Pectinase production by *Neurospora crassa*: purification and biochemical characterization of extracellular polygalacturonase activity

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The production of pectinase was studied in *Neurospora crassa*, using the hyperproducer mutant *exo-1*, which synthesized and secreted five to six times more enzyme than the wild-type. Polygalacturonase, pectin lyase and pectate lyase were induced by pectin, and this induction was glucose-repressible. Polygalacturonase was induced by galactose four times more efficiently than by pectin; in contrast the activity of lyases was not affected by galactose. The inducing effect of galactose on polygalacturonase was not glucose-repressible. Extracellular pectinases were separated by ion exchange chromatography. Pectate and pectin lyases eluted into three main fractions containing both activities; polygalacturonase eluted as a single, symmetrical peak, apparently free of other protein contaminants, and was purified 56-fold. The purified polygalacturonase was a monomeric glycoprotein (38% carbohydrate content) of apparent molecular mass 36.6–37.0 kDa (Sephadex G-100 and urea-SDS-PAGE, respectively). The enzyme hydrolysed predominantly polypectate. Pectin was also hydrolysed, but at 7% of the rate for polypectate. K_m and V_{max} for polypectate hydrolysis were 5.0 mg ml⁻¹ and 357 µmol min⁻¹ (mg protein)⁻¹, respectively. Temperature and pH optima were 45 °C and 6.0, respectively. The purified polygalacturonase reduced the viscosity of a sodium polypectate solution by 50%, with an increase of 7% in reducing sugar groups. The products of hydrolysis at initial reaction times consisted of oligogalacturonates without detectable monomer. Thus, the purified *Neurospora crassa* enzyme was classified as an endopolygalacturonase [poly(1,4- α -D-galacturonide) glycanohydrolase; EC 3.2.1.15].

Introduction

The biological decomposition of pectin is an important process in biomass degradation. In nature, pectin is degraded by enzymic systems produced by a wide variety of saprophytic and phytopathogenic micro-organisms, including bacteria and fungi (Rombouts & Pilnik, 1980). Pectinases hydrolyse pectin by different mechanisms, and are divided into those which attack methyl-esterified pectin (pectinic acids) and those which act on de-esterified pectin (pectic acids). Within each class can be distinguished hydrolases and lyases, which may cleave randomly (endo-acting) or at the extremities (exo-acting) of these polymers (White & Kennedy, 1988).

One of the best-characterized pectolytic activities is that of endopolygalacturonase (poly(1,4- α -D-galacturonide) glycanohydrolase; EC 3.2.1.15), a hydrolase which randomly cleaves glycosidic bonds of pectic acids or polygalacturonates, producing mono-, di-, and oligo-

galacturonates as final hydrolysis products. This enzyme has been purified and characterized from several fungi, including *Aspergillus* sp. (Aguilar & Huitron, 1987), *Botrytis cinerea* (Leone & Van Den Heuvel, 1987), *Fusarium moniliforme* (De Lorenzo *et al.*, 1987), *Rhizoctonia solani* Kuhn (Marcus *et al.*, 1986), *Rhizopus stolonifer* (Manachini *et al.*, 1987), *Trichoderma kőningii* (Fanelli *et al.*, 1978) and *Verticillium albo-atrum* (Wang & Keen, 1970).

The long-standing interest in pectinases resides in their role in the invasion of plant tissues by phytopathogens, the spoilage of fruit and vegetables and also in their food processing and plant biotechnology applications. The present investigation was attempted to characterize the pectolytic system of *Neurospora crassa*. This fungus is neither a phytopathogen, nor commonly regarded as of practical value in terms of enzymic productivity. Nevertheless, *Neurospora crassa* is probably the best-studied filamentous fungus in terms of biochemistry and genetics, and is also a valuable model system for molecular research. *Neurospora crassa*

Abbreviation: CMC, carboxymethylcellulose.

produces many hydrolytic enzymes, including cellulases (Eberhart *et al.*, 1977; Yazdi *et al.*, 1990), nucleases (Lindberg & Drucker, 1984), proteases (Cohen & Drucker, 1977), amylase (Sigmund *et al.*, 1985), xylanase (Mishra *et al.*, 1984), glucosidases (Eberhart & Beck, 1973) and others, which have been the focus of intense research. These studies are relevant to our understanding of the genetics, enzymology, regulatory properties and other characteristics of fungal hydrolases. Nevertheless, our knowledge of *Neurospora crassa* pectinases lags well behind that of other hydrolases. After an earlier study (Roboz *et al.*, 1952) describing an extracellular activity, designated 'pectin depolymerase', which hydrolyses pectin, producing mainly low-molecular-weight polyuronides, no further studies related to pectinase activity of *N. crassa* were published.

