Production and Catabolite Repression of *Penicillium italicum* β-Glucanases

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The filamentous fungus *Penicillium italicum*, grown in a defined liquid medium, produced β-1,3-glucanase, which remained essentially bound to the cells, and β-1,6-glucanase, an essentially extracellular enzyme. When glucose was depleted from the medium, when a limited concentration of glucose (0.2%) was maintained, or when the carbon source was galactose (3%) or lactose (3%), a significant increase in the specific activity of β-1,3-glucanase, in cell extracts, took place. This was paralleled by a very slow rate of growth, and under glucose limitation, the appearance of β-1,3-glucanase in the medium was also observed. On the other hand, when an excess of glucose, fructose, or sucrose was present, the specific activity remained constant and active growth was promoted. Laminarin, cellobiose, gentiobiose, and isolated *Penicillium italicum* walls were not capable of significantly inducing β-1,3-glucanase synthesis to a level beyond that attained by glucose limitation. A similar behavior was observed for β-1,6-glucanase. β-1,3-Glucanase and β-1,6-glucanase are therefore constitutive enzymes subjected to catabolite repression. The results are discussed in the context of the possible functions that have been suggested for glucanases and related enzymes.

Enzyme activities capable of hydrolyzing β-1,3- and β-1,6-linked glucans are present in the supernatant fluids from cultures of certain bacterial strains that are used to digest the cell walls of yeast and filamentous fungi to obtain protoplasts (24). β-1,3-glucanase and β-1,6-glucanase, purified from fungal wall lytic preparations, have also been shown to hydrolyze components of the fungal wall (14, 18, 19). The synthesis of β-1,3-glucanase by a thermophilic species of *Streptomyces* is semiconstitutive and subject to catabolite repression by metabolizable carbon substrates (10).

On the other hand, it has been found that β-glucanases are also produced by fungal cells, and this adds new interest to these enzymes. β-1,3-glucanase occurs in filamentous fungi as well as in yeast (16, 25); the enzyme is secreted into the culture medium and/or is located in the periplasmic space between the cell wall and the plasma membrane (7, 23). *Saccharomyces cerevisiae* protoplasts, under regeneration conditions, are also capable of secreting β-1,3-glucanase into the medium (2). β-1,6-glucanase is secreted by many species of filamentous fungi (17) and in the case of yeast, the existence of nonspecific glucanases capable of degrading both β-1,3 and β-1,6 linkages has been reported (7, 23).

Earlier workers suggested that extracellular β-1,3- and β-1,6-glucanase are produced constitutively by fungi (16, 4). However, very little is known about the factors that regulate the synthesis of these enzymes by filamentous fungi. This report concerns the production of β-1,3-glucanase (β-1,3-glucan-3-glucanohydrolase, EC 3.2.1.6) and β-1,6-glucanase (EC 3.2.1.75, endo-β-1,6-glucanase) by *Penicillium italicum* in a defined medium and the effect that different carbohydrates, used as carbon sources, exert on that production. A certain amount of β-1,3-glucanase is produced constitutively; carbon source conditions that determine a slow rate of growth favor a significant derepression of the enzyme, whereas this derepression is prevented by the presence of carbon sources that can support active growth. A similar pattern is observed with β-1,6-glucanase.

MATERIALS AND METHODS

Chemicals. Laminarin was purchased from Koch-Ligh Laboratories, Colnbrook-Bucks, England. Gentiobiose and Cellobiose were from Sigma Chemical Co., St. Louis, Mo. Pustulan was obtained from *Umbilicaria pustulata* (11), and *P. italicum* cell walls were prepared by the method of Mahadevan and Tatum (13). Glucose oxidase and peroxidase were from Sigma.

Organism, growth conditions, and preparation of the extracts. *P. italicum* CECT 2294 was maintained on slants of potato extract agar medium (3). Conidia were harvested by the addition of sterile distilled water, and the conidial suspension was filtered through glass wool. The number of conidia was determined by visual inspection and by counting in a phase-contrast microscope (Zeiss) using a hemocytometer slide (THOMA, E. Hartnack, Germany), and this suspension was used for inoculation. The concentration of conidia inoculated in each case will be indicated. Liquid cultures were grown in modified Czapek-Dox medium containing 3% glucose, 0.2% $NaNO_3$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$ (Czapek-glucose medium).

