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Regulation of the production of polygalacturonase by *Aspergillus terreus*

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Summary

The synthesis of polygalacturonase (PG) (EC 3.2.1.15) by a strain of *Aspergillus terreus* was induced by polygalacturonic acid and repressed by glucose, galactose or fructose even in the presence of the inducer. The production of PG increased when the mycelium was washed free of glucose and incubated in a glucose-free medium containing the inducer, a fact that indicated the reversibility of the repression mechanism. When Actinomycin D and cycloheximide were added to the culture medium, the synthesis of PG ceased. PG synthesis increased 43% with the addition of methionine and 64% both with leucine and with tyrosine. Specific productivity with leucine was 210% higher than that of the control as against 149% with methionine and 70% with tyrosine. The results obtained suggest that PG synthesis is regulated by leucine.

Introduction

Pectic substances are complex polysaccharides of plant origin that contain a large proportion of anhydrous galacturonic acid subunits linked by α 1–4 glycosidic linkages. Pectin and pectate are both pectic substances; the former is partially methylesterified while the latter is free of methyl groups. These compounds are hydrolysed by pectinolytic enzymes. Pectolytic capacity has been widely reported and thoroughly studied in bacteria and fungi because it is thought to play an important role in plant diseases. Pectolytic enzymes are used in the industrial production of fruit juice because the partial degradation of pectin and other related substances enhances the overall yield of the extractive process.

Catabolic repression of the synthesis of inducible enzymes by glucose, fructose and intermediates of the glycolytic pathway has been observed in many microorganisms (Leone & Van Den Heuvel 1986). Studies concerning polygalacturonase (PG) (EC 3.2.1.15) from *Aspergillus niger* have shown that PG synthesis is induced by pectin as well as by galacturonic and polygalacturonic acid (PGA) at the transcription level (Maldonado *et al.* 1989a). Glucose, on the other hand, represses PG synthesis by a catabolic repression mechanism at the translation level (Maldonado *et al.* 1989b).

Recently, numerous studies have been carried out concerning the molecular biology of pectinases in various microorganisms such as bacteria and fungi. Bussink *et al.* (1990, 1991b) have recently isolated the genes encoding the two major endopolygalacturonases. A pectin lyase gene family of *A. niger* has also been isolated lately (Gysler *et al.* 1990; Harmser *et al.* 1990). Another important point concerning the molecular biology of pectinases was the obtention by Khanh *et al.* (1990) of a cDNA clone of pectinesterase.

As pointed out by several investigators (Hornewer *et al.* 1987; Aguilar & Huitron 1990), most PGs are produced by a wide range of microorganisms, among them *A. terreus*.

We thought that *A. terreus*, given its environment and the decay it produces on vegetation, was likely to produce cellulases and pectinases. Once we had confirmed that it was so, we decided to use this microorganism in our investigation. This study describes the regulation of PG synthesis using different carbon sources and amino acids in order to maximize enzyme yields.

Materials and Methods

Microorganism

The strain *A. terreus* was obtained from the Microbiological Institute stock (Universidad Nacional de Tucumán), cultured in Czapek agar slant tubes, incubated at 30 °C and stored at 4 °C.

Fermentation medium

In g/l: $NaNO_3$: 3, K_2HPO_4: 1, KCl: 0.5, $MgSO_4$: 0.5, PGA: 5. The alternative carbon sources used were added

as indicated in Figure 1. Actinomycin D and cycloheximide were added at 15 and 20 μg/ml concentrations, respectively, as previous tests demonstrated that higher concentrations impaired the growth of the microorganism while lower ones had no significant influence on the synthesis of the enzyme. The amino acids were tested at a concentration of 0.02%. Fifty milliliter of the fermentation medium were inoculated with about 2.6×10^6 spore/ml from a stock culture and incubated in a shaker at 30 °C for 4 days.

Biomass determination

The mycelium obtained after filtration was rinsed with sterile distilled water and dried at 90 °C until constant weight was reached.

PG assay

One milliliter of filtrate was added to a solution containing 1 ml of 0.9% PGA in 0.1 M acetate buffer, pH 4.5. After incubation at 37 °C for 60 min, reducing sugars were determined by the dinitrosalicylic acid method (Miller 1959) using galacturonic acid as a reference. One unit of PG was defined as the amount of enzyme that releases one micromole of galacturonic acid per minute.

