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Cyclic AMP and Low Molecular Weight Effector(s) Present in Yeast Extract Are Involved in Pectin Lyase Production by *Penicillium griseoroseum* Cultured on Sucrose

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Abstract

Pectin lyase (PL) induction by organic and inorganic components of yeast extract (YE) was evaluated in *Penicillium griseoroseum*, cultured in a mineral medium containing sucrose, by determining PL activity (A_{235}) and mycelial growth (mycelial dry weight). The lowest YE concentration that promoted significant PL induction without acting as a carbon source for the fungus corresponded to 0.0075%. Neither calcined YE nor a nutrient solution containing micronutrients induced PL production, indicating that the inducer was an organic compound. Vitamins, phospholipid components, amino acids, and nitrogenous bases were tested in place of YE and promoted no significant PL induction. A PL inducer compound was found to be soluble in the nucleotide fraction obtained during extraction of YE. The inducer was shown to be a thermostable polar substance dialyzable at 2000 Daltons, hydrolyzable by HCl, and activated by boiling for up to 60 min. Cyclic AMP (cAMP) exogenously added to the culture medium at 5 and 10 m*M* was capable of inducing PL in *P. griseoroseum* grown on sucrose, suggesting that at least one compound may be present in YE acting in a cooperative fashion for the maintenance of high levels of cAMP into the cell. PL activity and the level of cAMP inside the fungal cells increased after the addition of YE to the

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culture medium, suggesting the participation of this messenger in this enzyme's synthesis.

Index Entries: Cyclic AMP; yeast extract; pectin lyase; pectinases; *P. griseoroseum*; sucrose.

Introduction

The enzymatic hydrolysis of plant fiber pectins by pectinases is a phenomenon called *maceration (1)* and has been studied in order to degum natural fibers, such as ramie and flax, for use in the textile industry *(2,3)*. Pectin lyase (PL) (EC 4.2.2.3) is the only known pectinase capable of lysing α-1,4 bonds of highly esterified pectin without the previous action of other pectinases *(4)* by means of β-eliminative cleavage of glycosidic linkages *(5)*.

Penicillium griseoroseum produces PL using sucrose as a carbon source *(6)*. Baracat-Pereira et al. *(7)* have demonstrated that besides sucrose, yeast extract (YE) is essential for PL production by this fungus. Sucrose is an inexpensive sugar that can be used as substrate whereas YE is a complex compound that contains simple substances such as amino acids and peptides, water-soluble vitamins, nitrogenous bases, and carbohydrates *(8)*. Therefore, the objective of this work was to investigate the process of PL production in *P. griseoroseum* cultured on sucrose and YE, in the hope that in future studies YE can be replaced by a less expensive inducer.

Materials and Methods

Organism, Inoculum Production, Culture Conditions, and Growth Determination

P. griseoroseum, isolated from seeds of forest trees at the Department of Plant Pathology, Universidade Federal de Viçosa—MG, Brazil, was subcultured on slants of oatmeal-agar at 25°C for 9 d and stored at 4°C *(6)*. The fungus was cultivated in 125-mL Erlenmeyer flasks containing 50 mL of a mineral medium (pH 6.3) with the following composition: 8 g/L KH_2PO_4; 2.48 g/L K_2HPO_4; 1.1 g/L $MgSO_4 \cdot 7H_2O$; and 1.0 g/L $(NH_4)_2SO_4$. In addition to the mineral components, sucrose at 0.4% and YE (Merck, Darmstadt, Germany) at either 0.06% or at increasing concentrations (0–0.06%) were added to the culture medium.

Calcined YE (400°C; 2 h) at 0.06% and a micronutrient solution were tested in place of YE. The micronutrient solution, added to the medium at 0.002, 0.02, and 0.1% (v/v) was composed of 7 µ*M* Mn, 19 µ*M* B, 2 µ*M* Zn, 0.0856 µ*M* Mo, and 0.5 µ*M* Cu. Twenty amino acids, each at 50 µg/mL, were also tested in place of YE. The amino acids were grouped in families according to the biosynthetic pathways that use glucose as a carbon source: glutamate, aspartate, aromatic amino acids, serine, pyruvate, and histidine families *(9)*. Similarly, nine vitamins, inositol, and choline were tested each at a different concentration. The nitrogenous bases adenine, guanine, uracil,

