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Regulation of the β-1,3-Glucanase System in *Penicillium italicum*: Glucose Repression of the Various Enzymes

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The microscopic fungus *Penicillium italicum* when grown in a synthetic liquid medium produced at least three enzymes with β-1,3-glucanase activity which were separated by diethylaminoethyl-Sephadex column chromatography. These were named β-1,3-glucanases I, II, and III respective to their order of elution from the column. A tentative characterization of these three enzymes indicated that they have different modes of action; the first one is an endoglucanase, the second is an exoglucanase, and the third probably has both mechanisms of action. Glucose had a repressive effect on all three enzymes. Only small amounts of β-1,3-glucanases II and III were present in the cells when they were actively growing in the presence of this sugar. However, when the cells were transferred to a medium low in glucose, a significant increase in the specific activity of β-1,3-glucanase took place; this was due in part to a much more active production of β-1,3-glucanases II and III and in part to the appearance of β-1,3-glucanase I, which could only be detected after more than 12 h of incubation in this medium. The results are discussed in the context of possible β-1,3-glucanase functions in the fungal cells.

Many species of fungi and yeast produce enzymes capable of hydrolyzing β-1,3-glucans (1, 6, 13, 16, 25). Of particular interest is the fact that these glucans are structural components of the cell wall of many β-1,3-glucanase producers (9). The combination of column chromatographic analysis with the use of different substrates has enabled workers to separate and identify β-1,3-glucanases with different modes of action. Laminarinases, which also hydrolyze the synthetic derivative *p*-nitrophenyl-β-D-glucoside (*p*-NPG), are considered to follow an exohydrolytic mechanism of action by attacking the polymer at the ends of the chain (8). Another substrate used is periodate-oxidized laminarin; periodate (11) modified the ends of the polymer and renders the substrate inadequate for those enzymes that follow an exohydrolytic mechanism. Periodate-oxidized laminarin is therefore the substrate employed for the detection of endo-β-1,3-glucanases, enzymes that act on intermediate points of the polysaccharide chain.

Reports by many workers disclose the existence of a great diversity among the β-1,3-glucanases produced by fungal species. Some enzymes are either exohydrolytic (14, 21) or endohydrolytic (1, 2), whereas others are capable of both types of action (7). Examples exist also of nonspecific glucanases that can degrade both β-1,3 and β-1,6 linkages (8, 26).

Previous work from our laboratory showed that the filamentous fungus *Penicillium italicum* produced a constitutive level of β-1,3-glucanase during active growth. This level was maintained at a minimum as long as glucose was present in the medium; however, the lack of a metabolizable carbon source, which is accompanied by slow growth, led to a significant increase in the specific activity of β-1,3-glucanase (23). In this communication we present evidence of the existence, in *P. italicum*, of at least three enzymes with β-1,3-glucanase activity. Two of them are produced when active growth takes place (logarithmic phase). A significant increase in the production of these two enzymes and the appearance of a new β-1,3-glucanase, not present in log-phase cells, account for the much higher level of specific activity attained in the absence of glucose.

MATERIALS AND METHODS

Chemicals. Laminarin was purchased from Koch-Light, and *p*-NPG was from Sigma Chemical Co. Periodate-oxidized laminarin was prepared as described by Hay et al. (11). All other reagents were of analytical grade from commercial sources.

Organism, growth conditions, and preparation of extracts. *P. italicum* CECT 2294 was the organism used throughout this study. The fungus was maintained on slants of potato extract medium (5). Conidia were obtained from these cultures and inocu-

lated (to a final concentration of 4×10^6/ml) into 1,000-ml flasks containing 250 ml of modified Czapek-Dox medium (150 mM glucose, 0.2% $NaNO_3$, 0.05% K_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4$). The flasks were incubated at 28°C in an orbital incubator (Gallenkamp), and after 48 h mid-log-phase mycelium was available for harvesting. Log-phase mycelium was either used for the preparation of extracts or transferred to 1,000-ml flasks with 250 ml of another medium of the same salt composition as described above but with a much lower concentration of glucose (10 mM). After an incubation period under the same conditions as used before, the mycelium was harvested and cell extracts were prepared.