All the operations for harvesting the mycelium and preparing the extracts were done between 0 and 4°C. The mycelium was harvested by filtration through Whatman no. 3 filter paper, washed three times with 50 mM acetate buffer, pH 5.1, and finally suspended in this buffer in a proportion of 0.5 g (wet weight) of mycelium to 5 ml of buffer. Unless otherwise indicated, cell-free extracts were obtained by mechanical breakage of the cells using a Braun homogenizer and Ballotini glass beads (0.2-mm diameter). Approximately 5 g of Ballotini beads was added to the suspension consisting of 5 ml of buffer and 0.5 g (wet weight) of mycelium. The mixture was homogenized for 1 min under refrigeration with dry ice, and the glass beads were decanted. The enzyme assays were performed either in the crude extracts or in the supernatant fluids obtained by the centrifugation of the extracts at 1,800 × *g* for 10 min.

Standard induction procedure. A standard induction system consisting of a two-step culture was used to study the effect of different carbon sources on the synthesis of β-glucanases. Conidia were first inoculated (to a final concentration of 4 × 10^6/ml) into a 1,000-ml flask containing 250 ml of Czapek-glucose medium. The culture was incubated at 28°C in a orbital incubator (Gallenkamp). After 48 h, when active growth was taking place, the mycelium was collected by filtration and treated with 0.1 M HCl for 5 min. This treatment destroys wall-bound enzymes, which might affect possible inducers, without affecting the viability of the cells (6). The treated mycelium was washed and suspended in several flasks containing fresh media. These media had the same composition as the previous one with regards to salts; however, the carbon source could either be different or the same (glucose), although present in a different concentration. The corresponding carbon source and its concentration will be indicated in each case. Approximately 1.5 g (wet weight) of mycelium was suspended in each 250-ml flask containing 65 ml of medium. The cultures were incubated as described above for a period of 22 h and at the indicated times, samples were withdrawn and used for growth and enzyme activity determinations. The concentration of the carbon sources in the media, as will be indicated, was either 3% when an excess was desired or 0.2% when a limited-carbon medium was

intended. During the incubation, more of the carbon source was added to the cultures either to maintain the excess or to avoid lysis of the mycelium that would take place, in the carbon-limited medium, upon complete exhaustion of the carbon source. After 8 h of incubation, 2% of the corresponding carbon source was routinely added to the medium with excess, and 0.2% was added to the limited-carbon medium.

Determinations. Under the conditions described above, *P. italicum* grows as a homogeneous suspension of mycelium in the culture medium rather than forming aggregates of cells. This makes it possible to estimate the total cell mass by measuring the dry weight of cells in aliquots of a known volume taken from the culture. Therefore, to measure growth, aliquots were taken from the culture and filtered through Whatman filter paper to separate the mycelium, and the filters were oven-dried at 80°C until a constant weight was attained.

Enzyme activities were assayed in the cell-free extracts, in their 1,800 × *g* supernatant fluids or in the culture medium. The assay of β-1,3-glucanase is based on the release of reducing sugar groups from laminarin. Assay mixtures contained a total of 2 ml and were made from 1 ml of the enzyme solution in 50 mM acetate buffer, pH 5.1, plus 1 ml of a solution of 0.5% laminarin in the same buffer (laminarin was dissolved by warming in a boiling-water bath for 1 min). Mixtures were incubated for 30 min at 37°C and the reaction was stopped by transferring the tubes to a bath of boiling water. After 3 min in the bath of boiling water, the protein was precipitated and ready to be removed by centrifugation. A 1-ml portion of the clear supernatant was taken and used for the determination of the reducing power released by the action of the enzyme by the method of Somogyi (21) and Nelson (15). The results were referred to glucose as a standard; two blanks were discounted in each case, one that had no substrate, to correct for the reducing power released by the enzyme preparation in the absence of substrate, and another one that had no enzyme and that corrected for the non-enzymatic hydrolysis of the substrate.