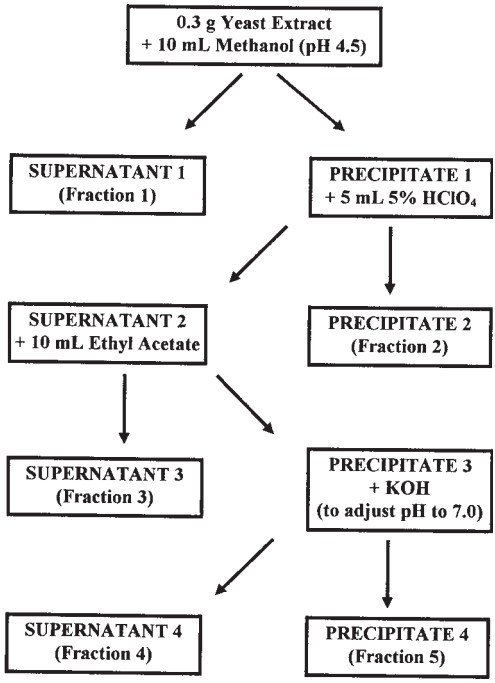

Fig. 1. Extraction sequence for soluble nucleotides present in YE. Fractions 1–5 were used at 0.03% instead of YE for PL production by *P. griseoroseum* grown on 0.4% sucrose.

thymine, xanthine, and hypoxanthine were tested individually at 20 µg/mL. Additionally, a mix composed of adenine, guanine, uracil, and xanthine, each nitrogenous bases at 20 µg/mL, was also evaluated as a PL inducer. Hypoxanthine was also tested in place of YE at 5, 10, 20, 40, and 80 µg/mL.

Soluble nucleotides were extracted according to Ingle *(10)* with some modifications (Fig. 1). For all solvents, three sequential extractions were performed and the mixtures were shaken for 10 min and centrifuged at 5000*g* for 10 min at room temperature. The supernatants of three replications were pooled and completely evaporated at 80°C under vacuum. The precipitate was resuspended to the same initial concentration of unmodified YE. The precipitate and supernatant fractions were tested as PL inducers in place of YE (fractions 1–5).

Acid hydrolysis was performed by adding 25 mL of 6*N* HCl to 0.125 g of YE in tubes with Teflon caps and incubating at 110°C for 24 h with subsequent neutralization with KOH. This solution was then used instead of YE in the culture medium. For precipitation with silver, 20 mL of a 1% $AgNO_3$ solution was added to 25 mL of 3% YE and centrifuged at 4°C and 8000*g* for 10 min. The supernatant was added to the culture medium in place of YE.

YE was dialyzed through a fine-pore membrane (2000-Dalton cutoff), and the internal and external dialysis fractions were added to the culture medium at 0.06% in place of YE.

cAMP at 5 and 10 m*M* was tested as PL inducer instead of YE, after which the level of cAMP was determined inside fungal cells cultured on 0.4% sucrose supplemented or not with 0.03% YE after 12 h of growth.

After inoculation with 5×10^4 spores/mL, cultures were incubated for 48 h at 25°C and 150 rpm on a rotary shaker. Growth was measured according to Calam *(11)* by harvesting the mycelium with a 400-mesh sieve and drying at 105°C. All experiments were done in triplicate.

Enzyme Assay

PL activity was determined in the culture filtrates at 235 nm, according to the method of Albersheim *(12)*, by adding 0.5 mL of culture filtrate to 3 mL of 0.25% citric pectin (Sigma, St. Louis, MO) in 50 m*M* Sörensen's phosphate buffer, pH 6.0. After incubation at 40°C for 30 min, 0.2 mL of 1*N* HCl was added to the reaction. One unit of PL activity was defined as nanomoles of unsaturated products produced per milliliter of culture filtrate per minute.

cAMP Determination Inside Fungal Cells

cAMP was determined according to Thevelein et al. *(13)*. Samples containing 35–450 mg of cells were quickly filtered on Millipore filters (0.22 µm, 25 mm diameter) and immediately immersed in liquid nitrogen. The filters were rapidly (without thawing) broken into pieces and transferred into 7-mL Teflon containers (Braun, Melsugen, Germany) prefrozen in liquid nitrogen and containing 3.3-g glass beads of 3 mm diameter, 2.5-g glass beads of 0.5 mm diameter, 1 mL of 1*M* $HClO_4$, and a small amount of liquid nitrogen. The containers were allowed to warm up to –20°C in a freezer and then vibrated in a Braun microdismembrator for about 7 min at an amplitude of 10 mm. Under these conditions the frozen mixture was first pulverized; when it subsequently thawed, the cells were killed and broken at a temperature substantially lower than 0°C. The perchloric acid extracts were centrifuged for 2 min at 10,000*g* to remove potassium perchlorate. Samples (250 µL) of the supernatant were transferred into tubes placed in ice and mixed with 10 µL of a saturated thymol blue solution. Thereafter, a volume of 5*M* K_2CO_3 (about 40–50 µL) was added until the color changed to blue. The tubes were left (open) in ice for about 15 min and subsequently centrifuged for 2 min at 10,000*g*; 200 µL of the supernatant were removed and 1*M* HCl was added until the color changed to yellow. Thereafter, 10 µL of 2*M* Tris/HCl, pH 7.5, was added, the tubes were centrifuged again for 2 min at 10,000*g*, and 50 µL of the supernatant were taken for cAMP determination. cAMP assay kits (Amersham, Amersham Place, UK), based on competition of cAMP with [^{3}H]cAMP for the regulatory subunit of cAMP-dependent protein kinase, were used for all cAMP determinations.