The operations for counting the conidia, harvesting the mycelium, and preparation of the extracts, by mechanical breakage in a Braun homogenizer, were as described previously (23). The extracts, prepared in 50 mM acetate buffer (pH 5.5), were centrifuged in a J-21B Beckman centrifuge with a JA-20 fixed-angle rotor for 30 min, and the supernatant fluids were dialyzed against 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.2) for 24 h. The dialyzed solutions were chromatographed on a diethylaminoethyl (DEAE)-Sephadex column.

Column chromatography. The enzyme solutions were applied to a DEAE-Sephadex A-50 column (18 by 2 cm; bed volume, 45 ml) previously equilibrated with 50 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.2. The column was washed with the same buffer until no UV-absorbing material eluted and was developed with a linear salt gradient (0 to 1 M NaCl) in the same buffer. The flow rate was 18 ml/h, and the fractions collected were 5 ml. Sephadex G-100 gel filtration was performed on a column (75 by 1.5 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 5.1. After the sample was applied, the column was eluted with the same buffer at a flow rate of 18 ml/h, and 5-ml fractions were collected with an Ultrorac (LKB) fraction collector.

Assays. The general assay for β-1,3-glucanases was based on the release of reducing sugar groups from laminarin (a β-1,3-glucan of plant origin). To characterize the mode of action of the enzyme, two other substrates were used. One was periodate-oxidized laminarin, which is hydrolyzed by endo-β-1,3-glucanases, leading to a release of reducing sugar groups. The other was *p*-NPG, which is hydrolyzed by exo-β-1,3-glucanases; in this case, the assay was based on the release of *p*-nitrophenol from *p*-NPG by the action of the enzyme. Two other substrates, cellobiose and salicin, were eventually used to test for enzyme activity against them.

Assay mixtures contained a total of 0.5 ml and were made from 0.25 ml of the corresponding column fraction as a source of the enzyme, plus 0.25 ml of a solution containing 0.5% of the substrate (laminarin or oxidized laminarin) in 50 mM acetate buffer, pH 5.5. The mixtures were incubated at 37°C for 120 min, and the reducing power released was determined by the method of Somogyi (24) and Nelson (20). The addition of these reagents stopped the enzymatic reaction. The results were compared with glucose as a standard; one unit of activity was defined as the amount of enzyme that catalyzed the release of reducing sugar groups equivalent to 1 nmol of glucose per min under the conditions of the reaction. For the assay with *p*-NPG, cellobiose, or salicin as the substrate, the conditions were the same except for the length of the incubation, which was 60 min. When *p*-NPG was the substrate, the reaction was stopped by the addition of 4.5 ml of 4% Na_2CO_3, and the amount of *p*-nitrophenol released was determined spectrophotometrically by measuring optical density at 410 nm. In this case, one unit of enzyme catalyzed the release of 1 nmol of *p*-nitrophenol per min under the conditions of the reaction. When the substrate was cellobiose or salicin, free glucose released by the action of the enzyme was determined with glucose oxidase coupled to peroxidase. Again the enzymatic unit was defined as the amount of enzyme catalyzing the release of 1 nmol of glucose per min. Protein determinations were carried out by the method of Lowry et al. (18).

RESULTS

β-1,3-Glucanase activity was present in *P. italicum* mycelium under conditions of active growth in the presence of glucose. However, when log-phase mycelium was transferred to a medium low in glucose (10 mM), derepression took place, and the specific activity of the enzyme increased significantly (up to ca. sixfold) after incubation for 24 h (23). The percent activity for oxidized laminarin also increased when the mycelium was transferred to the medium low in glucose (Fig. 1). In the log-phase mycelium, activity for oxidized laminarin amounted to 10% of that for laminarin, and no change in this value was observed after a 12-h incubation in the low-glucose medium. However, after 24 h it had increased to 25%. The increase in percent activity for oxidized laminarin, taking place after 12 h incubation under these conditions, was repeatedly observed. Since oxidized laminarin is a substrate that can only be degraded by endo-β-1,3-glucanases, these results suggested that there must be more than one enzyme with β-1,3-glucanase activity and that the proportion of enzymes with endo- and exohydrolytic mechanisms must have been altered under the conditions of carbon source starvation. It also seemed quite feasible, according to these results, that the observed derepression of β-1,3-glucanase was at least partially due to the appearance of a new enzyme, an endo-β-1,3-glucanase, not present in log-phase cells. This hypothesis was confirmed by the evidence shown in Fig. 2. DEAE-Sephadex column chromatography of extracts of log-phase *P. italicum* mycelium revealed two peaks of activity against laminarin eluting at salt concentrations of 0.18 and 0.29 M. No other peak of activity appeared even when the assay mixtures of the column fractions were incubated for 16 h to check for any possible minor peak. Of the two peaks that constituted

