JOURNAL OF BACTERIOLOGY, Feb. 1978, p. 542-548
0021-9193/78/0133-0542$02.00/0

Vol. 133, No. 2
Printed in U.S.A.

Regulation of β-1,3-Glucanase Synthesis in *Penicillium italicum*

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Received for publication 19 August 1977

The filamentous fungus *Penicillium italicum* produced a certain level of β-1,3-glucanase during active growth in a glucose-supplemented medium; however, at a low glucose concentration (2 to 10 mM), derepression took place and the specific activity of the enzyme increased significantly. Derepressed cells (incubated in a glucose-limited medium) accumulated a capacity for the synthesis of β-1,3-glucanase, which led to a subsequent increase in the specific activity even when the cells were transferred to a medium with an excess of glucose (180 mM). Two protein synthesis inhibitors, cycloheximide and trichodermin, immediately stopped the increase in specific activity when added to derepressed cells. On the other hand, 8-hydroxyquinoline, an RNA synthesis inhibitor, acted differently, since it permitted the specific activity to increase for some time after being added to derepressed cells. Moreover, the concentration of glucose did not affect the 8-hydroxyquinoline-insensitive synthesis of β-1,3-glucanase. It is concluded that the glucose repression effect on β-1,3-glucanase production must be exerted at a pretranslational level that could be either mRNA synthesis or some stage of the process involved in its maturation or stabilization.

Fungi and yeasts as well as bacteria produce enzymes capable of hydrolyzing components of their own cell walls. Examples of these enzymes are β-1,3- and β-1,6-glucanases, which appear in fungal cells and which are also liberated by the cells into the culture medium. Very little is known about the factors that regulate the synthesis of fungal wall-lytic enzymes or about the role that these enzymes may play in the fungal cell.

In a previous report (17), we analyzed the production of β-1,3-glucanase and β-1,6-glucanase by the filamentous fungus *Penicillium italicum* and showed that it is influenced by catabolite repression. Both enzymes were produced constitutively; an excess of glucose or excesses of other metabolizable carbon sources maintained the level of specific activity at a minimum, whereas, at a low glucose cocentration, this level was significantly elevated, suggesting that derepression of synthesis of the enzyme had taken place. Transcriptional and translational controls have been proposed as the possible mechanisms through which catabolite repressors exert control on enzyme synthesis (13). A transcriptional control seems to operate in the case of the nitrate reductase of *Ustilago maydis* and *Neurospora crassa*, whose production is repressed at the level of mRNA synthesis (7, 18). On the other hand, a translational control has been suggested for the synthesis of polygalacturonase and other degradative enzymes in *Aspergillus nidulans* (19).

With inhibitors of RNA and protein syntheses, we have studied the mechanisms of derepression of β-1,3-glucanase synthesis in *P. italicum*. In this communication, we present evidence that suggests that glucose represses β-1,3-glucanase synthesis at a level before translation.

MATERIALS AND METHODS

Chemicals. Laminarin was from Koch-Light Laboratories (Colnbrook, England), cycloheximide was from Sigma Chemical Co. (St. Louis, Mo.), and 8-hydroxyquinoline was from E. Merck AG (Darmstadt, Germany) (stock solutions of 10 mg/ml in ethanol were prepared). Trichodermin was a gift of W. O. Godtfredsen (Leo Pharmaceutical Products, Ballerup, Denmark); stock solutions of 10 mg/ml in ethanol-water (1:1 [vol/vol]) were used. [*U*-^{14}C]glucose (3 mCi/mmol), [*U*-^{14}C]leucine (150 mCi/mmol), and [*U*-^{14}C]uracil (60 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). Before use, they were diluted with cold material to specific activities of 6.5 μCi/mmol, 10 μCi/mmol, and 114 μCi/mmol, respectively. All other reagents were of analytical grade.

