Extracellular polygalacturonases from *Penicillium frequentans*: separation and regulatory aspects

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Extracellular polygalacturonase activities of *Penicillium frequentans* were induced by pectin and repressed, but not inactivated, by glucose. The absence of a carbohydrate source derepressed 50% of viscosity-diminishing activity and 36% of reducing-groups-releasing activity compared to production in the presence of pectin. High concentrations (30 mM and 50 mM) of D-galacturonic acid reduced only the viscosity-diminishing activity, and under these conditions the fungus grew poorly. Neither effect was observed if the mycelium had been previously induced by pectin. Polygalacturonase activities produced in the presence of pectin were separated by ion exchange chromatography. These enzymes eluted in six peaks, characterized as exopolygalacturonases I, II and III and endopolygalacturonases, I, II and III.

Introduction

Fungal pectinolytic complexes are generally constituted by deesterifying enzymes (pectin esterases) and depolymerizing enzymes (endopolygalacturonase, exopolygalacturonase, exopectate lyase, endopectate lyase and endopectin lyase) (Rombouts & Pilnik, 1980). These enzymes have commercial interest for the food and textile industries, and are also involved in phytopathogenic processes. Fungal pectinase production depends on environmental conditions and the characteristics of the organism (Strzelczyk *et al.*, 1987; Manachini *et al.*, 1988). It has been asked how pectic substances which cannot permeate into the cytoplasm can induce the synthesis of pectinases. A likely explanation is that micro-organisms produce low constitutive levels of extracellular enzyme activities. These partially degrade pectic substances, releasing low-molecular-mass products which induce the pectinase synthesis (Leone & Van den Heuvel, 1987). *Aspergillus* constitutively produces exo-pectinase when grown on glucose, sucrose, fructose, glycerol or galacturonic acid (Aguilar & Huitron, 1990).

In this work we investigated some regulatory aspects of the production of polygalacturonase by *Penicillium frequentans*, and the biochemical heterogeneity of these enzymes.

Methods

Micro-organism. *Penicillium frequentans* was isolated from Brazilian soil samples (Said *et al.*, 1991), and deposited in the collection of the Fundação Tropical de Tecnologia e Pesquisa 'André Tosello' (Campinas São Paulo, Brazil). The organism was maintained by weekly transfers on slants of PDA medium, incubated at 30 °C.

Enzyme production conditions. The culture medium contained (g l⁻¹): KH₂PO₄, 2.0; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 1.0; Na₂HPO₄·2H₂O, 0.9; yeast extract (Difco), 1.0; supplemented with 0.5% pectin, or according to the experimental protocol. The initial medium pH was adjusted to 2.5. The medium was inoculated with 5 × 10⁶ spores ml⁻¹ and incubated with agitation (100 r.p.m.) at 30 °C. The mycelium was separated from the culture fluid by filtration and the culture filtrate was dialysed against 25 mM-Tris/acetate buffer (pH 6.5) containing 1 mM-EDTA (buffer A).

Enzyme assays. Polygalacturonase activity was quantified by measuring the release of reducing sugar groups, by the dinitrosalicylic acid method (Miller, 1959) at pH 4.8 and 50 °C using 1.0% (w/v) polygalacturonic acid sodium salt (NaPP) in 25 mM-sodium acetate buffer (buffer B). One activity unit was defined as the amount of enzyme which released 1 µmol of reducing sugar groups per min, using D-galacturonic acid as standard. Polygalacturonase activity was also assayed by changes in viscosity. Viscosity-diminishing activity was determined by measuring the decrease in relative viscosity of a 0.5% (w/v) solution of pectin in buffer B, at 50 °C using a Ostwald viscometer with a water flow time of 23 s, calculated according to Mill & Tuttobello (1961). One relative viscometric unit was defined as the amount of enzyme which reduced the initial viscosity of pectin solution by 50% in 1 min of reaction. Pectin esterase activity was assayed with a 1% (w/v) pectin solution in buffer A as substrate, by continuous titrimetric determination of the carboxyl groups liberated from the methylester bonds after 2 h incubation at 50 °C. One unit was the amount of enzyme which released one microequivalent of carboxyl

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Abbreviation: NaPP, polygalacturonic acid sodium salt.