Results and Discussion

Low concentrations of YE were capable of inducing PL in *P. griseoroseum* grown on sucrose. As indicated by the Scott-Knott test, the lowest concen-

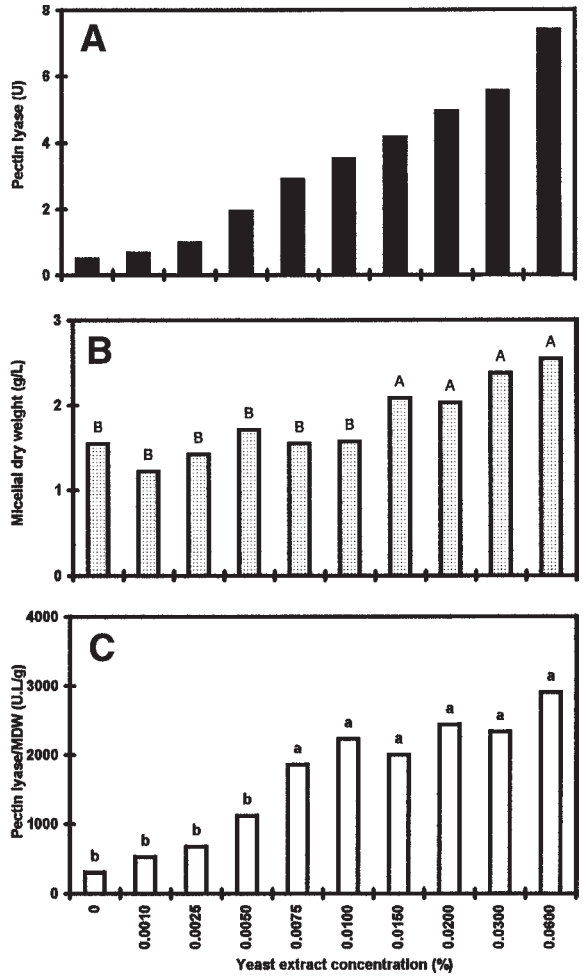

Fig. 2. **(A)** PL activity, **(B)** mycelial dry weight (MDW), and **(C)** relative PL activity (PL activity/MDW) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0–0.06% YE. Treatments followed by the same letter do not differ statistically from each other at 5% probability according to the Scott-Knott test.

tration of YE that induced significant PL amounts without being used by the fungus as a carbon source corresponded to 0.0075% (Fig. 2B,C). These results confirmed the inducing capacity of YE earlier demonstrated by Baracat-Pereira et al. *(7)*. PL activity increased concomitantly with the increases in the concentration of YE added to the culture medium (Fig. 2A). However, the growth rate of the fungus was lower than the rate of PL production (Fig. 2B,C), which demonstrated that the effect of YE on PL synthesis was not simply a result of increases in mycelial mass.

To determine whether PL-inducing components present in YE were either organic or inorganic, calcined YE or a mineral solution containing Mn, B, Zn, Mo, and Cu were added to the culture medium in place of YE.

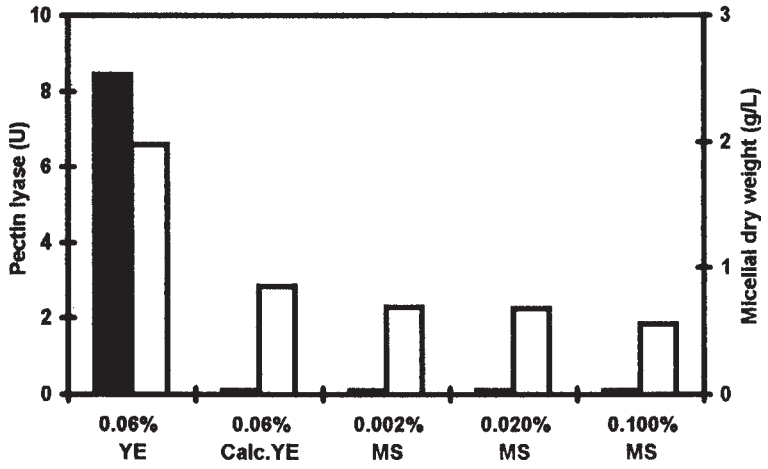

Fig. 3. PL activity (■) and MDW (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE, calcined or not, and with a mineral solution (MS) containing Mn, B, Zn, Mo, and Cu.