The procedure for assaying β-1,6-glucanase was identical to the one used for β-1,3-glucanase, except that pustulan was substituted for laminarin as the substrate of the reaction. One unit of activity is defined as the amount of enzyme that catalyzes the release of reducing sugar groups equivalent to 1 nmol of glucose per min under the conditions of the reaction. Glucose in the culture medium was determined with glucose oxidase coupled to peroxidase, and protein determination was done by the method of Lowry et al. (12), using bovine albumin as a standard.

Sephadex gel filtration. Sephadex G-100 gel filtration was performed on a column (45 by 1.5) previously equilibrated with 50 mM sodium acetate buffer, pH 5.1. The sample, 1 ml of 1,800 × *g* supernatant fluid of a *P. italicum* extract, was applied to the column and eluted with the same buffer at a flow rate of 18 ml/h. Fractions of 5 ml were collected using a fraction collector (Ultrorac, LKB), and the

absorbance at 280 nm was determined in a spectrophotometer (Beckman).

RESULTS

Production and catabolite repression of β-glucanases. It is apparent from Fig. 1 that *P. italicum*, grown in a defined liquid medium, produces β-1,3- and β-1,6-glucanase. These two activities were separated by Sephadex G-100 gel filtration, as shown in Fig. 2; the heavier peak corresponded to β-1,3-glucanase, whereas β-1,6-glucanase appeared in a lighter one. We conclude from this result that the two activities are due to two different enzymes. A number of other characteristics of the production of β-glucanases by *P. italicum* are shown in Fig. 1 and in Table 1. Most of the β-1,3-glucanase that was produced remained bound to the cells; only at the maintenance (stationary) phase of growth could significant amounts of this enzyme be detected in the culture medium. In contrast, β-1,6-glucanase was essentially an extracellular enzyme; although it could be assayed in cell extracts and their 1,800 × *g* supernatant fluids (Fig. 1b), the majority of the enzyme that was produced appeared in the culture medium (Fig. 1c). It should be further noted from Fig. 1 that the level of specific activity of both enzymes in extracts and the amount of β-1,6-glucanase in the culture medium increased significantly during the maintenance phase after the carbon source (glucose) had been exhausted. The drop in the amount of extracellular β-1,6-glucanase that took place between 120 and 144 h must be due to the rise in the pH that occurred at the same time (Fig. 1a), which caused the inactivation of this enzyme (Larriba and Villanueva, unpublished data).

Figure 3 shows the results of a similar experiment to that of Fig. 1, although in this case the maintenance phase was reached in a shorter time, due to the heavier inoculum that was used. Note that the increase in the specific activity that took place at the time of glucose exhaustion could be partially prevented in a parallel culture by supplementing citrate (up to 3%) at that time or fully prevented by the additions of glucose. We interpret these results as meaning that derepression of glucanase synthesis does not take place in the presence of an excess of glucose and that citrate has only a partial effect.

Induction properties of β-glucanases. A standard system was used to determine the effect of different carbon source conditions on the production of β-glucanases. Figure 4 provides further evidence for the role of glucose limitation on β-1,3-glucanase synthesis. When
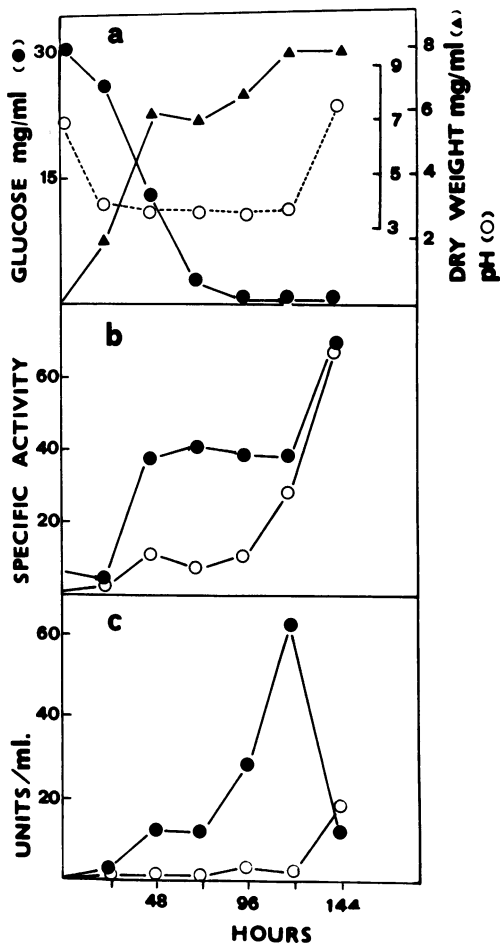