Fig. 1. Effect of glucose on the production of polygalacturonase by *Penicillium frequentans*, using 0.5% (w/v) pectin as inducer (●) and with addition of 30 mM-glucose at zero time (○) or after 12 h incubation (□). (a) Reducing-groups-releasing activity; (b) viscosity-diminishing activity.

groups per min under the above conditions. Transeliminase activity was assayed by measuring at 50 °C the increase in A_{235} of the unsaturated products of degradation of 1% (w/v) pectin or NaPP in 25 mM-Tris/HCl buffer, pH 8.5, according to Albersheim (1966). One activity unit was defined as the amount of enzyme which released one μmol of unsaturated product per minute at 50 °C. Protein was determined by the Lowry method using bovine serum albumin (Sigma) as standard or by measuring A_{280} . For dry weight determinations, the mycelium was rinsed and dried at 70 °C until constant weight was attained.

Separation of pectic enzymes. The enzyme activities in dialysed culture filtrates were separated by two steps of column chromatography. Samples (50 ml) of the crude filtrate were initially applied to a DEAE-Sephacel column (1.6×25 cm) equilibrated with buffer A and eluted by stepwise (20, 40, 150 and 300 mM) increases in NaCl concentration in the same buffer. Fractions (5.5 ml) were collected at a flow rate of 48 ml h^{-1} , and the enzyme activities were determined. The protein not adsorbed on to the DEAE-Sephacel column (Pool I) was dialysed against 25 mM-sodium acetate buffer pH 5.0, containing 1 mM-EDTA (buffer B) and was applied to a CM-Cellulose column (1.6×25 cm) equilibrated with the same buffer. The protein was eluted by increasing NaCl concentrations (50 and 150 mM) in buffer B. Fractions (5.5 ml) were collected at a flow rate of 33.5 ml h^{-1} . The products of enzymic degradation of NaPP were examined by TLC (Fonseca *et al.*, 1991).

Fig. 2. Effect of D-galacturonic acid on growth (upper panels) and production of polygalacturonase (lower panels) by *Penicillium frequentans*. (a) Conidia ($5 \times 10^6 \text{ ml}^{-1}$) incubated for 17 h in a medium supplemented with 0.5% (w/v) pectin (P), or D-galacturonic acid (GA), or without carbohydrate source (WCS). (b) Mycelia obtained from 17 h cultures supplemented with 0.5% (w/v) pectin, rinsed and transferred to fresh media containing 0.5% (w/v) pectin, D-galacturonic acid or without carbohydrate source, and reincubated for a further 6 hours. Activities are expressed in percentages relative to those of a culture grown for 17 h (a) or 6 h (b) in the presence of 0.5% (w/v) pectin. □, Viscosity-diminishing activity; ■, reducing-groups-releasing activity.

Chemicals. Pectin, D-galacturonic acid, polygalacturonic acid sodium salt, CM-Cellulose and DEAE-Sephacel were from Sigma. All other chemicals were of the best quality available.

Results and Discussion

Carbon source regulation of polygalacturonase production

When *Penicillium frequentans* was grown for 12 h in a medium supplemented with 80 mM-glucose, reducing-group-releasing and viscosity-diminishing activity levels were very low, but fungal growth was good (not shown). When the cultures were first grown on 0.5% pectin for 12 h, and 30 mM-glucose was then added, the enzyme activities already present in the culture medium remained stable for about 12 h, increasing again thereafter (Fig. 1). This result suggested that glucose repressed the synthesis but did not cause inactivation of these enzymes. If pectin and glucose were simultaneously present at the inoculum time, the fungus grew rapidly

Fig. 3. Separation profiles on DEAE-Sephacel (a) and CM-Cellulose (b) of the extracellular polygalacturonases produced by *Penicillium frequentans*. Details are described in the text. The dialysed culture fluid was applied to a column (1.5 × 27 cm). The columns were eluted by stepwise gradients of NaCl as indicated. Fractions of 4.8 ml were collected and analysed for protein (●), viscosity-diminishing activity (○) and reducing-sugar-releasing activity (□).

