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Tea extract as an inexpensive inducer of pectin lyase in *Penicillium griseoroseum* cultured on sucrose

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Extracts of tea, coffee, cocoa, and yeast induced pectin lyase (PL) in *Penicillium griseoroseum* cultured in a mineral medium with sucrose as the carbon source. PL activity and fungal growth were similar in the treatments with 0.5% tea extract, the highest concentration tested, and 0.03% yeast extract. When tea extract was added singly to the culture medium, *P. griseoroseum* produced 59% and 17% of the PL activity and mycelial mass, respectively, obtained in a treatment with tea extract and sucrose. These results suggest that the production of the enzyme was not proportional to mycelial growth. No PL was produced in the medium with sucrose and without inducers. The small amounts of pectic substances present in the tea extract could not be responsible for PL induction. PL activity was detected after 12 h of growth in the medium containing sucrose and tea extract added at time zero, and after 48 h of growth when tea extract was added at times 12 and 24 h. Mycelial mass in all treatments was similar after 48 h of incubation. However, the addition of tea extract at time zero increased PL activity by 20–25%. Cyclic AMP at 5 and 10 mM in the culture medium induced 20 and 30%, respectively, of the PL activity obtained with 0.03% yeast extract, suggesting that PL induction brought about by either yeast extract or tea extract might involve the intracellular metabolism of cAMP.

Keywords: cocoa; coffee; pectin lyase; *Penicillium griseoroseum*; sucrose; tea

Introduction

The action of pectic enzymes on plants results in the rapid loss of tissue coherence and the separation of plant cells [8], a phenomenon that has been studied for the degumming of natural fibres in the textile industry [10,16]. Pectin lyase (E.C. 4.2.2.10) (PL) is important for degumming since it is the only pectic enzyme that can cleave the α-1,4 bonds of highly esterified pectins without the previous action of other enzymes [1].

Since purified pectin is an expensive substrate for the production of cellulase-free pectinases, alternative carbon sources and inducers have been tested to produce these enzymes. In earlier studies in our laboratory, we observed that *P. griseoroseum* was capable of producing PL in a medium containing the non-pectic substances, sucrose and yeast extract [4].

The objective of this study was to investigate the effect of inexpensive inducers on the production of PL by *P. griseoroseum*.

Materials and methods

Microorganism, inoculum production and culture conditions

Penicillium griseoroseum strain (Dierckx), originally isolated from forest tree seeds at the Departamento de Fitopatologia, Universidade Federal de Viçosa, was cultured on slants of oatmeal-agar for 9 days at 25°C and stored at 4°C [6]. The mineral medium used for cultivation consisted of (g L^{-1}): KH_2PO_4, 8.0; K_2HPO_4, 2.48; $MgSO_4 \cdot 7H_2O$, 1.1 and $(NH_4)_2SO_4$, 1.0, pH 6.3. Sucrose (0.4%) and 0.03% yeast extract (Merck, Rio de Janeiro, RJ, Brazil) or extracts of tea, coffee or cocoa were added at different concentrations to 50 ml of mineral medium contained in 125-ml Erlenmeyer flasks. Extracts (5% wt/vol) were obtained by boiling 5 g of tea (Matte Leão, Curitiba, PR, Brazil), coffee, and cocoa (Garoto, Vitória, ES, Brazil) powder for 10 min in water. The extracts were then filtered with qualitative filter paper and the volume was made up to 100 ml with distilled water. Cyclic AMP was added to the culture medium at 5, 10, 15, and 20 mM to verify whether PL could be induced by the exogenous addition of this nucleotide.

After inoculating the medium with 5×10^4 spores ml^{-1}, cultures were incubated at 25°C and 150 rpm for 48 h on a rotary shaker. Cultures were then harvested by filtering them through a 400-mesh sieve, and the filtrates were used for enzyme determinations. The experiments were carried out with three replications.