Here, we describe pectolytic enzymes produced by *Neurospora crassa* mycelium incubated in pectin as the inducer carbon source, and report the purification and partial biochemical characterization of an extracellular polygalacturonase activity.

Methods

***Neurospora crassa* strains and culture conditions.** The *N. crassa* strains used in this work, FGSC 424 (wild-type) and FGSC 2256 (*exo-1*), were a gift from the Fungal Genetics Stock Center (Kansas City, Kansas, USA). These strains were propagated on slants of solid Vogel (1964) medium supplemented with 2% (w/v) sucrose. Liquid cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml Vogel's minimal medium supplemented with 2% (w/v) glucose or other carbon sources, as indicated for each experiment. The inoculum consisted of suspensions of macroconidia (5×10^5 ml⁻¹). Cultures were incubated with agitation (140 r.p.m.) at 30 °C. For induction of pectolytic activities a two-step culture procedure was used: the fungus was first grown in glucose medium for 24 h (step I) and was then transferred to induction medium and incubated for an additional 48 h (step II). The cultures were harvested by filtration (Whatman no. 1), and the culture filtrates and mycelia were saved for enzyme determination. Before the enzyme assay, the crude preparations were dialysed overnight against 50 mM-sodium acetate buffer, pH 5.5. The purification procedure for extracellular pectolytic activities is presented in Results.

Preparation of crude extracts. Mycelial pads were ground in a porcelain mortar with twice their weight of sea sand at 0 °C. After cell breakage of more than 90% was achieved, 10 ml sodium acetate buffer was added, and the slurry was centrifuged at 15900 g for 20 min. The crude extract supernatant was used for enzyme determinations.

Enzyme assays. Polygalacturonase activity was assayed with sodium polypectate as a substrate by measuring the amount of reducing sugar released (Miller, 1959), as previously described (Polizeli *et al.*, 1990). An enzyme unit is the amount which releases reducing sugar at an initial rate of 1 µmol min⁻¹ at 37 °C, using monogalacturonic acid as the standard. Viscosity measurements were carried out at 37 °C in Ostwald viscosimeters with 2% sodium polypectate solution, according to Roboz *et al.* (1952). The enzyme action was expressed as a percentage change in viscosity. Pectate lyase activity was monitored in a Beckman DU-7 recording spectrophotometer, by measuring the increase in A_{232} due to 4,5-unsaturated reaction products (Nagel & Anderson, 1965)

according to Collmer *et al.* (1988). One unit of enzyme forms 1 µmol 4,5-unsaturated product min⁻¹ under the conditions of the assay. Pectin lyase was measured colorimetrically by the reaction between unsaturated products of pectin degradation and thiobarbituric acid (Ayers *et al.*, 1966) according to the procedure described by Pitt (1988). One enzyme unit causes a change in absorbance of 0.01 under the conditions of the assay.

Polyacrylamide gel electrophoresis. Electrophoresis under non-denaturing conditions was carried out by the method of Davis (1964). Urea (8%) sodium-dodecyl-sulphate-polyacrylamide (5%) gel electrophoresis (urea-SDS-PAGE) was carried out according to Swank & Munkres (1971). Molecular mass markers were: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; deoxyribonuclease, 31 kDa and trypsin inhibitor, 20.1 kDa.

Gel filtration. The molecular mass of the purified polygalacturonase was estimated by filtration in a Sephadex G-100 (1.5 × 60 cm) column equilibrated with 10 mM-Tris/HCl (pH 7.5) plus 100 mM-KCl. Elution (12.0 ml h⁻¹) was carried out with the same buffer and fractions (1.5 ml) were collected and analysed for polygalacturonase activity. Void volume (34 ml) was determined with blue dextran. The following proteins were used as molecular mass markers: alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa and cytochrome *c* (12.4 kDa).

Chromatographic separation of the hydrolysis products. Hydrolysis products from sodium polypectate by the purified polygalacturonase were analysed by thin-layer chromatography on silica gel G-60, using ethyl acetate/acetic acid/formic acid/water (9:3:1:4, by vol.) as the mobile phase system. Sugars were detected with 0.2% orcinol in sulphuric acid-methanol (10:90 ml) (Fontana *et al.*, 1988).