FIG. 1. *Production of β-1,3-glucanase and β-1,6-glucanase by Penicillium italicum grown in liquid medium. Six 250-ml flasks containing 65 ml of Czapek-glucose medium were inoculated with 2.5 × 10⁷ conidia per flask. The flasks were incubated as described under Materials and Methods and at the times indicated, the total content of one flask was used for determinations. Harvesting of the mycelium and preparation of the extracts was carried out as described in Materials and Methods, except for the breaking of the cells, which was done by stirring the mixture of buffer, mycelium and glass beads in a Sorvall Omnimixer for 10 min in an ice bath. (a) Growth, glucose concentration, and pH in the culture medium. (b) Specific activities of β-1,3-glucanase (○) and β-1,6-glucanase (●) in 1,800 × g supernatant fluids of P. italicum extracts. (c) β-1,3-glucanase (○) and β-1,6-glucanase (●) in the culture medium.*

a low concentration of glucose (around 0.2%) was maintained, slow growth was accompanied by a four- to fivefold increase in the specific activity of β-1,3-glucanase. On the other hand, when an excess of glucose, which permitted
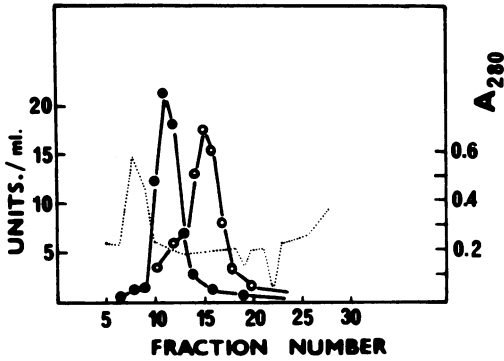
FIG. 2. *Sephadex G-100 gel filtration of a 1,800 × g supernatant fluid from a Penicillium italicum extract. Symbols: β-1,3-glucanase (●), β-1,6-glucanase (○), absorbance at 280 nm (-----).*

TABLE 1. *Distribution of β-glucanases during growth of Penicillium italicum* [a]

Days of growth	β-1,3-Glucanase		β-1,6-Glucanase	
	Cell bound	Culture fluid	Cell bound	Culture fluid
1	5.44	0	3.20	0
3	210	54	326	781
5	365 (70%)	160	331 (8%)	3,802

[a] Conditions as in Fig. 1. Figures express units per flask. Values in parentheses represent percentage of total (cell-bound plus culture fluid activity units).

active growth to take place, was present, the level of specific activity remained constant. These results suggest that during active growth, the amount of β-1,3-glucanase that is produced is only that needed for the specific activity to remain constant and that slow growth promotes a derepression of β-1,3-gluca canase synthesis, so that the specific activity increases to a significant extent.

The effect of other carbon sources on the synthesis of β-1,3-glucanase was examined in the experiments shown in Fig. 5. Sucrose and fructose at concentrations of 3% acted as effectively as glucose in preventing any increase in specific activity. However, in the presence of 3% lactose or galactose, a sixfold increase in the specific activity was again observed. As in the previous experiments, active growth was promoted in the presence of sucrose and fructose, which acted as repressors, whereas slow growth accompanied the induction of enzyme synthesis when galactose or lactose was used as the carbon source (data not shown).