Growth determination and enzyme assay

Growth was measured by estimating cell dry weight according to Calam [7]. PL activity was determined according to Albersheim [2] by adding 0.5 ml of culture filtrate to 3.0 ml of 50 mM phosphate buffer, pH 6.0, containing 0.25% citric pectin (Sigma, St Louis, MO, USA). After incubation at 40°C for 30 min, 0.2 ml of 1 M HCl was added to the reaction mixture. One unit of PL activity (U) was defined as that amount of enzyme which produced one nmol of unsaturated uronides per ml of culture filtrate per minute.

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Pectin concentration in tea extract

Tea extract was treated with a commercial pectinolytic complex for 12 h. At the beginning and at the end of each reaction, reducing sugars and glucose were determined by the dinitrosalicyclic acid [14] and glucose oxidase [9] methods, respectively. To determine pectin concentration in tea extract, glucose content was subtracted from the total reducing sugars.

Results and discussion

Penicillium griseoroseum produced pectin lyase (PL) when cultured in mineral medium with sucrose supplemented with yeast, tea, coffee or cocoa extracts (Figure 1).

Tea contains 1.5–4.0% caffeine, 0.2–0.4% theobromine and about 0.02% theophylline [11]. The caffeine content of roasted coffee beans varies from 1 to 5% according to the blend and yield. Other methylxanthines, theobromine and theophylline, are present at low concentrations [3]. Caffeine is the main physiologically active component of tea and coffee. Cocoa powder is used for chocolate production and contains purines, theobromine, and caffeine [12].

In *Thermomonospora curvata*, theophylline and caffeine inhibit cyclic phosphodiesterase, an enzyme that degrades cAMP [19]. Tröger and Meyer [18] have also reported that theophylline is an inhibitor of phosphodiesterase in animal cells. Cyclic AMP acts as a second messenger in the cell, starting a cascade of protein kinases that influence many processes by means of protein-phosphorylations. The enzyme cascade sequence up to the generation of phosphoprotein is well understood, but the signal transmission that follows and leads to the triggering of a biological effect is not [18]. There are many isolated reports that cAMP is involved in the regulation of a host of cellular processes in bacteria, including the synthesis of induced enzymes such as cellulases [5]. In *Neurospora crassa*, Terenzi *et al* [17] showed that cAMP enhances invertase production. We suggest that the alkaloids present in tea, coffee, and cocoa extracts probably act as inducers of PL in *P. griseoroseum* (Figure 1) by promoting increases in the intracellular level of cAMP.

The highest PL activity was obtained with the addition of coffee extract to the medium (Figure 1). Since coffee is more expensive than tea, tea extract was used as an inducer in our studies.

The addition of 0.3% tea extract to the culture medium at 0, 12 or 24 h of cultivation showed that tea extract had a real inducing effect on PL activity (Figure 2). When no tea extract was added to the medium, no PL activity was detected. However, after 48 h of cultivation, mycelial mass reached 72% of the value obtained when tea extract was added at the beginning of incubation. Mycelial mass was similar for all cultures to which tea extract was added. The addition of tea extract at time zero induced PL activity after 12 h of incubation and an increase of 20–25% in PL activity was obtained after 48 h of growth. When tea extract was added to the cultures at other times, PL activity was detected only after 48 h of incubation (Figure 2).

The fungus also produced PL in the presence of tea extract in medium with no sucrose (Figure 3). In the medium with sucrose but without tea extract, the fungus was able to grow but did not produce PL. The higher the concentrations of tea extract added to the medium, the higher the amounts of PL synthesised by the fungus (Figure 3). The use of sucrose and 0.5% tea extract yielded levels of PL activity that were similar to those obtained with sucrose and 0.03% yeast extract (Figure 3), which is in accordance with results we described [4] under similar conditions. Mycelial growth did not increase proportionally to PL activity as a function of tea extract concentration. Thus, tea extract was really an inducer since the addition

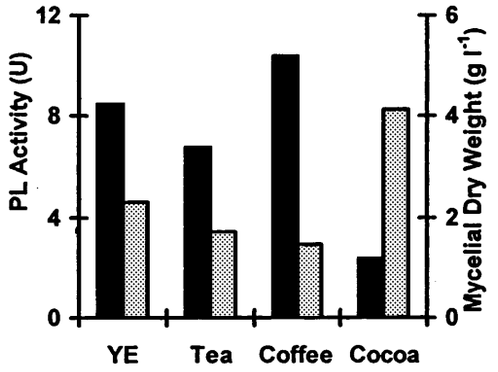

Figure 1 Pectin lyase (PL) activity (■) and mycelial dry weight (▨) of *P. griseoroseum* cultured on sucrose and yeast (YE), tea, coffee or cocoa extract.