Table 1
Amino Acids as Inducers of PL on *P. griseoroseum*

Amino acid family	Compounds	MDW (g/L)	PL activity (U)	PL activity (% of control)
Glutamate	Glu, Gln, Arg, Pro	1.190	0.533	6.7
Aspartate	Asp, Asn, Met, Thr, Ile, Lys	1.414	0.400	5.1
Aromatic	Trp, Phe, Tyr	1.340	0.533	6.7
Serine	Ser, Gly, Cys	1.288	0.533	6.7
Pyruvate	Ala, Val, Leu	1.242	0.000	0.0
Histidine	His	1.254	0.000	0.0
Mix	20 amino acids	1.330	0.267	3.4
	YE (300 µg/mL)	1.950	7.911	100.0

Note: *P. griseoroseum* was cultured on 0.4% sucrose supplemented with 0.03% YE or the 20 standard amino acids, each at 50 µg/mL. In the mix, all amino acids were simultaneously added at 50 µg/mL each.

No PL induction was obtained (Fig. 3), indicating that the inducers present in YE were organic.

Among the main organic components of YE, Crueger and Crueger *(8)* cite amino acids, peptides, and water-soluble vitamins. The 20 standard amino acids of proteins, grouped in families (Table 1), were tested as substitutes for YE and promoted fungal growth in the range of 61–72.5% of that obtained with 0.03% YE. However, PL activity was very low when compared with that obtained with the control (0.03% YE) (Table 1). Similar results were obtained when all 20 amino acids were tested simultaneously at the same concentrations, indicating that these compounds were not the PL-inducing components present in YE.

Table 2
Vitamins or Phospholipid Components as Inducers of PL in *P. griseoroseum*

Compound	Concentration (µg/mL)	MDW (g/L)	PL activity (U)	PL activity (% of control)
Thiamine	5.0	1.422	0.000	0.0
Pyridoxine	10.0	1.022	0.000	0.0
Pyridoxal	3.0	0.934	0.000	0.0
Ca pantothenate	5.0	1.130	0.000	0.0
Riboflavin	5.0	1.030	0.000	0.0
Niacin	10.0	1.078	0.000	0.0
PABA	10.0	0.988	0.000	0.0
Biotin	0.025	1.000	0.000	0.0
Folic acid	1.0	1.006	0.000	0.0
Inositol	50.0	0.900	0.000	0.0
Choline	50.0	0.874	0.000	0.0
YE	300.0	2.012	9.778	100.0

Note: *P. griseoroseum* was cultured on 0.4% sucrose supplemented with 0.03% YE, vitamins, or phospholipid components, each at a different concentration.

Table 3
Nitrogenous Bases as Inducers of PL on *P. griseoroseum*

Compound	Concentration (µg/mL)	MDW (g/L)	PL activity (U)	PL activity (% of control)
Adenine	20	1.082	0.489	5.9
Guanine	20	0.856	1.067	12.8
Uracil	20	0.910	1.200	14.4
Xanthine	20	1.064	0.355	4.3
Thymine	20	0.816	0.711	8.6
Hypoxanthine	20	0.768	1.644	19.7
Adenine, guanine, uracil, xanthine	80	0.944	1.956	23.5
YE	300	1.454	8.311	100.0

Note: *P. griseoroseum* was cultured on 0.4% sucrose supplemented with 0.03% YE or nitrogenous bases, added individually at 20 g/mL or added as a mix composed of adenine, guanine, uracil, and xanthine, each at 20 µg/mL.

Nine water-soluble vitamins and the phospholipid components inositol and choline were tested in place of YE and none induced PL (Table 2). This suggests that these compounds present in YE were not capable of inducing PL synthesis.

Continuing the evaluation of the organic compounds present in YE, six nitrogenous bases (four purines and two pyrimidines) were tested separately, and four of these (adenine, guanine, uracil, and xanthine, generally present in culture medium for microorganisms) *(9)* were tested simultaneously. In both experiments, PL activity levels were less than a quarter of those obtained with the control (Table 3), although mycelial weight

Table 4
Hypoxanthine as Inducer of PL on *P. griseoroseum*

Compound	Concentration (μg/mL)	MDW (g/L)	PL activity (U)	PL activity (% of control)
Hypoxanthine	5	1.110	2.222	26.7
	10	0.996	2.311	27.8
	20	1.034	2.578	31.0
	40	1.000	2.267	27.3
	80	0.960	2.222	26.7
YE	300	1.454	8.311	100.0

Note: *P. griseoroseum* was cultured on 0.4% sucrose supplemented with 0.03% YE or hypoxanthine, added at different concentrations.

remained between 53 and 74% of the value obtained with 0.03% YE. The simultaneous addition of adenine, guanine, uracil, and xanthine (each at 20 μg/mL), and hypoxanthine added singly to the culture medium (20 μg/mL) promoted 23.5 and 19.7% of the absolute PL activity obtained with 0.03% YE, respectively (Table 3). The addition of hypoxanthine at 5, 10, 20, 40, and 80 μg/mL induced up to 31% of the PL activity obtained with 0.03% YE (Table 4). Among the nitrogenous bases tested at 20 μg/mL, hypoxanthine promoted the best results for PL induction. In the cell, free purines are in large part salvaged and used to make nucleotides *(14)*. Free guanine and hypoxanthine are recycled in a salvage pathway by the enzyme hypoxanthine-guanine phosphoribosyltransferase *(14)*.