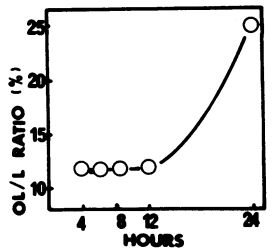

FIG. 1. *Ratio of β-1,3-glucanase activity against two different substrates, oxidized laminarin and laminarin, during incubation of P. italicum mycelium in a medium low in glucose. Actively growing mycelium was harvested and transferred to the low-glucose medium. Samples were taken at the times indicated and used for enzymatic determinations. In this case, the enzymes were assayed in cell extracts. L, laminarin; OL, oxidized laminarin.*

the β-1,3-glucanase system, the one that eluted first was much more intense than the other.

The same situation was observed when extracts of cells that had been incubated for 12 h in the low-glucose medium were chromatographed. Activity for laminarin appeared in two peaks that eluted from the column at essentially the same salt concentrations as described before. In this case, the relative proportions of both peaks were also the same as before although the number of units per 50 mg of protein had increased significantly.

Longer periods of incubation in the medium low in glucose resulted in a different pattern; chromatography of extracts of mycelium incubated for 18 h led to the separation of three peaks of laminarinase activity. A new peak of laminarin-hydrolyzing activity which eluted at 0.10 M salt was clearly apparent and was followed by two others whose relative proportions were the same as those already described for the extracts of log-phase mycelium. These results constitute evidence for the existence in *P. italicum* of at least three proteins that hydrolyze β-1,3-glucans. One of them only appeared in prevailing conditions of glucose starvation, and the synthesis of the other two, which were present in log-phase mycelium, was also stimulated by the lack of the carbon source. This stimulation took place in such a manner that the relative proportions of these two enzymes were maintained. For convenience we shall name these three enzymes β-1,3-glucanases I, II, and III, respectively, in order of their elution from the column.

Figure 3 offers a tentative characterization of β-1,3-glucanases II and III present in log-phase mycelium by assaying them against different substrates. The peak corresponding to β-1,3-glucanase II coincided with an intense peak of *p*-NPG-hydrolyzing activity and with a much less intense one of activity when assayed against oxidized laminarin. This indicated that β-1,3-glucanase II was probably an enzyme with a predominantly exohydrolytic mechanism of action. On the other hand, the fractions corre-

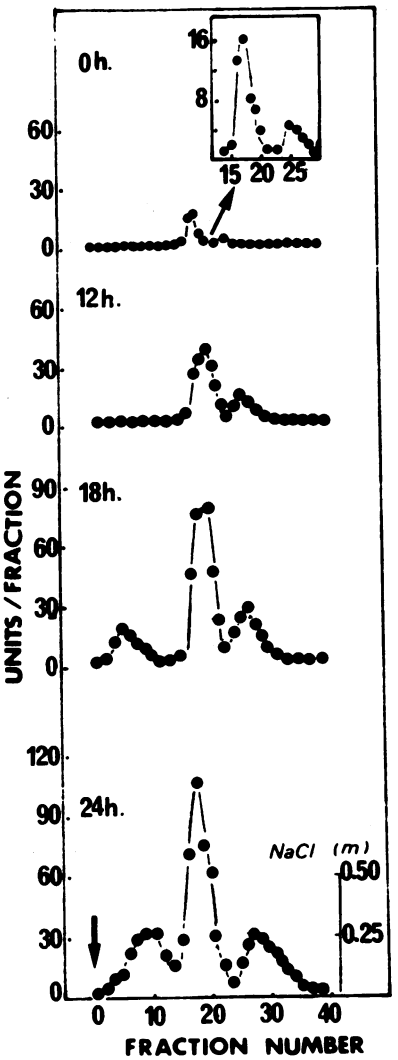

FIG. 2. *Analysis by DEAE-Sephadex column chromatography of β-1,3-glucanases present in P. italicum mycelium after different periods of incubation in a medium low in glucose. The amount of protein applied to the column in each case was as follows: 31.7 mg of extract of log-phase mycelium (0 h), 57 mg of extract of mycelium incubated for 12 h, 75 mg of extract of mycelium incubated for 18 h, and 35.5 mg of extract of mycelium incubated for 24 h. Values correspond to units per fraction if 50 mg of protein had been applied to the column in each case. Insert, Expanded-scale representation of the two peaks of activity present in extracts of log-phase mycelium.*