The standard induction system was also employed to study the possibility of the existence of positive inducers, that is, substances capable

of inducing β-1,3-glucanase synthesis to a level beyong that which is attained in a glucose-limited medium. In the case of a thermophilic *Streptomyces*, gentiobiose, a molecule
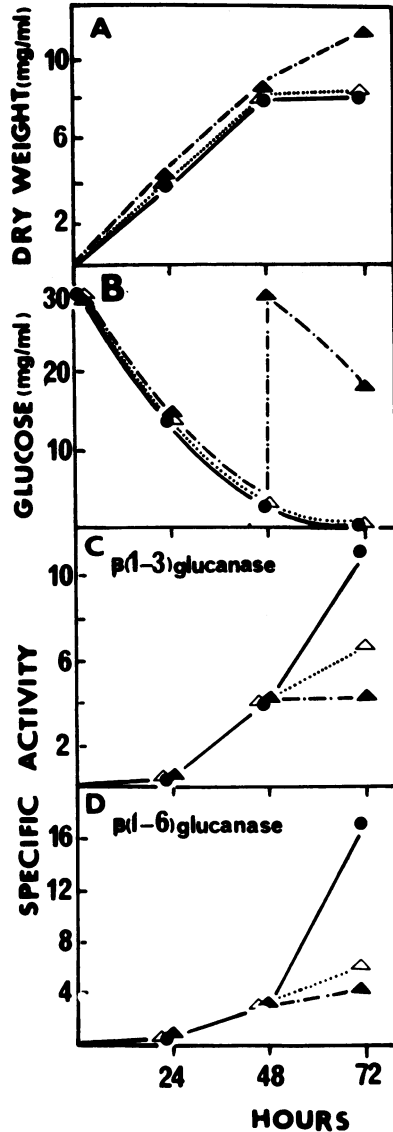

FIG. 3. *Catabolite repression of β-1,3-glucanase and β-1,6-glucanase. Penicillium italicum conidia were inoculated, up to a concentration of 4 × 10⁶/ml, into three 6,000-ml flasks containing 1,500 ml of Czapek-glucose medium. The cultures were incubated in parallel at 28°C with shaking, and at the time of glucose exhaustion one of the flasks was supplemented with glucose (up to 3%) (▲), another received citrate (up to 3%) (△), and nothing was added to the third one (●). Determinations were done in aliquots withdrawn at the indicated times. Crude extracts were used for enzyme assays.*
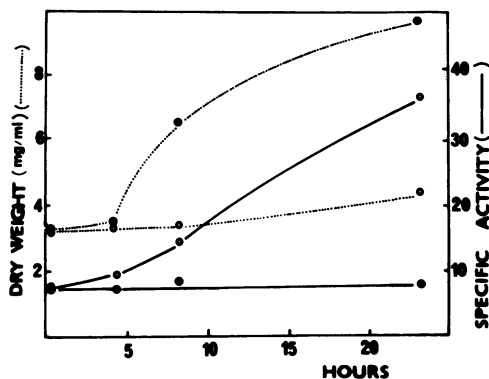
FIG. 4. *Growth and production of β-1,3-glucanase by Penicillium italicum under standard induction conditions. Symbols: 3% glucose in the medium (●), 0.2% glucose in the medium (○).*
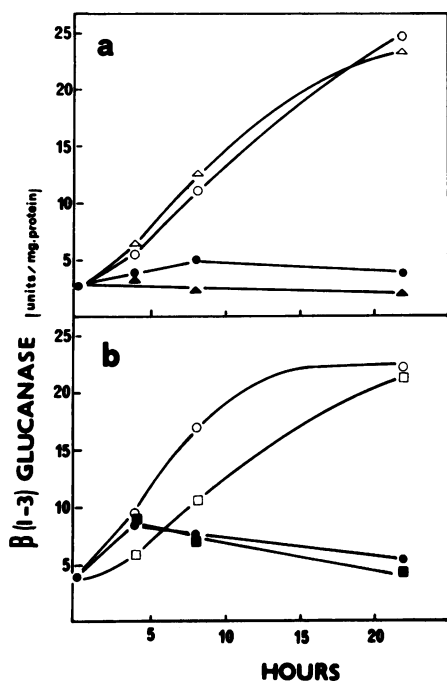