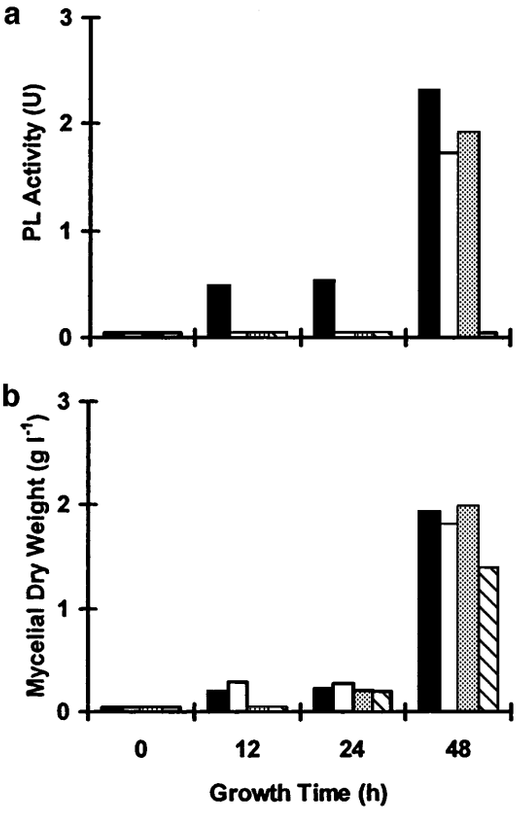

Figure 2 (a) Pectin lyase (PL) activity and (b) mycelial dry weight of *P. griseoroseum* cultured for 48 h on sucrose, in the absence (▨) or presence of 0.3% tea extract added at zero (■), 12 (□) or 24 h (▩) of growth.

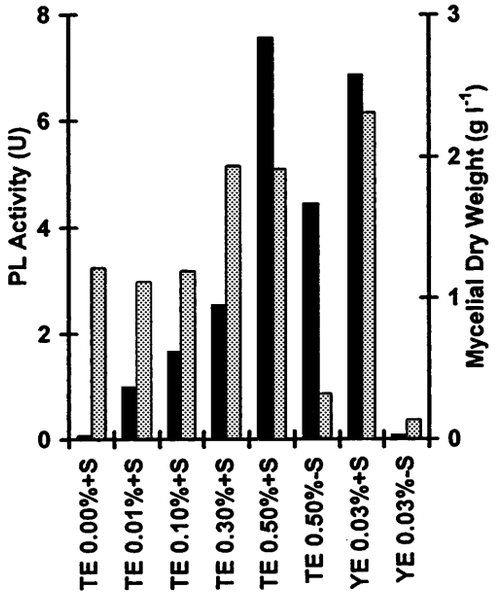

Figure 3 Pectin lyase (PL) activity (■) and mycelial dry weight (▨) of *P. griseoroseum* cultured on medium containing (+S) or lacking (–S) sucrose and yeast extract (YE) or different concentrations of tea extract (TE).

of either 0.01 or 0.1% tea extract induced PL and promoted mycelial growth similarly to sucrose in the absence of tea extract (no PL induced). Despite the reduced growth obtained when the fungus was grown in a medium with 0.5% tea extract alone (17% of the mycelial mass produced in a medium with sucrose), the fungus produced 59% of the PL activity observed with 0.4% sucrose and 0.5% tea extract (Figure 3). In this case, the PL activity determined per g of dry mycelium weight was 3.5 times that determined with sucrose and 0.5% tea extract, and 4.7 times that obtained with sucrose and 0.03% yeast extract. In this way, we concluded that PL production was affected by the concentration of tea extract and that the induction by tea extract differed from that obtained with yeast extract since it did not require the presence of sucrose.