SDS–polyacrylamide gel electrophoresis (PAGE)

Crude cell-free samples were concentrated by lyophilization and resuspended in distilled water to obtain a protein concentration of 150 μg/ml. SDS–PAGE was performed according to Laemmli (1970), with a resolving gel containing 10% acrylamide and 2.7% bis-acrylamide. The stacking gel contained 4% acrylamide and 2.7% bis-acrylamide. The SDS concentration was 0.1% in the gel and in the running buffer. Twenty microliter samples were maintained at 100 °C for 60 s in a sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris–HCl, pH 6.8 and 0.005% bromophenol blue). Samples were electrophoresed at 80 V for 2–2.5 h through 1.5 mm gel in a vertical slab gel unit Mini Protean II Electrophoresis Cells (Bio-Rad Laboratories). The gels were stained with silver stain (Oakley *et al.* 1980).

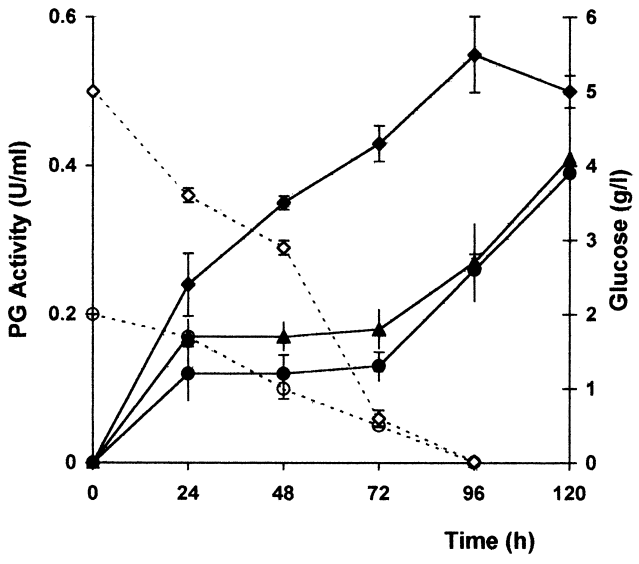

Figure 1. Effect of the initial concentration of glucose on PG production. The fermentation medium contained 5 g/l PGA as an inducer. Initial glucose in g/l: (◆), 0; (▲), 2; (●), 5. Dashed lines indicate the course of glucose uptake during fermentation.

Agarose–PGA overlays for in situ *detection of pectinolytic activity*

The agarose–PGA overlays (Ried & Collmer 1985) were prepared by dissolving the agarose by heating in 0.1 M acetate buffer, pH 4.5; then PGA was added and mixed. The mixture was then cast on a plate sandwich with 0.75 spacers in a gel caster previously heated at 37 °C. Final concentrations within the gels were 1% agarose and 0.2% PGA. After incubation with polyacrylamide gel the agarose overlays were stained with a solution of 0.05% ruthenium red for 30 min.

The results reported in this work are the average of duplicate determinations.

Results and Discussion

Maximum PG production in liquid cultures of *A. terreus* was reached after a 4-days incubation period. The fermentation medium containing 5 g/l fructose, glucose or galactose as a carbon source produced low PG levels, similar results being obtained when using glucose 5 g/l plus different PGA concentrations (0.1 to 5 g/l). Enzymatic activity increased at higher PGA concentrations and reached a maximum when the fungus was grown in PGA 5 g/l in the absence of glucose (Table 1).

On the basis of the above, we considered that the nature and concentration of the carbon source might have a strong influence on PG production. According to the results shown in Table 1, PG is an inducible enzyme.

Table 1. Effect of different carbon sources on PG production by *A. terreus*.