FIG. 5. *Production of β-1,3-glucanase by Penicillium italicum, under standard induction conditions in the presence of different carbon sources. (a) and (b) Results obtained in two different experiments. Symbols: 3% glucose (●), 0.2% glucose (○), 3% sucrose (■), 3% fructose (▲), 3% galactose (□), and 3% lactose (△).*

structurally unlike the substrate, has been shown to act as an inducer of this enzyme (10); and cellobiose, laminaribiose, and gentiobiose induce the synthesis of an aryl-β-glucosidase in *Neurospora crassa* (6). The effect of cellobiose, gentiobiose, laminarin, and *P. italicum* cell

walls on the production of β-1,3-glucanase is shown in Fig. 6. In all cases, no significant induction was observed beyond that which is caused by a glucose-limited medium. Therefore, none of these substances acts as a positive inducer of β-1,3-glucanase.

Finally, Fig. 7 shows the production of extracellular β-1,3-glucanase and β-1,6-glucanase. Extracellular β-1,3-glucanase was detected in the glucose-limited medium, and it could not be found when an excess of glucose is present. Extracellular β-1,6-glucanase was actively synthesized in the presence of a low concentration of glucose, cellobiose, and gentiobiose, as is shown by a linear increase in the amount of enzyme per milligram of cells (dry weight). The production of this last enzyme, although repressed in the first 8 h, was significant between 8 and 22 h, reaching a level that was half of that of the glucose-limited medium. Excess of glucose was also a repressor of the production of extracellular β-1,6-glucanase, although the concentration required in this case is probably higher than that needed for repression of β-1,3-glucanase, and this allows some production of
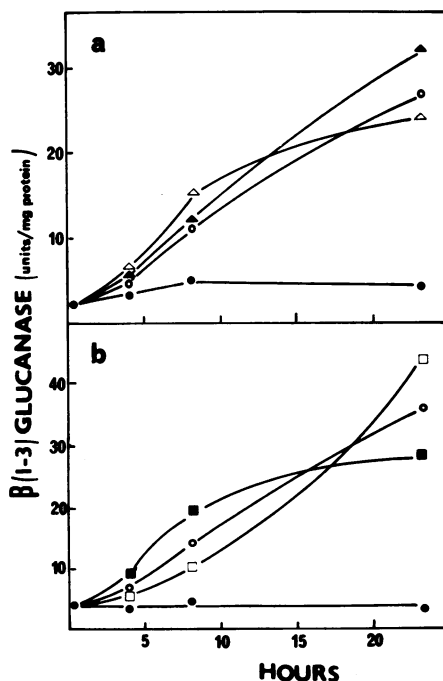

FIG. 6. *Production of β-1,3-glucanase by Penicillium italicum under standard induction conditions in the presence of different carbon sources. (a) and (b) Results of two different experiments. Symbols: 3% glucose (●), 0.2% glucose (○), 0.2% cellobiose (▲), 0.2% gentiobiose (△), 0.2% laminarin (□), and 0.2% P. italicum cell walls (■).*
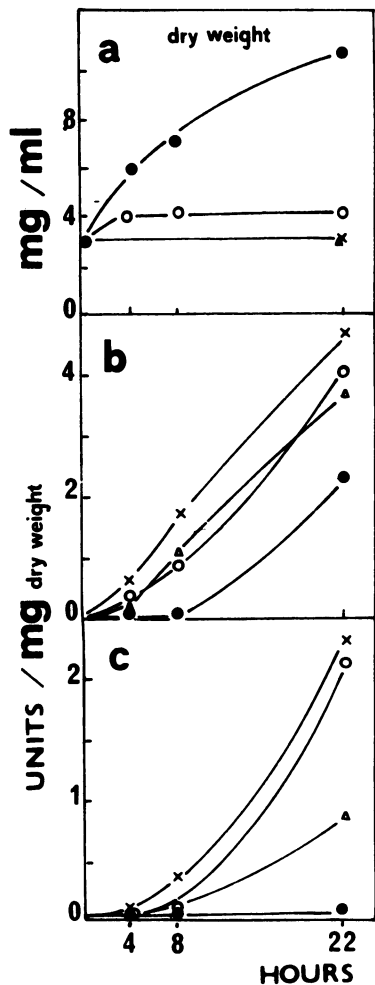
FIG. 7. *Growth and production of extracellular β-glucanases under standard induction conditions. Enzymes were assayed in aliquots of the culture fluid dialyzed versus 50 mM acetate, pH 5.1. (b) β-1,6-Glucanase; (c) β-1,3-glucanase. Symbols: 3% glucose (●), 0.2% glucose (○), 0.2% cellobiose (×), and 0.2% gentiobiose (△).*

the enzyme at the end of the induction period when part of the glucose has been used.