Organism and growth conditions. *P. italicum* CECT 2294 was the organism used throughout this study. The fungus was maintained on slants of potato

extract medium (2), and liquid cultures were grown in modified Czapek-Dox medium containing 180 mM glucose, 0.2% $NaNO_3$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and 0.001% $FeSO_4$ (Czapek-glucose medium). Conidia were inoculated (to a final concentration of 4×10^6/ml) into a 1,000-ml flask containing 250 ml of medium, and the culture was incubated in an orbital incubator (Gallenkamp & Co. Ltd., London, England) at 28°C. After 48 h, mid-log-phase mycelium was available for harvesting. All operations for counting the conidia and harvesting the mycelium were as described (17). For the incubation of the cells in all cases, modified Czapek-Dox medium was used; the only modification affected the carbon source (glucose) concentrations, which will be indicated. Many of the experiments involved incubation of the cells in a certain medium and transfer to another medium for further incubation. In all cases, the cells were washed before transfer with portions of the medium to which they were to be transferred.

Effect of glucose or different drugs on the derepression of β-1,3-glucanase synthesis. Under conditions of low glucose concentration, derepression of β-1,3-glucanase synthesis takes place and leads to a significant increase in the level of specific activity of the enzyme (17). For the study of the effect that either glucose or different drugs exert on that derepression, two types of experiments were performed. The first type involved a 24-h incubation; approximately 1.5 g (wet weight) of log-phase cells was added, per flask, to a group of 250-ml flasks with 60 ml of medium, usually containing 10 mM glucose as the only carbon source. During the incubation, a drug was added to the flasks and samples were withdrawn and used for determination of β-1,3-glucanase. The other type of experiment was completed in a much shorter time (a maximum of 6 h). In this case, the experiment began with either log-phase cells or derepressed cells, that is, cells that had been previously incubated in a medium containing 2 mM glucose. This incubation led to an accumulation of the capacity for synthesizing β-1,3-glucanase. Log-phase cells or derepressed cells (approximately 1.5 g, wet weight) were transferred to 250-ml flasks with 60 ml of medium containing 180 or 2 mM glucose or 2 mM glucose plus 150 μg of 8-hydroxyquinoline per ml. The flasks were shaken at 28°C, and samples were withdrawn and used for determination of β-1,3-glucanase.

Determination of protein and RNA syntheses. Protein and RNA syntheses were followed by measuring the incorporation of [^{14}C]leucine and [^{14}C]uracil into trichloroacetic acid-insoluble material. Log-phase cells (300 mg, wet weight) were harvested by filtration, washed with sterile distilled water, and transferred to a 250-ml flask with 50 ml of Czapek-glucose medium containing either [^{14}C]leucine (2 mM) for the determination of protein synthesis or [^{14}C]uracil (0.09 mM) for the measurement of RNA synthesis. The flasks were incubated in an orbital incubator (Gallenkamp), and the radioactivity incorporated into trichloroacetic acid-insoluble material was measured from samples taken at the times indicated. The samples were processed as follows. Five milliliters of ice-cold 20% trichloroacetic acid was added to a 5-ml sample; the mixture was maintained at 0°C for 30 min and finally filtered through a preweighed GF/C (Whatman, Inc., Clifton, N. J.) filter. The filter was washed with 50 ml of ice-cold 5% trichloroacetic acid and dried in an oven at 90°C until a constant weight was attained. The filter was weighed again for the dry weight of the cells, and its radioactivity was counted in a Packard Tri-Carb scintillation counter. Three-milliliter vials with 2 ml of scintillation solution [0.35% 2,5-diphenyloxazole and 5% 1,4-bis-(5-phenyloxazolyl)-benzene in toluene] was used, and the counting efficiency for ^{14}C was calculated to be 87%. Two controls were run, in some cases, to exclude the possibility that the precipitation of radioactively charged tRNA was contributing significantly to the value of the protein being measured. One of the controls consisted of maintaining the sample in 10% trichloroacetic acid for 15 min at 90 instead of 0°C. In the other control, the sample was treated with 1 N KOH for 60 min at room temperature, neutralized with 1 N HCl, and maintained in 10% trichloroacetic acid for 30 min at 0°C. These samples were then washed and processed as above, and no significant differences were observed among the values obtained by these three procedures.