In another attempt to verify the participation of nitrogenous bases and, possibly, nucleotides in PL induction, fraction 5 containing extracted nucleotides (Fig. 1) was tested in place of YE and promoted 11% of the absolute PL activity and 52% of the mycelial mass produced in the control treatment (Fig. 4). These results suggest that the nucleotides present in YE could have a role in the induction of PL in *P. griseoroseum*. Also, methanol at pH 4.5 (Fig. 1, fraction 1) removed part of the PL-inducing capacity of YE (Fig. 4), suggesting the existence of two inducing fractions present in YE—fractions 1 and 5.

The extractions of YE with compounds presenting different polarity showed that butanol extracted about 28% of the inducing capacity (in PL activity) (Fig. 5) present in YE. Similar results were not obtained for chloroform (Fig. 5), confirming the polar nature of the inducers present in YE. The highest extraction efficiency when methanol was used as extractant was obtained at pH 8.5, followed by pH 5.5 and 4.5 (Fig. 6). Methanol at pH 4.5 extracted 76.3% of the total mass of YE (data not shown). The precipitate also induced PL, showing that the extraction was not totally efficient at removing the inducer. The partial extraction of YE by methanol was in agreement with the PL activities induced by fractions 1 and 5 obtained during the extraction of nucleotides (Fig. 1).

To estimate the size of the inducer molecule, YE was dialyzed through a fine-pore membrane (2000-Dalton cutoff). The external dialysis fraction

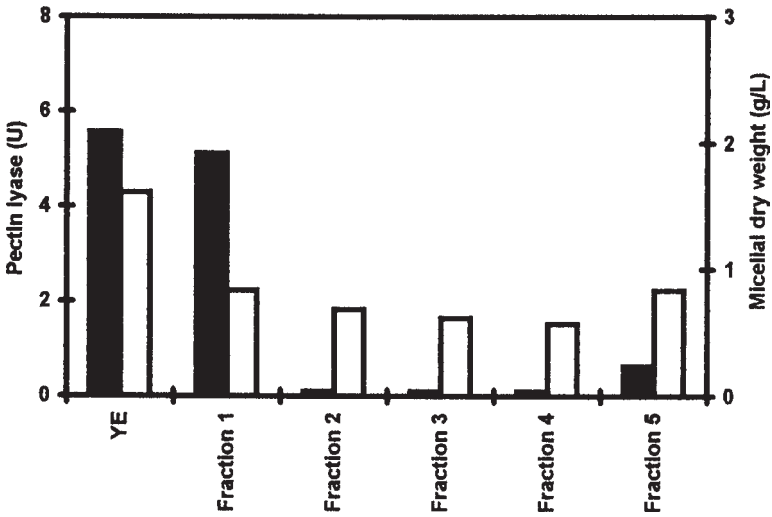

Fig. 4. PL activity (■) and MDW (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE or with fractions 1–5 obtained from YE during nucleotide extraction (Fig. 1).

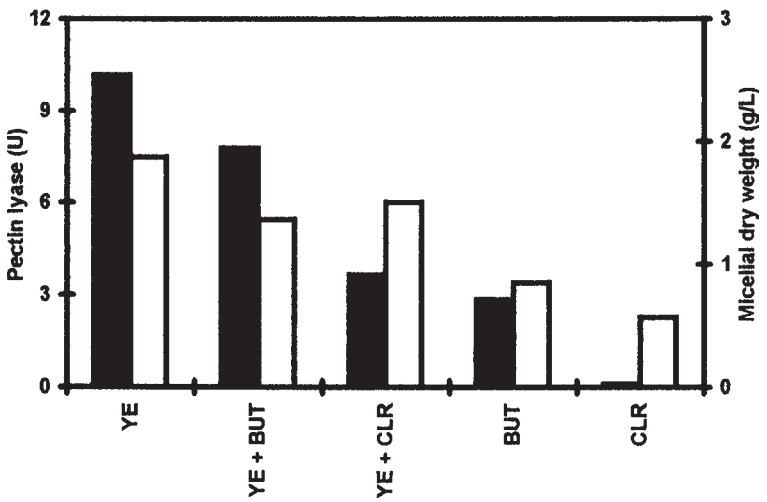

Fig. 5. PL activity (■) and MDW (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE or with one of the following extraction fractions: butanol aqueous fraction (YE + BUT); chloroform aqueous fraction (YE + CLR); butanol fraction (BUT); chloroform fraction (CLR).

promoted a PL activity three times higher than that promoted by the internal fraction when 0.03% dialyzed YE was added (Fig. 7). PL activity was detected only with the use of the external fraction when 0.0075% dialyzed YE was used (Fig. 7). Based on these results, we concluded that the organic inducers of PL present in YE had a low molecular weight.