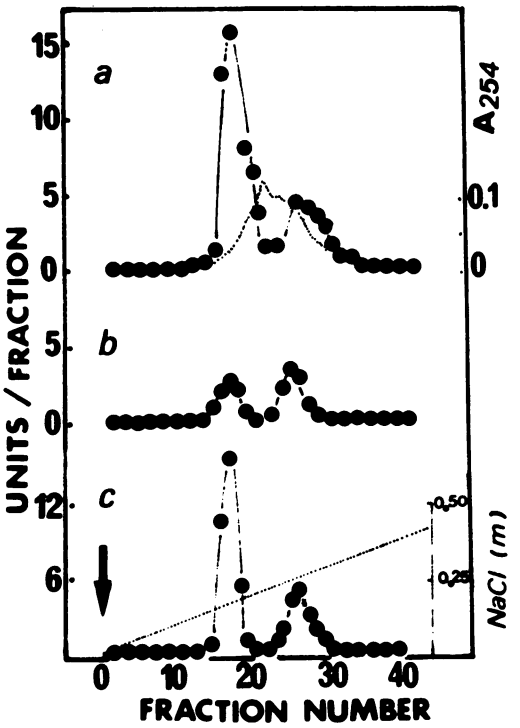

FIG. 3. *Characterization of β-1,3-glucanases present in extracts of log-phase P. italicum mycelium. Protein (37.1 mg) of extract of log-phase mycelium was applied to a DEAE-Sephadex column. (a) Activity against laminarin. (b) Activity against oxidized laminarin. (c) Activity against p-NPG. Values are in units per fraction. Symbol: ●, enzyme units. A_{254}, Absorbance at 254 nm.*

sponding to β-1,3-glucanase III also had activity when assayed against both *p*-NPG and oxidized laminarin, and, in this case, the intensity of the action against the three substrates was essentially the same. β-1,3-glucanase III therefore appeared not to be very specific, acting both as an endo- and an exohydrolase. The same analysis was carried out for β-1,3-glucanases present in extracts of glucose-starved mycelium (Fig. 4). The first one eluting from the column, referred to as β-1,3-glucanase I, appeared in an area totally devoid of any activity when assayed against P-NPG. Thus, β-1,3-glucanase I seemed to be an endoglucanase.

Although many of the exo-β-1,3-glucanases that have been studied are active against *p*-NPG (8, 25), the possibility that in the present case this action was due to β-glucosidases, eluting with β-1,3-glucanase II and/or III, could not be excluded. To clarify this, new portions of extracts of glucose-starved mycelium were chromatographed on the DEAE-Sephadex column and, after the three peaks of β-1,3-glucanase were located, the fractions were assayed against two other substrates, cellobiose and salicin. Only the fractions corresponding to the third peak (β-1,3-glucanase III) hydrolyzed these two substrates, the other two peaks being free from any activity against them (data not shown). The fractions of this third peak were pooled, concentrated, and filtered through a Sephadex G-100 column (Fig. 5). The fractions of the peak with laminarinase activity were again capable of hydrolyzing both oxidized laminarin and *p*-NPG, but this peak was also clearly separated from the peak of activity against cellobiose. These results clearly indicate that the second and third peaks of Fig. 4 correspond to β-glucanases active against *p*-NPG but are devoid of any activity against cellobiose or salicin.