The effects of different drugs on both protein and RNA syntheses were studied by adding the corresponding drugs to the flasks at the times indicated.

Determinations. β-1,3-Glucanase was assayed in cell-free extracts of *P. italicum* obtained by mechanical breakage of the cells in a Braun homogenizer. The assay is based on the release of reducing sugar groups from laminarin (a β-1,3-glucan of plant origin). The conditions for the preparation of the extracts and for the enzymatic assay have been previously described (17). 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. Protein determinations were carried out by the method of Lowry et al. (8). Specific activity was defined as the number of units per milligram of protein.

RESULTS

Effect of glucose on β-1,3-glucanase synthesis. The glucose repression effect on glucanase production is shown in Fig. 1a. When log-phase *P. italicum* mycelium was harvested and incubated in a synthetic medium with an excess of the carbon source (180 mM glucose), the specific activity level of β-1,3-glucanase remained constant and active growth took place. However, the opposite was observed when the glucose concentration in the medium was low (10 mM); in this case, very slow growth was accompanied by a significant increase in the specific activity of the enzyme. When log-phase cells were incubated in media with either 180 or 2 mM glucose, the same phenomenon was observed (Fig. 1b). The specific activity of β-1,3-glucanase in the cells incubated under glucose limitation increased more than fivefold, and in this case it took a much shorter time due to the lower initial concentration of glucose. How-

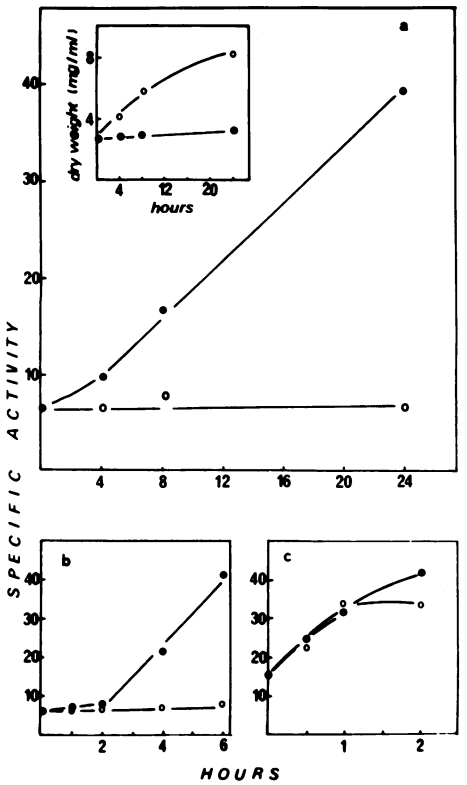

FIG. 1. *Effect of glucose on the production of β-1,3-glucanase by P. italicum. (a) Log-phase cells transferred to a medium with either 180 (○) or 10 (●) mM glucose; (insert) growth of the fungus in these two media. (b) Log-phase cells transferred to a medium with either 180 (○) or 2 (●) mM glucose. (c) Derepressed cells (incubated for 4 h in a medium with 2 mM glucose) transferred to the same media as in (b).*

ever, completely different results were obtained in experiments in which derepressed cells (cells incubated for 4 h in a 2 mM glucose medium) were substituted for log-phase cells (Fig. 1c). In derepressed cells transferred to media with either 180 or 2 mM glucose, the specific activity continued to increase, independently of the glucose concentration, for a period of at least 1 h.

These results clearly show that glucose represses the synthesis of β-1,3-glucanase; when the cells were actively growing in the presence of this sugar, only enough enzyme was produced to maintain a constant level of specific activity, and, on the other hand, when the concentration of glucose was low, the enzyme was actively produced and the specific activity increased significantly. Incubation of the cells in a medium with a low concentration of glucose also led to an accumulation of the capacity to synthesize the enzyme, and, after 4 h at 2 mM glucose, the specific activity continued to increase even when the cells were transferred to a medium with an excess of the sugar. This was also true for a wide range of concentrations of derepressed cells in the medium with excess glucose. Diluting the cells five times more than in the standard procedure (that is, 0.3 g of wet weight up to 60 ml of medium) made the excess of glucose even bigger, but the increase in specific activity was the same (data not shown).