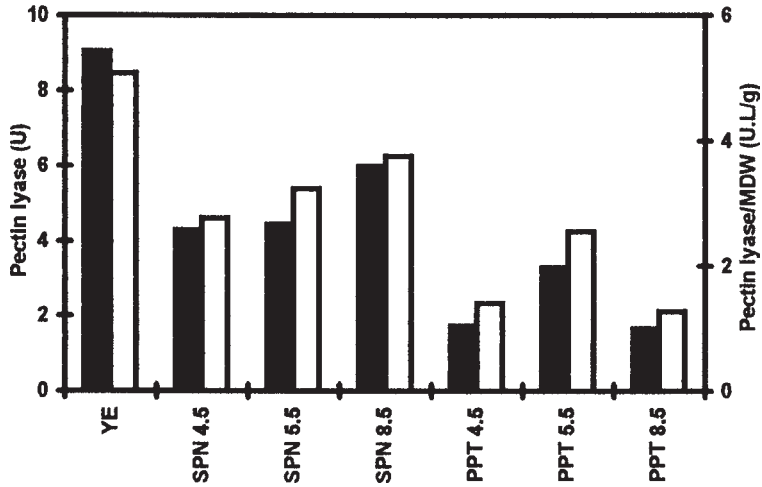

Fig. 6. PL activity (■) and relative PL activity (PL activity/MDW) (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE or with the supernatants (SPN) or precipitates (PPT) obtained during the extraction of YE with methanol at pH 4.5, 5.5, and 8.5.

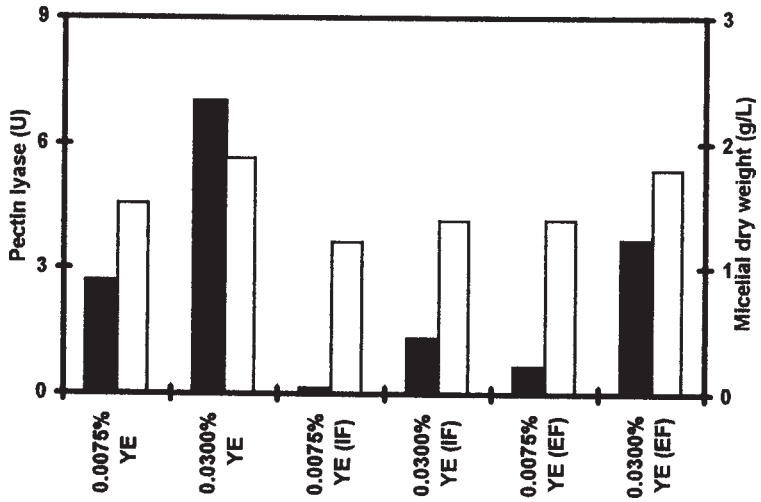

Fig. 7. PL activity (■) and MDW (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE or with the internal (IF) or external (EF) YE dialysis fractions at 0.03%.

Acid hydrolysis of YE completely eliminated its ability to induce PL, although the hydrolysate allowed a mass production by the fungus that was 36.4% superior to that obtained with YE (Fig. 8). This can be explained by the higher availability of easily metabolized substrates in the hydrolysate after YE hydrolysis. The patterns of mass pellets produced with the addition of YE hydrolysate or nonhydrolyzed YE to the medium differed

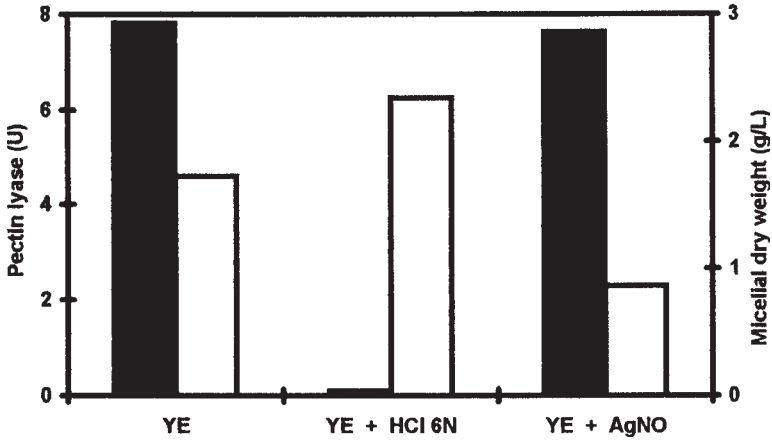

Fig. 8. PL activity (■) and MDW (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE or with acid-hydrolyzed or $AgNO_3$-precipitated YE.