Since tea extract was obtained by boiling plant material, pectic substances in the solution could act as inducers. The action of a commercial pectinase on tea extract showed that it contains only 0.05% pectic substances (data not shown). Therefore when 0.5% tea extract was added to 50 ml of mineral medium, the amount of pectic substances present was 0.005%. The addition of 0.0001–0.005% citric pectin to the culture medium promoted no significant induction of PL (Figure 4b). Increasing amounts of the enzyme were induced by the addition of 0.01–0.5% tea extract (Figure 4a) that contained amounts of pectic substances, equivalent to 0.0001 and 0.005%, respectively. Therefore, PL was not induced by pectic substances present in the tea extract.

Since tea constituents contain high concentrations of caffeine, theobromine, and theophylline [11], and such methylxanthines act as phosphodiesterase inhibitors which increase the levels of endogenous cAMP [15], these compounds could be inducing PL activity via cAMP metabolism [13]. Pall [15] reported that very high concentrations of

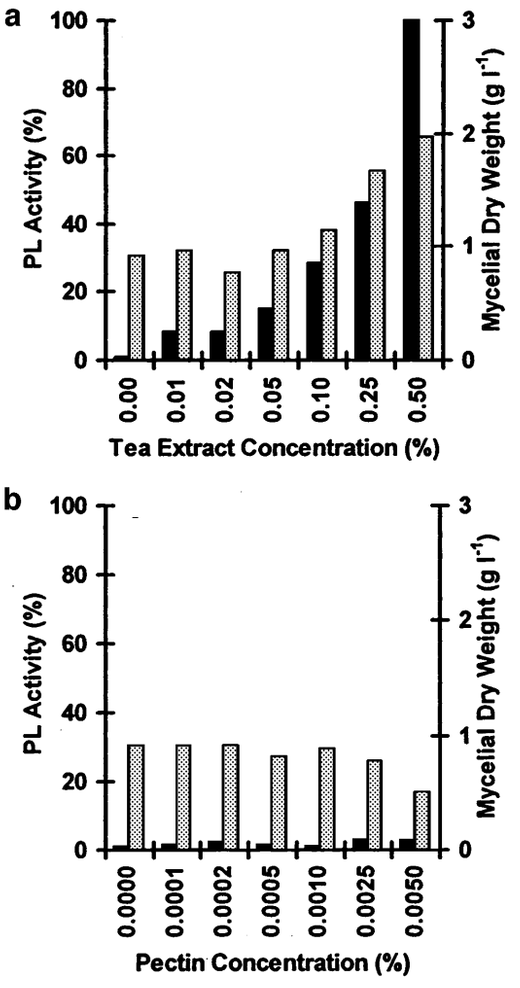

Figure 4 Pectin lyase (PL) activity (■) and mycelial dry weight (▨) of *P. griseoroseum* cultured on sucrose and (a) tea extract from zero to 0.5% or (b) citric pectin (Sigma) from zero to 0.0050%.

cAMP (5–10 mM) are required to obtain only partial phosphodiesterase inhibition as a result of the low permeability of cells to cAMP and of its fast intracellular turnover. Exogenous concentrations of 5 and 10 mM cAMP induced 20 and 30%, respectively, of the PL activity obtained by cultivating *P. griseoroseum* in 0.03% yeast extract (Figure 5), suggesting the occurrence of partial phosphodiesterase inhibition. Higher concentrations of cAMP (15 and 20 mM) also induced PL, albeit at lower levels than those obtained with 10 mM cAMP. Cyclic AMP at 15 and 20 mM induced 80 and 50%, respectively, of the PL activity induced by 10 mM cAMP (data not shown).

We suggest that methylxanthines present in tea extract may induce PL by inhibition of phosphodiesterases with a consequent increase in the levels of endogenous cAMP. Our results indicate that tea extract is a good substitute for yeast extract as a PL inducer in *P. griseoroseum*. Additionally, tea extract is inexpensive and is therefore an economical substrate for the industrial production of PL.