DISCUSSION

Like many other species of fungi and yeast (16, 17, 23, 25), *P. italicum* produces β-1,3-glucanase and β-1,6-glucanase. The former remains bound to the cell, whereas the latter is essentially an extracellular enzyme. The carbon source exerts a similar effect on the production of both enzymes in a defined medium. A certain amount of β-glucanase is produced constitutively; however, a significant increase in

the level of specific activity detected in extracts follows glucose exhaustion or accompanies the carbon source conditions that determine slow growth. Glucose limitation and slow growth are also paralleled by the appearance of extracellular β-1,3-glucanase and by a higher production of extracellular β-1,6-glucanase. These results are consistent with the notion that a derepression of glucanase synthesis takes place, leading to a selective increase in the rate of synthesis of β-glucanases and therefore in the level of specific activity. Excess of glucose or other easily metabolizable carbon sources prevents derepression and under those conditions, only enough enzyme is synthesized for the specific activity to remain constant. This behavior is typical of a number of carbohydrases, produced by bacteria and fungi, which are subjected to catabolite repression and whose synthesis is stimulated under conditions that favor a less than maximal rate of growth. Examples of these are β-1,3-glucanase of a thermophilic *Streptomyces* (10), β-1,3-glucanase of the basidiomycete QM 806 (8), and cellulase of *Myrothecium verrucaria* (9). Studies are under way to clarify the mechanism of derepression of β-glucanases with the use of ribonucleic acid and protein synthesis inhibitors.

Of more general interest is the question of the function or functions that β-1,3-glucanase and β-1,6-glucanase might serve in the cell that produces them. Morphogenetic changes in some filamentous fungi take place under carbon limitation and are accompanied by a sharp increase in several degradative enzymes. For example, cleistothecium formation in *Aspergillus nidulans* starts when glucose is depleted from the medium, and α-1,3-glucanase seems to play a role in this morphogenetic event by hydrolyzing reserve α-1,3-glucan and supplying the energy needed for the process (26, 27). Aryl-β-glucosidase of *N. crassa* is another example of an enzyme whose formation parallels conidiation (6). Our results are compatible with a metabolic role for β-1,3-glucanase and β-1,6-glucanase of *P. italicum*; these enzymes might supply energy from reserve or extracellular β-glucans (5) under carbon limitation. The derepression of β-1,3-glucanase and β-1,6-glucanase in *P. italicum* could be coupled to conidia formation.

The hypothesis that postulates a participation of lytic enzymes in growth and extension of the cell wall is also very well known (20). According to this hypothesis, β-glucanase could collaborate in the extension of the cell wall by acting on the structural glucan and could be one of the determinants of its shape. This would be quite feasible, since the cell wall of penicillia

has been shown to contain β-1,3- and β-1,6-glucan (22); moreover, β-1,3-glucanase and β-1,6-glucanase, when partially purified from *P. italicum*, act on the isolated cell walls of this organism, releasing glucose (T. Santos, C. Nombela and J. R. Villanueva, unpublished data). Our results might also be compatible with the notion of a morphogenetic role for β-glucanases, since β-1,3-glucanase and β-1,6-glucanase are produced in significant amounts during active growth.

The major difficulty in determining the function of β-glucanases, in the fungal and yeast cells that produce them, stems from the fact that no mutants with an altered content of these enzymes have so far been isolated. On the other hand, the variety of β-glucanases that is being described could mean that they do not all have the same role. In any case, we believe that research on the regulation of the production of β-glucanases should contribute a great deal to the clarification of this central problem: the role of fungal wall lytic enzymes in the fungal cell.

ACKNOWLEDGMENT

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