These results suggest that the glucose repression effect on glucanase production must be exerted at a level before the synthesis of the enzymatic protein in the ribosomes; this hypothesis would be consistent with the observations that, at a low glucose concentration, the cells accumulate the capacity for the synthesis of the enzyme and that the expression of that capacity is unaffected by the concentration of glucose.

Effect of protein and RNA synthesis inhibitors on derepression of β-1,3-glucanase synthesis. The effect of cycloheximide, an inhibitor of protein synthesis, on derepression of β-1,3-glucanase is shown in Fig. 2a. The addition of this drug, at different times, to cells that were being incubated under derepression conditions (10 mM glucose) brought about an instantaneous halt in the increase of the β-1,3-

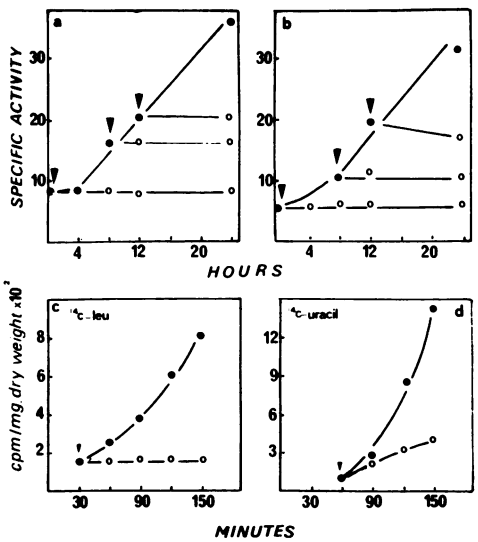

FIG. 2. *Effect of cycloheximide (a) and trichodermin (b) on the production of β-1,3-glucanase by P. italicum grown under glucose limitation. (a) and (b) correspond to the long-incubation experiment described in the text. Cycloheximide (50 μg/ml) or trichodermin (15 μg/ml) added (○); control (●). Incorporation of [¹⁴C]leucine (c) and [¹⁴C]uracil (d) in the presence (○) and in the absence (●) of trichodermin. In all cases, the triangles (▼) indicate times of addition of the corresponding drug.*

glucanase level. Very similar results were obtained with trichodermin (21), another inhibitor of translation, which blocks protein synthesis in *P. italicum* while at the same time permitting RNA synthesis to continue for a period (Fig. 2c and d). Trichodermin (15 μg/ml) prevented any increase in the specific activity of β-1,3-glucanase, and the value that had been attained at the time of drug addition remained constant (Fig. 2b). We conclude from these results that protein synthesis is necessary for the specific activity of β-1,3-glucanase to increase in derepressed cells; when the former is blocked, the latter is immediately prevented.

A transcription inhibitor, 8-hydroxyquinoline (3, 4), was also used for these studies. This drug, at concentrations of 100 to 150 μg/ml, inhibited RNA synthesis in *P. italicum*, reducing it to a level of 25 to 5% of the control (Fig. 3a, b, c, and f). The effect was observed not later than 15 min after the addition of the drug, and protein synthesis was much less affected (Fig. 3d and e). As shown in Fig. 4, the effect of this inhibitor on glucanase derepression was different from that of cycloheximide or trichodermin. The addition of 8-hydroxyquinoline to log-phase cells