Carbon source	Enzymatic activity (U/ml)	Standard errors
5 g/l fructose	0.003	0.0090
5 g/l glucose	0.005	0.0009
5 g/l galactose	0.009	0.0014
5 g/l glucose + 0.1 g/l PGA	0.060	0.0110
5 g/l glucose + 0.2 g/l PGA	0.161	0.0070
5 g/l glucose + 0.5 g/l PGA	0.230	0.0190
5 g/l glucose + 1 g/l PGA	0.315	0.0170
5 g/l glucose + 2 g/l PGA	0.496	0.0300
5 g/l glucose + 5 g/l PGA	0.530	0.0110
Control (5 g/l PGA)	0.836	0.0110

However, it is also apparent that there is a basal level of constitutive enzyme, since it was produced in a medium containing sugars other than PGA. The inducer plus glucose or galactose affected PG synthesis. Even when the PGA concentration was the same as that of glucose, the PG level was lower than that of the control, a fact that showed that the rate of production of the enzyme was not completely restored. The catabolic repression effect was studied on the basis of these results.

The effect of different glucose concentrations (0.5, 2 and 5 g/l) on PG activity was determined in order to differentiate it from the effect of glucose on the synthesis of the enzyme, the same enzymatic activity values (0.38 U/ml) being obtained with the three glucose concentrations assayed. This fact suggests that the decrease in the content of enzyme, together with the increase in the initial concentration of glucose in the fermentation medium, was due to the negative effect of the sugar on the synthesis of the enzyme. Moreover, when the level of available glucose decreased as a result of culture growth, the synthesis of the enzyme increased (Figure 1) until 96 h of culturing. From then onwards there was a decrease in enzymatic activity; no further enzymes were synthesized and there was a loss in the activity of the existing enzymes. The effect of glucose was further investigated with mycelium harvested during the stationary phase in order to minimize the influence of growth (Figure 2). When glucose was added at the beginning of the experiment, PG production ceased even though the inducer, PGA, was also present. However, when after 6 h of incubation the mycelium was washed free of glucose and placed in a glucose-free medium containing PGA, the synthesis of PG started again, thus proving the reversible characteristic of the repression mechanism of the synthesis of PG by glucose.

Table 2. Effect of different amino acids (0.02%) on PG production by *A. terreus*.

Aminoacids	Enzymatic activity (U/ml)	Standard errors
Control	0.633	0.0127
Histidine	0.496	0.0135
Asparagine	0.160	0.0055
Arginine	0.314	0.0140
Proline	0.107	0.0045
Alanine	0.047	0.0075
Cysteine	0	0
Methionine	0.903	0.0040
Leucine	1.036	0.0135
Tyrosine	1.036	0.0210
Isoleucine	0.229	0.0040
Serine	0.175	0.0066
Phenylalanine	0.120	0.0040

The addition of a repressor of transcription such as actinomycin D reduced PG production during the first 4 h, after which time synthesis of the enzyme ceased. This event suggests that translation occurs as long as there is active mRNA available in the system. When cycloheximide, a repressor of translation, was added instead of actinomycin D, PG production decreased markedly. The above results led us to the following

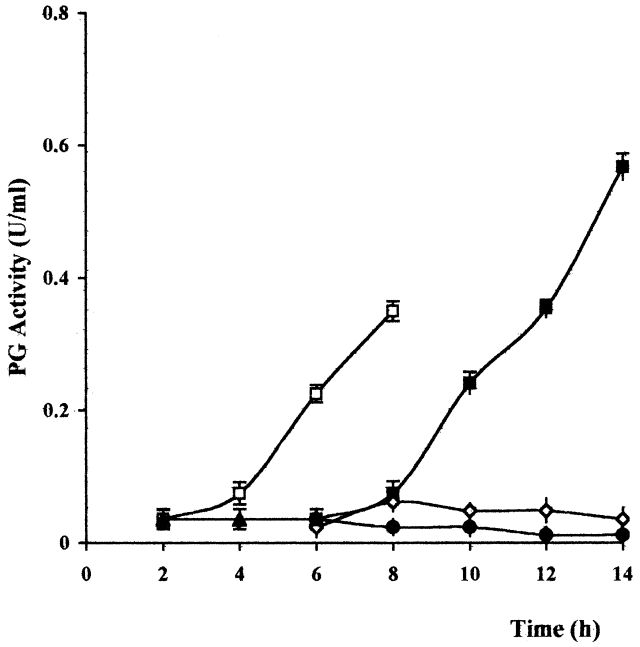

Figure 2. Reversibility of the repression by glucose on PG production. Effect of actinomycin (Am) and cycloheximide (CH). The microorganisms was grown in a fermentation medium containing PGA as an inducer at 30 °C. At the stationary phase of growth (110 h) the mycelium was collected by filtration through SSN595 filter paper, carefully washed with distilled water and incubated in a fermentation medium containing the inducer, either with glucose (5 g/l) (▲) or without it (□). After 6 h the mycelium incubated in the presence of glucose was collected and washed as described above. Three aliquots of the mycelium were then incubated, one in the fermentation medium containing the inducer (■), another in the medium with the inducer plus CH (◇) and the third in the medium with the inducer plus Am (●).