Determination of neutral carbohydrate and protein. Total neutral carbohydrate in protein samples was estimated by the phenol/sulphuric acid method of Dubois *et al.* (1956) using mannose as standard. Protein was determined by the Lowry method using bovine serum albumin as standard.

Chemicals. Polygalacturonic acid sodium salt, pectin (8.9% methoxy content), other polysaccharides and monosaccharides, DEAE-cellulose, carboxymethyl-cellulose, PAGE reagents and molecular mass markers were purchased from Sigma. Sephadex G-100 and Bio-Gel P-60 were obtained from Pharmacia and Bio-Rad, respectively. Silica Gel G was obtained from Merck. All other reagents were of analytical grade.

Results

Production of pectolytic activity

A significant level of polygalacturonic acid-degrading activity [1.6 units (mg wet wt)⁻¹] was present, among other hydrolases, on the outer surface of intact conidiospores. Most, if not all, of this activity was extractable with water; e.g. two cycles of vortexing and centrifugation of a conidial suspension removed 84% of the original activity present on the cells. Interestingly, the polygalacturonic-acid-degrading activity of conidia increased considerably [5.0 units (mg wet wt)⁻¹] when the conidiospores were grown in pectin-supplemented solid medium. This substrate induction effect was not observed for other conidial exoenzymes tested, includ-

Fig. 1. Time-course development of polygalacturonic-acid-degrading activity in wild-type *N. crassa* cultures incubated with 2% glucose (a) or 1% pectin (b) as carbon source. Symbols: (○) activity in the culture filtrate; (●) activity in mycelium extracts; (▲) glucose concentration. Panel (c) growth (mg total protein) of cultures grown in glucose (Δ) and in pectin (□). Enzyme activity was estimated on the basis of reducing sugar production using sodium polypectate as substrate, as described in Methods.

ing amylase, cellulases, glycosidases and alkaline phosphatase (data not shown).

In liquid medium supplemented with pectin as the sole carbon source, *N. crassa* grew slowly (Fig. 1c) and at the end of the growth phase (65 h) the mycelial yield was one-fourth of that of cultures grown in glucose-supplemented medium. For cultures grown with glucose as carbon source, polygalacturonic-acid-degrading activity was detected even before total disappearance of the sugar (Fig. 1a), and maximal activity was attained after 72 h incubation. On the other hand, polygalacturonic-acid-degrading activity in pectin-supplemented cultures increased faster than in the glucose-supplemented ones (Fig. 1b), and reached its maximum value after 50 h incubation. At that time, the difference in specific polygalacturonic-acid-degrading activity between glucose-grown and pectin-grown cultures after 50 h of incubation was approximately 16-fold and decreased to about 3.3-fold by the end of the incubation period.

In view of the relatively poor mycelium growth in pectin-supplemented cultures, resulting in low yields of enzyme for purification purposes, a two-step culture procedure was employed for inducing the enzymes (see Methods). In this way, the yield of extracellular polygalacturonic-acid-degrading activity was improved from an average of 0.08 units ml⁻¹ (cultures grown directly on pectin) to about 0.2 units ml⁻¹ (two-step cultures). Further improvement in enzyme yield was

achieved by using the *exo-1* mutant strain, reportedly an hyperproducer of other hydrolases (Gratzner & Sheenan, 1969). Under induction conditions, the production of polygalacturonic-acid-degrading activity of the mutant (about 1.1 U ml⁻¹ in two-step cultures) was five to six times higher than that of the wild-type. All results presented hereafter were obtained with the *exo-1* mutant strain.

Effect of the carbon source on the production of pectolytic activities

Table 1 shows the results of a series of two-step incubations of the *exo-1* mutant in several carbon sources. The culture filtrates were assayed for polygalacturonase, pectin lyase and pectate lyase. Incubation in pectin, polygalacturonate or monogalacturonate increased polygalacturonase activity about 13-fold over the basal level (glucose-grown cultures or cultures incubated without a carbon source). Other carbon sources including starch, microcrystalline cellulose, carboxymethyl-cellulose, xylan, trehalose, raffinose or sucrose had no effect (not shown). The inductive effect of pectin (Table 1), polygalacturonate and monogalacturonate (not shown) was abolished if glucose was present in the medium. Galactose was a very effective inducer of polygalacturonic-acid-degrading activity (62-fold over basal values). Interestingly, the inductive effect of galactose was not abolished by the presence of glucose.

Table 1. *Effect of carbon sources on the production of extracellular polygalacturonase, pectate lyase and pectin lyase activities by the *exo-1* mutant strain*

A two-step culture procedure as described in Methods was used. Carbon sources were added at 1% (w/v) concentration. Specific activities were calculated by dividing total enzyme units of the culture filtrate by the total protein (mg) of the crude mycelial extract.