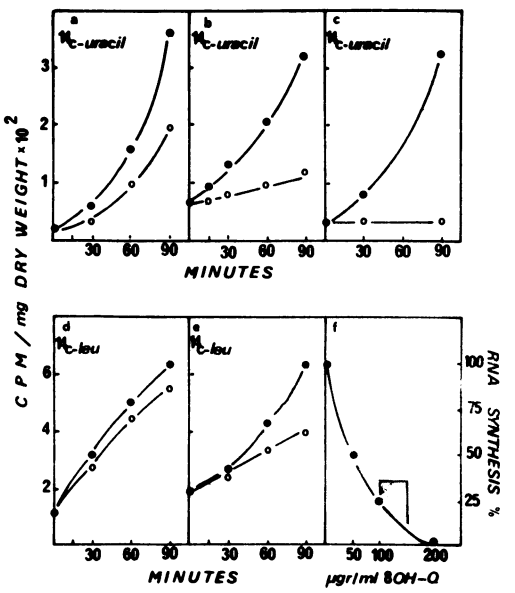

FIG. 3. *Effect of 8-hydroxyquinoline on RNA (a, b, c, f) and protein (d, e) syntheses in P. italicum. 8-Hydroxyquinoline added* (○); *control* (●). *The concentration of the drug was 50 μg/ml in (a) and (d), 100 μg/ml in (b) and (e), and 200 μg/ml in (c). In (f), the percentages of inhibition of RNA synthesis at different concentrations of the drug are represented; the shadowed area represents the range of concentrations of 8-hydroxyquinoline (8OH-Q) used in the experiments of Fig. 4.*

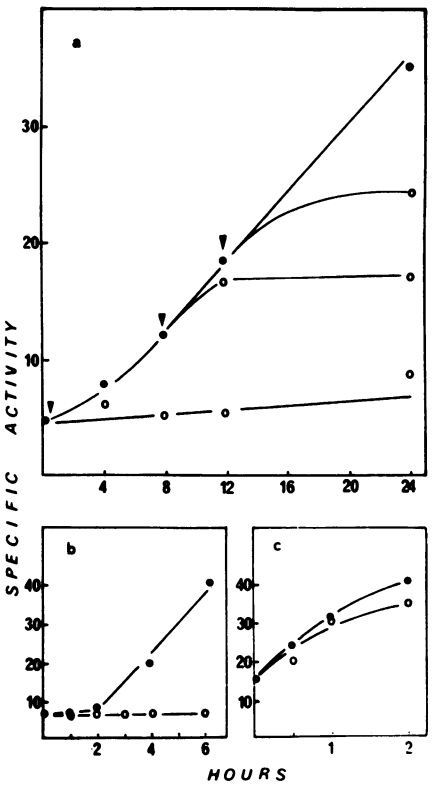

FIG. 4. *Effect of 8-hydroxyquinoline on the production of β-1,3-glucanase by P. italicum grown under glucose limitation. (a) Long-incubation experiment (see text); log-phase cells were incubated in flasks with 10 mM glucose medium, and 8-hydroxyquinoline* (○) *was added to a concentration of 100 μg/ml at the times indicated* (▼); *control* (●). *(b) Log-phase cells transferred to a medium with 2 mM glucose; 8-hydroxyquinoline added (150 μg/ml)* (○); *control* (●). *(c) Derepressed cells (incubated for 4 h in a 2 mM glucose medium) transferred to a medium with 2 mM glucose; 8-hydroxyquinoline (150 μg/ml) added* (○); *control* (●).

at the time they were transferred to the glucose-limited medium (either 10 or 2 mM glucose) immediately halted the increase in specific activity of β-1,3-glucanase (Fig. 4a and b); however, when the drug was added to the medium after a period of incubation under derepression conditions (10 mM glucose), a delayed inhibition of β-1,3-glucanase production was observed and the specific activity increased for some time before leveling off. The value reached indicated that the synthesis continued for about 2 h. When derepressed cells (incubated for 4 h at 2 mM glucose) were transferred to a medium with a very low concentration of glucose (2 mM), the synthesis of β-1,3-glucanase was practically unaffected by 8-hydroxyquinoline for a period of at least 1 h (Fig. 4c).