greatly from each other, suggesting the occurrence of modifications in the metabolism of the fungus when grown on the hydrolysate. YE hydrolysate promoted the formation of abundant small pellets, whereas nonhydrolyzed YE normally promoted growth as a few large pellets with the appearance of mycelial strands on the walls of the flasks in which the fungus was cultivated. On the other hand, precipitation with $AgNO_3$ did not affect PL induction (Fig. 8) but decreased fungal growth by 50%. Considering that $AgNO_3$ precipitates alkalies, bromide, carbonate, chloride, iodide, tartarate, hypophosphite, and phosphate, among others *(15)*, our results indicated that the PL inducers present in YE probably did not belong in this group of chemical compounds.

The inducing power increased by up to 80% when YE was heated to boiling for 0–60 min before autoclaving (Fig. 9), suggesting the occurrence of reactions activated by heat that led to the production of PL inducers. It is noteworthy that after boiling YE, concentration was returned to the initial value by adding distilled water. Mycelial mass did not increase significantly after boiling, which showed that the increase in PL activity was owing to an increase in PL production rather than to increases in fungal growth.

The results obtained so far indicate that a PL inducer present in YE can be characterized as an organic, thermostable, low molecular weight, polar substance that is activated by heat, hydrolyzable by HCl, and soluble in the nucleotide fraction during the extraction of YE. Since pertinent literature cites many reports on the involvement of nucleotides in enzymatic regulation, especially cAMP *(16)*, we suspected the involvement of this second messenger in PL induction by the fungus. Whereas in prokaryotes cAMP receptor is a protein that regulates transcription, in eukaryotes the receptor is a cAMP-dependent protein kinase *(17)*. Some articles report the role of cAMP in the expression of extracellular enzymes of fungi, such as invertase *(18)* and PL *(19)*. To investigate the ability of cAMP to induce PL in

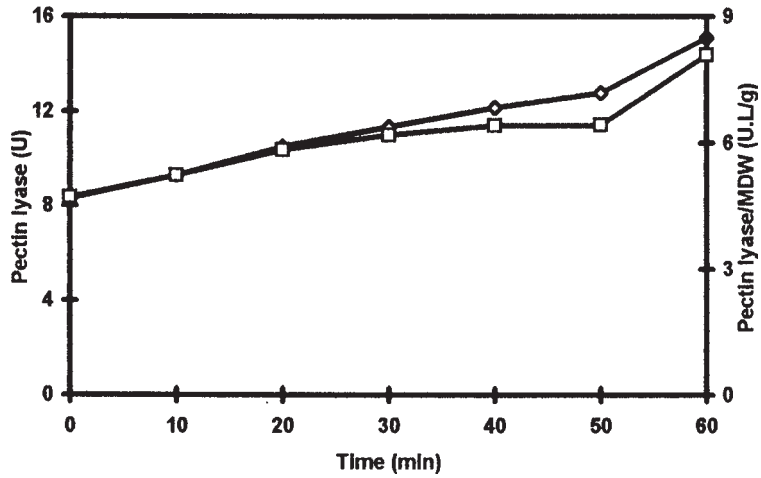

Fig. 9. PL activity (◇) and relative PL activity (PL activity/MDW) (□) of *P. griseoroseum* cultured on 0.4% sucrose supplemented with 0.03% YE heated to boiling for 0–60 min.

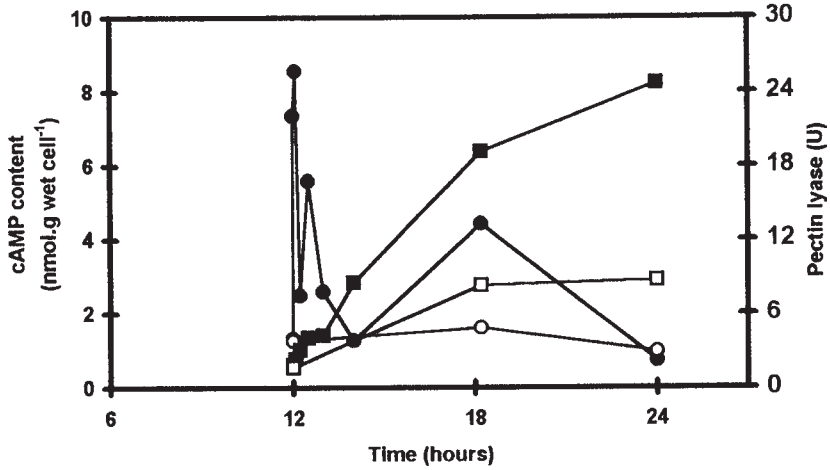

Fig. 10. Extracellular PL activity (■, □) and cAMP level inside cells (●, ○) of *P. griseoroseum* cultured on 0.4% sucrose supplemented (solid symbols) or not (open symbols) with 0.03% YE.