Finally, Fig. 6 shows the kinetics of production of β-1,3-glucanases by *P. italicum* during the 24-h period of glucose starvation. These conditions stimulated active production of the three enzymes, and the specific activity (units in the fractions of the peaks divided by the amount of protein applied to the column) increased significantly. The specific activity of β-1,3-glucanases II and III increased linearly and in parallel dur-

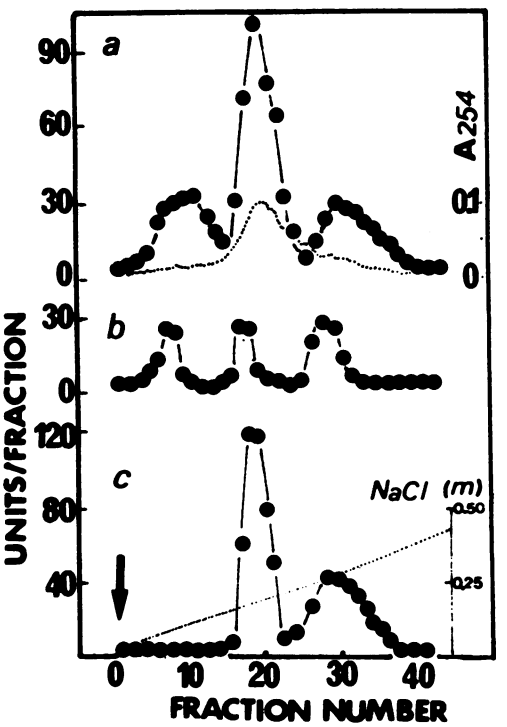

FIG. 4. *Characterization of β-1,3-glucanases present in extracts of P. italicum after 24 h of incubation in a medium low in glucose. Protein (35.5 mg) was applied to a DEAE-Sephadex column. (a, b, and c) As in Fig. 3. A_{254}, Absorbance at 254 nm.*

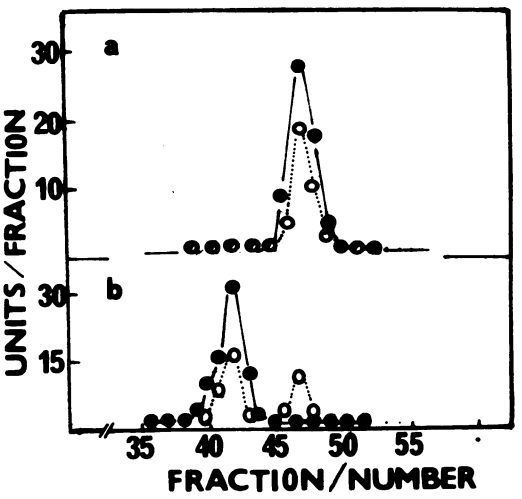

FIG. 5. *Sephadex G-100 gel filtration of β-1,3-glucanase III from a DEAE-Sephadex column. The fractions corresponding to β-1,3-glucanase III from a column run exactly as that of Fig. 4 were pooled, concentrated to 2 ml, dialyzed against 50 mM acetate buffer (pH 5.1), and applied to the Sephadex G-100 column. (a) ●, Activity against laminarin; ○, activity against oxidized laminarin. (b) ●, Activity against cellobiose; ○, activity against p-NGP.*

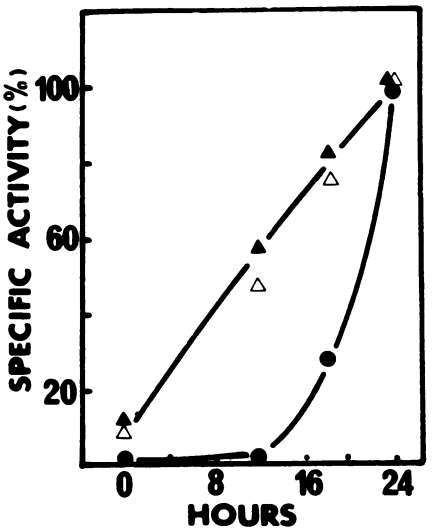

FIG. 6. *Kinetics of the production of different β-1,3-glucanases by P. italicum incubated in a medium low in glucose. The enzymes were separated by DEAE-Sephadex column chromatography as described in the text. Values express the number of units in the fractions corresponding to each peak of glucanase divided by the amount of protein applied to the column. 100% corresponds to the value after 24 h of incubation in the medium low in glucose. Symbols: ●, β-1,3-glucanase I; ▲, β-1,3-glucanase II; △, β-1,3-glucanase III.*

ing the whole period, whereas that of β-1,3-glucanase I, undetected in the first 12 h, increased in a manner suggesting a faster rate of synthesis.