These results suggest that, in the glucose limited-medium, the cells must have accumulated stable β-1,3-glucanase mRNA, whose translation permitted the specific activity to increase for some time after the addition of 8-hydroxyquinoline. This mRNA cannot be accumulated in log-phase cells, since the addition of 8-hydroxyquinoline to those cells prevented the increase in specific activity from the beginning. The results presented so far indicate that the glucose repression effect must be exerted at some level before translation. Further evidence in favor of this interpretation may be deduced from Table 1. The increase in specific activity taking place after the addition of 8-hydroxyquinoline to derepressed cells was not affected by the glucose concentration. Therefore, glucose did not affect the 8-hydroxyquinoline-insensitive synthesis of β-1,3-glucanase by derepressed cells, indicating that derepressing conditions (a medium low in glucose content) led to the accumulation of mRNA specific for glucanase whose translation was not influenced by the concentration of the sugar. As a control, experiments were run to follow the uptake of glucose in the presence of 8-hydroxyquinoline and to eliminate the possibility that the drug might be blocking the transport of the carbon source. As shown in Fig. 5, in the presence of 8-hydroxyquinoline, uptake of glucose does take place and is proportional to the concentration of the sugar in the medium.

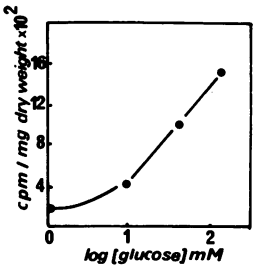

FIG. 5. *Uptake of glucose by P. italicum in the presence of 8-hydroxyquinoline. Log-phase cells (1.5 g, wet weight) were transferred to 250-ml flasks containing 60 ml of media with 150 μg of 8-hydroxyquinoline per ml and the indicated concentrations of [U-^{14}C]glucose. After 60 min of incubation, a 1-ml sample was taken, filtered through a GF/C (Whatman) filter, and washed with 100 ml of distilled water. The filter was finally dried, weighed, and counted as described in the text.*

DISCUSSION

The evidence presented in this paper and in a previous one (17) discloses the existence of a mechanism of regulation, by the carbon source, of β-1,3-glucanase synthesis in *P. italicum*. When this fungus is grown in a synthetic liquid medium, two situations can be clearly defined with regard to the production of the enzyme. The first is that of active growth (log phase), when an excess of glucose is present in the medium; under these conditions, a low, although measurable, and constant level of specific activity occurs, suggesting that only enough enzyme is synthesized for maintaining that constitutive level. Alternatively, glucose could stimulate turnover of the enzyme (10), but this is less probable in view of the other results. The second situation, to which we refer as derepressive conditions, is that of glucose limitation (2 to 10 mM), which determines a very slow growth and is characterized by a constant increase in the level of specific activity. The synthesis of many other carbohydrases by fungi is also favored under conditions of glucose limitation. This is the case, for example, of the cellulase of *Myrothecium verrucaria* (6), the β-1,3-glucanase of the basidiomycete QM806 (5), and the α-1,3-glucanase of *A. nidulans* (22). In *P. italicum*, glucose limitation also led to an accumulation of the capacity to synthesize β-1,3-glucanase, and derepressed cells continued to increase their levels of the enzyme for a considerable period, even when the excess of glucose was restored.

TABLE 1. *Effect of glucose on the 8-hydroxyquinoline-insensitive synthesis of β-1,3-glucanase*[a]

Time (min)	Sp act of β-1,3-glucanase in cells incubated in a medium containing:		
	2 mM glucose	2 mM glucose + 8-hydroxyquinoline	180 mM glucose + 8-hydroxyquinoline
0	15.60	15.60	15.60
30	20.80	21.10	21.00
60	32.40	30.60	36.12
120	40.50	37.40	34.50

[a] Derepressed cells (incubated for 4 h in a medium with 2 mM glucose) were transferred to media containing 8-hydroxyquinoline and/or glucose as indicated. The flasks were shaken at 28°C, and the enzyme was assayed in samples withdrawn at the times indicated. The concentration of the drug was 150 μg/ml.