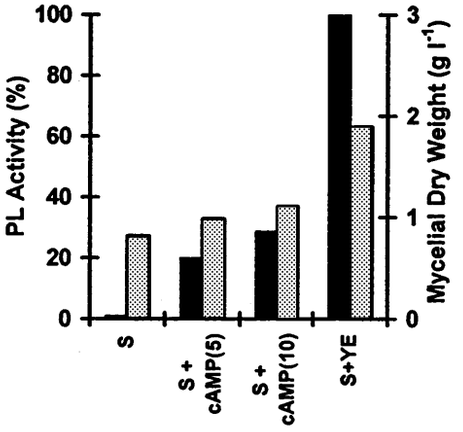

Figure 5 Pectin lyase (PL) activity (■) and mycelial dry weight (▨) of *P. griseoroseum* cultured on sucrose.

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References

1 Alanã A, MJ Llama and JL Serra. 1991. Purification and some properties of the pectin lyase from *Penicillium italicum*. FEBS Lett 280: 335–340.
2 Albersheim P. 1966. Pectin lyase from fungi. In: Methods in Enzymology (Neufeld ES and V Ginsburg, eds), Vol 8, pp 628–635, Academic Press, New York.
3 Arnaud MJ. 1987. The metabolism of coffee constituents. In: Coffee — Physiology (Clarke RJ and R Macrae, eds), Vol 3, pp 33–55, Elsevier Applied Science, London.
4 Baracat-Pereira MC, JLC Coelho and DO Silva. 1994. Production of pectin lyase by *Penicillium griseoroseum* cultured on sucrose and yeast extract for degumming of natural fibres. Lett Appl Microbiol 18: 127–129.
5 Botsford JL and JC Harman. 1992. Cyclic AMP in prokaryotes. Microbiol Rev 56: 100–122.
6 Brumano MHN, JLC Coelho, EF Araujo and DO Silva. 1993. Production of pectin lyase by *Penicillium griseoroseum* as a function of the inoculum and culture conditions. World J Microbiol Biotechnol 9: 225–228.
7 Calam CT. 1969. The evaluation of mycelial growth. In: Methods in Microbiology (Norris JR and DW Ribbons, eds), Vol 1, pp 567–591, Academic Press, London.
8 Chesson A. 1980. A review — Maceration in relation to the post-harvest handling and processing of plant material. J Appl Bacteriol 48: 1–45.
9 Day DS and WE Workman. 1982. A kinetic assay for cellulases. Anal Biochem 126: 205–207.
10 Deshpande KS and K Gurucharanam. 1985. Degumming of ramie fibres: role of wall degrading enzymes of *Aspergillus versicolor*. Indian J Bot 8: 79–81.
11 Finger A, S Kuhr and UH Engelhardt. 1992. Review — Chromatography of tea constituents. J Chromatog 624: 293–315.
12 Heath HB. 1981. Source Book of Flavors. The Avi Publishing Company, Westport.
13 Hunter T. 1987. A thousand and one protein kinases. Cell 50: 823–829.
14 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426–428.
15 Pall ML. 1981. Adenosine 3′, 5′-phosphate in fungi. Microbiol Rev 45: 462–480.
16 Sharma HSS. 1988. Microorganisms in processing of flax. Biotechnology 6: 746–755.
17 Terenzi H, HF Terenzi and JA Jorge. 1992. Effect of cyclic AMP on invertase activity in *Neurospora crassa*. J Gen Microbiol 138: 2433–2439.
18 Tröger U and FP Meyer. 1995. Influence of endogenous and exogenous effectors on the pharmacokinetics of theophylline – focus on biotransformation. Clin Pharmacokinet 28: 287–314.
19 Wood WW, DG Neubauer and FJ Stutzenberger. 1984. Cyclic AMP levels during induction and repression of cellulase biosynthesis in *Thermomonospora curvata*. J Bacteriol 160: 1047–1054.