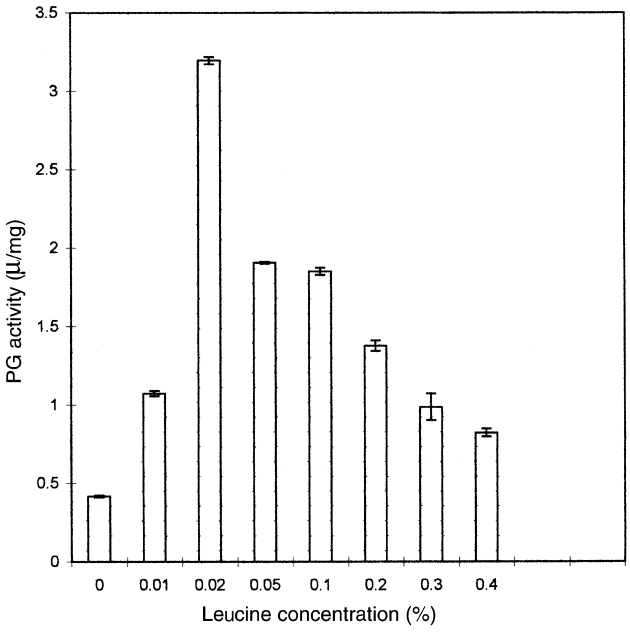

Figure 3. Effect of different leucine concentrations on PG production by *A. terreus*.

conclusions: (a) the regulation of PG synthesis in the strain under study takes place at the transcription level; (b) the enzyme was induced by PGA and repressed by glucose, fructose and galactose.

The effect of different amino acids on the synthesis of PG was also studied (Table 2). Results showed an increase of 43% in enzymatic synthesis when using methionine and of 64% with either leucine or tyrosine. The biomass values reached when methionine and leucine were added to the medium were 4.3 and 3.5 g/l, respectively. These values were very similar to that of the control (4.5 g/l) whereas, when using tyrosine, the value reached was 10.5 g/l. When comparing these biomass values with those of enzymatic activity, it can be seen that the most remarkable effect was produced by the addition of leucine. Histidine and arginine had no effect on PG synthesis while proline, alanine, asparagine, cysteine, isoleucine, serine and phenylalanine were inhibitory. The fact that cysteine inhibited PG production in *A. terreus* is in agreement with a previous study of *A. niger* by Chopra & Metha (1985). For accurate comparison, it was necessary to calculate the specific activity of the enzyme as the level of enzyme was similar in cultures grown with leucine, tyrosine and methionine, but the biomass concentration was different. Maximum specific productivity (210% higher than that of the control) was obtained with leucine, while the values obtained with methionine and tyrosine were 149 and