The addition of protein synthesis inhibitors (cycloheximide and trichodermin) to derepressed cells immediately prevented the specific activity from reaching a higher value, and this suggested that the observed increase was due to de novo synthesis of the enzyme. The fact that the level of specific activity remained constant after the addition of the protein synthesis inhibitors, even for long periods of time, emphasizes the stability of the enzyme. An RNA synthesis inhibitor (8-hydroxyquinoline) acted dif-

ferently from cycloheximide or trichodermin. Although it immediately halted the increase in specific activity in log-phase cells, the effect was delayed in derepressed cells. In this case, the specific activity continued to increase for some time after the addition of this drug. This indicates that glucose limitation leads to an accumulation of mRNA specific for β-1,3-glucanase, which can be translated in the presence of 8-hydroxyquinoline. Therefore, the effects of glucose and 8-hydroxyquinoline on the production of β-1,3-glucanase by derepressed cells appear to be very similar. Moreover, the concentration of glucose did not affect the 8-hydroxyquinoline-insensitive synthesis of β-1,3-glucanase.

These results are consistent with the notion that the regulation of β-1,3-glucanase production by glucose is exerted at a pretranslational level. The lack of glucose in the medium must lead to some differential activation of synthesis or processing of β-1,3-glucanase mRNA that, in a later stage, results in an increase in the specific activity. In any case, it is interesting to note that the basal level, which is produced during active growth, is not affected by glucose. The existence of similar controls in other fungal systems has been shown by other workers with the use of RNA and protein synthesis inhibitors for a temporal separation of transcription and translation. For example, nitrate acts at the transcriptional level, inducing nitrate reductase synthesis in *U. maydis* (7) and in *N. crassa* (18), and a similar mechanism of control exists in the case of the kynureninase of *N. crassa* (20).

The phenomenon of specific inhibition of enzyme synthesis in the presence of glucose, called the "glucose effect" (11) or "catabolite repression" (9), is widespread among microorganisms and has been known for many years. In *Escherichia coli*, glucose represses the synthesis of many proteins; among them there are not only enzymes involved in the sugar catabolism, but also other degradative enzymes and even some structural proteins, such as those that form part of flagella (14). Glucose repression also affects the production of β-*N*-acetylglucosaminidase, a bacterial wall-lytic enzyme produced by *Bacillus subtilis* (12). The glucose repressor effect in *E. coli* is frequently exerted at the transcriptional level and is mediated by cyclic AMP, which, in the absence of glucose, acts as a positive regulatory element by turning on gene expression. In a eucaryotic system, such as *Dictyostelium discoideum*, glucose represses the biochemical events involved in development, an effect that also seems to be mediated by cyclic AMP (15). One important feature of the proteins that are subjected to catabolite repression is the fact that they are not needed for survival in a glucose-supplemented medium (14). According to the results discussed above, there could be more than one role for β-1,3-glucanase in *P. italicum*. The very high amount of enzyme that appears upon exhaustion of the carbon source and that is not needed during active growth may well have a metabolic function directed towards the mobilization of wall or external glucan for the purpose of obtaining energy. This enzyme could also participate in the autolysis of the mycelium that eventually takes place under these conditions. On the other hand, the enzyme that is produced at a basal level during active growth could be the one that participates in growth and extension of the cell wall according to the theory that postulates that elongation of the wall should be the result of a lysis of its components at certain points and resynthesis of new material (1). In view of these two functions, it is quite possible that there is more than one enzyme with β-1,3-glucanase activity in *P. italicum* and that derepression is, at least partially, due to the appearance of a new enzyme that is not present during active growth. Experiments to be described elsewhere show that, in fact, under glucose limitation, a new enzyme with β-1,3-glucanase activity appears (16).

ACKNOWLEDGMENTS

T. S. acknowledges a fellowship from Ministerio de Educación y Ciencia, Spain. This investigation was aided by a grant from Comisión Asesora de Investigatión Científica.

We are indebted to W. O. Godtfredsen of Leo Pharmaceutical Products for a generous gift of trichodermin.

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