DISCUSSION

Almost no data exist in the literature about the regulation of production of β-1,3-glucanases, enzymes that are widely distributed in nature and that are produced by many bacterial and fungal species (27). We believe that such information will be useful in understanding of the role of these enzymes, especially in the case of the cells that produce them and have, at the same time, β-1,3-glucans as structural components of their cell wall. It has been hypothesized that β-1,3-glucanases and other cell wall lytic enzymes play a role in vitally important proccesses for fungal growth and morphogenesis such as daughter cell separation, bud emergence, conjugation, and cell wall expansion (3, 4, 10, 15, 19). However, the evidence in favor of this hypothesis is at best only indirect and, to our knowledge, no data have been published that would unequivocally attribute a role in the fungal cell to any of the described β-1,3-glucanases. The feasibility of isolating mutants with an altered content of these enzymes is being tested to contribute to clarification of their roles.

β-1,3-Glucanases are commonly assayed with a substrate such as laminarin, which is simple and readily available. Our results, based on DEAE-Sephadex column chromatography, disclose the existence, in *P. italicum*, of at least three enzymes capable of hydrolyzing laminarin which we refer to as β-1,3-glucanases I, II, and III. Only the last two were present in actively growing cells. The significant increase in the specific activity which took place when these cells were transferred to a medium low in glucose was due in part to a more active production of β-1,3-glucanases II and III and in part to the production of β-1,3-glucanase I, which could only be detected after more than 12 h of incubation in the medium low in glucose. From these results, we can conclude that glucose exerts a partial repression of the production of β-1,3-glucanases II and III, maintaining their level at a low value during active growth. They also suggest that a derepression of both enzymes in the absence of glucose takes place in a coordinated manner, since the specific activity of both enzymes increased linearly and in parallel and their relative proportions were maintained. It is also clear that glucose fully represses the production of β-1,3-glucanase I, whose pattern of derepression in the absence of sugar seems to be different from those of the other two; it started after 12 h in the medium low in glucose, and its specific activity increased at a faster rate.

A tentative characterization of these three enzymes indicates that they have different modes of action. β-1,3-Glucanase I is probably an endoglucanase, β-1,3-glucanase II is a predominantly exohydrolytic enzyme, and β-1,3-glucanase III probably has both mechanisms of action. The existence of β-1,3-glucanases with different modes of action is well known; endo-β-1,3-glucanases (1, 2), exo-β-1,3-glucanases (14, 21), and endo-exo-β1,3-glucanases (7) are produced by fungi, and the production of more than one of these enzymes by a given species is quite common. This is not surprising, since the cooperative action of more than one glucanase may be needed to exert a lytic effect on the cell wall (16). The diversity among cell wall lytic enzymes might also indicate that evolution tended to confer on them a certain degree of specificity for the cell wall of the organism that produces them. Evidence in favor of this specificity for homologous cell walls has been obtained in the case of *N*-acetylmuramic acid L-alanine amidase, a bacterial wall lytic enzyme (12). This amidase, isolated from *Bacillus subtilis* W-23, binds poorly to cell walls of *B. subtilis* ATCC 6051, whereas it binds very tightly to homologous cell walls. These differences in affinity seem to be due, to a large extent, to differences in peptidoglycan arrangement; moreover, a comparison between amidases isolated from both strains showed that the two proteins, which are of the same size and cleave the same bond, do not cross-react immunologically and are therefore not closely related in structure (17). Although no such complete data have been obtained for glucanases, it is interesting that, among the five β-1,3-glucanases produced by *B. circulans* WL-12, some are lytic for yeast cell walls and at least one is not, indicating the existence of a certain degree of specificity among β-1,3-glucanases (22).

Our results seem to imply that the different β-1,3-glucanases produced by *P. italicum* can serve specific functions in the cell. If the theory postulating the involvement of lytic enzymes in growth and extension of the cell wall is correct (3), that role should be accomplished by β-1,3-glucanases II and III, which are produced by actively growing mycelium. On the other hand, the activation of β-1,3-glucanase I must be directed towards a different function, since it takes place in the absence of glucose when the fungus is not growing. Among other possibilities, one can think of a mobilization of reserve or wall glucan for catabolic purposes or a participation of the enzymes in a morphogenetic change such as conidiation. Studies are now in progress to further purify and characterize *P. italicum* β-1,3-glucanases.

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