microbiol., in press).

ACKNOWLEDGMENTS

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