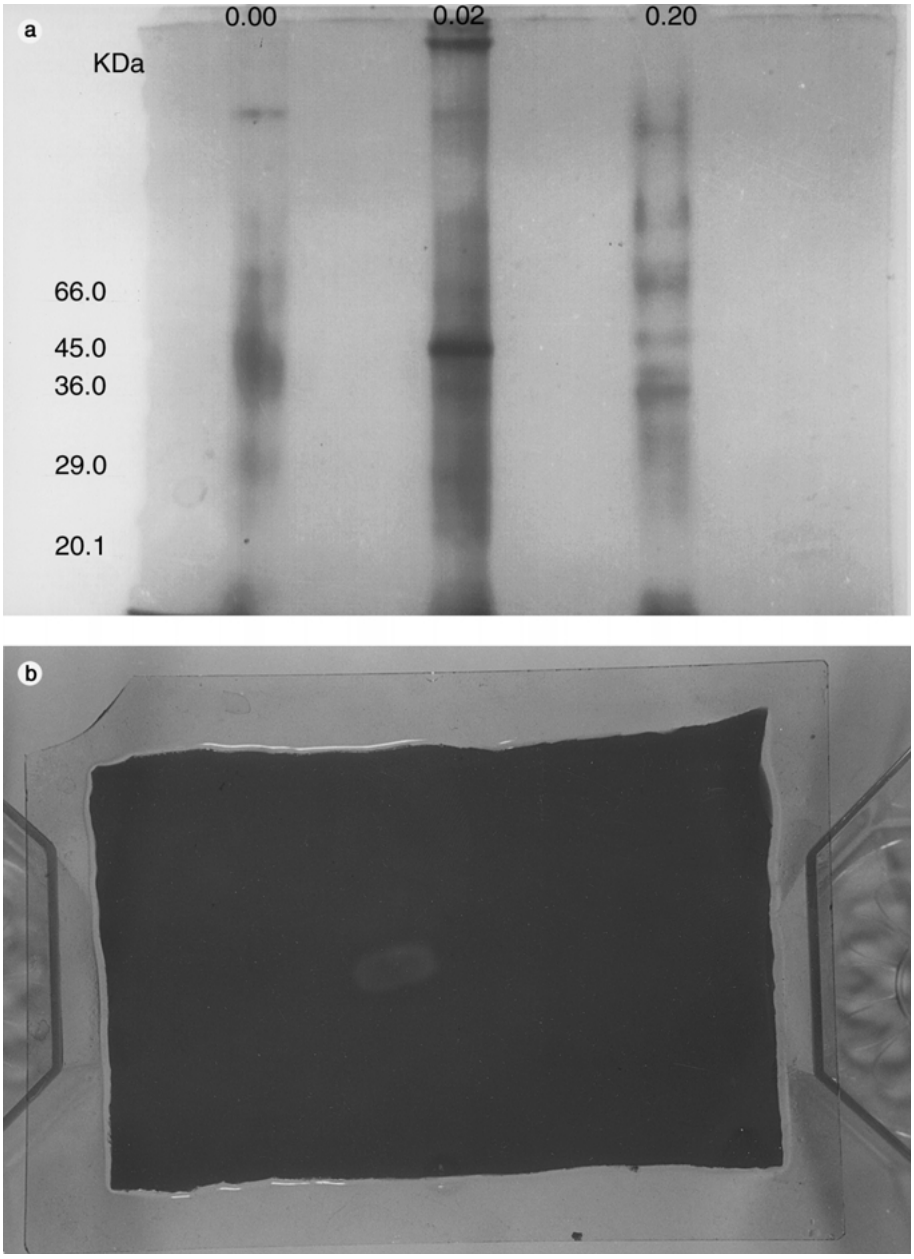

Figure 4. (a) SDS–PAGE of culture filtrates of *A. terreus* at 0, 0.02 and 0.2% leucine. Electrophoresis on 10% acrylamide slab gel was according to Laemmli, run at 80 V constant current for 2.5 h. Crude cell-free samples were concentrated by lyophilization and applied to each well without further treatment. (b) PG activity on PGA–agarose overlays of a culture filtrate of *A. terreus* at 0.02% leucine.

70% higher, respectively.

On the basis of the above results, different concentrations of leucine (from 0.01 to 0.4%) were tested, the highest stimulatory effect being obtained with 0.02% leucine (Figure 3). Similar results were obtained by Astapovich & Rozhkova (1983), who studied pectolytic enzyme synthesis by *Sclerotina sclerotium*, the highest stimulatory effect being obtained with 0.02% histidine. The effect of the different above-mentioned amino acids on the synthesis of PG appears not to be related to the structure of the R group since, while tyrosine is polar, neither leucine nor methionine are.

In order to find out whether the increase in PG production was due to the effect of leucine on the synthesis of the enzyme, the samples of the supernatants obtained after incubation for 120 h were electrophoresed (Figure 4 (a)).

The 45 kDa band belongs to PG (Aguilar *et al*. 1991; Blanco *et al*. 1994) (Figure 4(b)). This band exhibited a remarkable increase in the culture supplemented with 0.02 or 0.2% leucine with respect to the control without leucine. From these results, it can be concluded that PG synthesis could be regulated by the presence of leucine in the culture medium.

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