P. griseoroseum when supplied extracellularly in the culture medium, very high quantities of cAMP must be available since the fungus has difficulty absorbing this compound. In this way, the addition of cAMP at 5 and 10 m*M* induced 20.2 and 28.6%, respectively, of the PL activity obtained when the fungus was grown on 0.03% YE *(20)*.

Based on all the results obtained, we suggest that cAMP precursors or inducers may be present in YE and may be required for the process of induction or derepression of PL synthesis in *P. griseoroseum*. The substance(s) with the characteristics described previously may be present in YE acting in a cooperative fashion for the maintenance of high levels of cAMP in the cell.

The cAMP level increased about sixfold in the first minute after the addition of YE, with a second increase after 6 h of addition (Fig. 10), coinciding with the production of PL, which suggests its responsibility for the regulation of PL.

Other studies in our laboratory have shown that tea extract activates PL synthesis in *P. griseoroseum* and have also demonstrated the inductive effect of alkaloids on the synthesis of this enzyme *(20)*. Since alkaloids present in tea extract at high concentrations reduce phosphodiesterase activity, thereby increasing the intracellular concentrations of cAMP *(17)*, we suggest that there is a similar effect of YE on the levels of cAMP that accounts for the activation of PL synthesis in *P. griseoroseum* grown in the presence of YE and sucrose.

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References

1. Chesson, A. (1980), *J. Appl. Bacteriol.* **48,** 1–45.
2. Deshpande, K. S. and Gurucharanam, K. (1985), *Indian J. Botany* **8,** 79–81.
3. Sharma, H. S. S. (1988), *Biotechnology* **6,** 746–755.
4. Alaña, A., Llama, M. J., and Serra, J. L. (1991), *FEBS Lett.* **280,** 335–340.
5. Spagna, G., Pifferi, P. G., and Gilioli, E. (1995), *Enzyme Microbiol. Technol.* **17,** 729–738.
6. Brumano, M. H. N., Coelho, J. L. C., Araujo, E. F., and Silva, D. O. (1993), *World J. Microbiol. Biotechnol.* **9,** 225–228.
7. Baracat-Pereira, M. C., Coelho, J. L. C., and Silva, D. O. (1994), *Lett. Appl. Microbiol.* **18,** 127–129.
8. Crueger, W. and Crueger, A. (1982), *Biotechnology: A Textbook of Industrial Microbiology*, Sinauer Associates, Sunderland, MA.
9. Stanier, R. Y., Ingraham, J. L., Wheelis, M. L., and Painter, P. R. (1986), *The Microbial World*, 5th ed., Prentice Hall, Englewood Cliffs, NJ.
10. Ingle, J. (1963), *Phytochemistry* **2,** 353–370.
11. Calam, C. T. (1969), in *Methods in Microbiology*, vol. 1, Norris, J. R. and Ribbons, D. W., eds., Academic, London, pp. 567–591.
12. Albersheim, P. (1966), in *Methods in Enzymology*, vol. 8, Neufeld, E. S. and Ginsburg. V., eds., Academic, New York, pp. 628–635.
13. Thevelein, J. M., Beullens, M., Honshoven, F., Hoebeeck, G., Detremerie, K., Hollander, J. A. D., and Jans, A. W. H. (1987), *J. Gen. Microbiol.* **133,** 2191–2196.
14. Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1993), *Principles of Biochemistry*, 2nd ed., Worth Publishers, New York.
15. Budavari, S., O'Neil, M. J., Smith, A., Heckelman, P. E., and Kinneary, J. F., eds. (1996), *The Merck Index—An Encyclopaedia of Chemicals, Drugs, and Biologicals*, Merck, Rahway, NJ, p. 1462.
16. Botsford, J. L. and Harman, J. G. (1992), *Microbiol. Rev.* **56,** 100–122.
17. Pall, M. L. (1981), *Microbiol. Rev.* **45,** 462–480.
18. Terenzi, H., Terenzi, H. F., and Jorge, J. A. (1992), *J. Gen. Microbiol.* **138,** 2433–2439.
19. Mikhailova, R. V., Sapunova, L. I., and Lobanok, A. G. (1994), *World J. Microbiol. Biotechnol.* **10,** 457–461.
20. Baracat-Pereira, M. C., Minussi, R. C., Coelho, J. L. C., and Silva, D. O. (1997), *J. Ind. Microbiol. Biotechnol.* **18,** 308–311.