and viscosity-diminishing activity and reducing-groups-releasing activity were very low for the first 12 h. After this time the enzyme levels increased, probably as a consequence of glucose exhaustion. On the other hand, when the organism was incubated in a medium without carbohydrate source, after 17 h viscosity-diminishing and reducing-groups-releasing activities were 50% and 36%, respectively, of those produced in cultures supplemented with 0.5% (v/v) pectin, (Fig. 2a). These results suggested that the enzymes were partially derepressed in the absence of carbohydrate, in agreement with the results reported by Aguilar & Huitron for *Aspergillus* (1990). The effect of D-galacturonic acid on the production of pectinases was also investigated (Fig. 2a). High concentrations of D-galacturonic acid negatively affected the level of viscosity-diminishing activity, but not that of the reducing-groups-releasing activity, which was the same as that of a culture incubated in a medium without a carbohydrate source. Under these conditions fungal growth was poor. The different effects of galacturonic acid on viscosity-diminishing activity and reducing-groups-releasing activity was also reported by Aguilar & Huitron for *Aspergillus* sp. (1987). On the other hand, Leone & Van der Heuvel (1987) reported that the number of pectinases produced by *Botrytis cinerea* was influenced by the type and concentration of the substrate and consisted of polygalacturonase and pectinesterase isoenzymes. Our results suggested that a high concentration of galacturonic acid added at the start of the culture repressed only the synthesis of viscosity-diminishing activity, probably because polygalacturonase activity produced by *Penicillium frequentans* consisted of various enzymes whose synthesis was independently controlled. When a culture previously grown for 17 h in a medium supplemented with 0.5% pectin was transferred to a fresh medium containing D-galacturonic acid at various concentrations, the mycelium was able to grow further and to secrete pectinolytic enzymes after 6 h (Fig. 2b). These results suggest that induction by pectin may be a requirement for induction of D-galacturonic acid metabolism, and that this substance is perhaps not the only, or the best, inducer of the pectinolytic system in *Penicillium frequentans*.

Separation of polygalacturonase activities

A crude enzyme preparation was applied to a DEAE-Sephacel column and five peaks containing pectinolytic activities were resolved (Fig. 3a). Peak I was eluted with the equilibrating buffer. Elution of the column with a NaCl stepwise gradient yielded four other peaks containing pectinolytic activities. Peaks I, III, IV and V contained reducing-groups-releasing and viscosity-diminishing activities. The enzyme present in peak II

showed predominantly reducing groups-releasing activity; but some viscosity-diminishing activity could be detected after long periods of incubation (1–5 h). The recovery of reducing-groups-releasing and viscosity-diminishing activities initially present in the culture filtrate was 52.7% and 91.7%, respectively. The pooled fractions of peak I were dialysed against buffer B and rechromatographed on a CM-cellulose column (Fig. 3b). The stepwise gradient elution of the column with NaCl yielded two peaks (I.1 and I.2) containing reducing-groups-releasing and viscosity-diminishing activities, with a yield of 99.4% and 64.6% respectively, as compared to that of peak I. Transeliminase activity was not detected, probably because the initial pH of the culture was 2.5 and, as we previously reported, at this pH, lyase activity is not induced in *Penicillium frequentans* (Said *et al.*, 1991). The endo- or exo-nature of the enzymes present in the resolved peaks was determined by comparing the release of reducing groups and the viscosity changes of a pectin solution as a function of time. The enzymes present in peaks I.1, I.2 and V hydrolysed 3.3%, 14% and 12.5% of glycosidic bonds of NaPP, respectively, at the time when the viscosity of the reaction mixture was decreased by 50%. These polygalacturonases appeared to be endopolygalacturonases, since they promoted a sharp drop in viscosity (half value at 10 to 15% hydrolysis) with only a slow increase of reducing end groups, (Rombouts & Pilnik, 1980). The enzymes of peaks I.1, I.2 and V were named endo-PG I, II and III, respectively. The enzymes present in peaks II, III and IV hydrolysed 69%, 40% and 35% of glycosidic bonds, respectively, at the time when the viscosity of reaction mixture was decreased by 50%, and thus these enzymes appeared to be exopolygalacturonases, and were named exo-PG I, II and III. These results were confirmed by examining the products of NaPP degradation by TLC, which showed that the hydrolysis by enzymes from peaks I.1, I.2 and V produced little or no D-galacturonic acid, while those of peaks II, III and IV produced high concentration of this sugar after 30 min reaction. When 1% (w/v) NaPP was the substrate, reducing-groups-releasing activity in all peaks was higher than when 1% (w/v) pectin was used as substrate, suggesting that NaPP was the preferred substrate of these enzymes and, since lyase activity was not detected under these environmental conditions, it can be concluded that these enzymes are polygalacturonases.

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