Carbon source	Specific activity [U (mg protein) ⁻¹]		
	Polygalacturonase	Pectate lyase ($\times 10^3$)	Pectin lyase
Pectin	0.79	12.3	3.79
Sodium polypectate	0.54	4.3	ND
Monogalacturonic acid	0.66	10.5	ND
Galactose	3.12	4.5	ND
Galactose plus pectin	2.13	7.7	1.77
Glucose	0.05	3.1	ND
Pectin plus glucose	0.05	2.1	ND
Galactose plus glucose	4.02	2.5	ND
No carbon source added	0.09	ND	ND

ND, not detected.

The activities of pectate lyase and pectin lyase were higher than basal activity in pectin-supplemented cultures. Glucose repressed the induction by pectin, while galactose had little or no effect on the level of these enzymes.

Separation of pectolytic activities and purification of polygalacturonase

All steps were carried out at 4 °C or below. Pectin-induced cultures of the *exo-1* mutant were harvested by filtration. Routinely, 600 ml culture filtrate was used for enzyme purification. The culture filtrate was added to three vols absolute ethanol (−20 °C) and stored overnight. Precipitated material was collected by centrifugation and dissolved in 10 ml 50 mM-sodium acetate buffer, pH 5.5. The highly viscous solution was centrifuged to remove insoluble material, and the supernatant was passed through a Bio Gel P-60 column (4.3 × 51.0 cm) equilibrated with the same buffer. Pectolytic activities (polygalacturonase, pectate lyase and pectin lyase) co-eluted in the void volume of the column (not shown). At this stage, the enzyme preparation was substantially free of carbohydrate (mostly pectin from the culture medium) and contained associated invertase and amylase activities. The pooled active fractions were dialysed overnight against 0.01 M-Tris/HCl buffer, pH 7.5, and applied to a DEAE-cellulose column (1.7 × 45.0 cm) which had been equilibrated and washed with the same buffer. The pectolytic activities co-eluted with amylase in the void volume of the column while

invertase was bound to the resin and was eluted by increasing the ionic strength of the buffer (110 mM-NaCl). The pooled active fractions containing pectolytic activities were dialysed overnight against 10 mM-sodium acetate buffer, pH 5.5, and applied to a CM-cellulose column (1.2 × 17.0 cm) equilibrated with the same buffer. The column was first eluted with three vols of the equilibrating buffer followed by 200 ml of a linear sodium chloride gradient (0–0.5 M) dissolved in the same buffer (Fig. 2). Amylase activity did not bind to the resin and was eluted with a bulk of protein, in the equilibrating buffer. The salt gradient led to separation of pectolytic activities into four peaks (Fig. 2). Polygalacturonase eluted at 0.235 M-NaCl as a single, symmetrical peak (peak III) which contained 33.5% of the activity initially present in the crude filtrate. Pectin lyase and pectate lyase co-eluted in a minor and two major peaks of activity at 0.125 (peak I), 0.152 (peak II) and 0.264 (peak IV) M-NaCl, respectively. The relative activities of pectate lyase and pectin lyase in peaks I, II and IV, was variable (Table 3). Pectate lyase was predominant in peak II (62% of total activity eluted from the column, with a purification factor of 33.3) while pectin lyase was evenly distributed into peaks II and IV. The sum of activities of peaks I, II, and IV demonstrates that the recovery of pectate lyase and pectin lyase, relative to the activity in the crude filtrate, was approximately 25% and 12%, respectively. The pooled fractions of peak IV also contained considerable polygalacturonase activity (15% of total in CMC eluates). The combined fractions with polygalacturonase activity (fractions 35–39) of peak III exhibited a specific activity of 298.65 U (mg protein)⁻¹ and a purification factor of approximately 56 (Table 2). PAGE (6.0%, w/v) of the purified polygalacturonase revealed a single protein band after staining with Coomassie Blue (not shown), suggesting that the enzyme preparation was homogeneous.

The carbohydrate content of the purified polygalacturonase was estimated as 38% by the phenol/sulphuric acid method. Urea-SDS-PAGE produced an apparent molecular mass of approximately 37 kDa for the purified enzyme (results not shown). Gel filtration in Sephadex G-100 indicated an apparent molecular mass of approximately 36.6 kDa (results not shown). These results suggested that the enzyme was a monomeric glycoprotein.

Catalytic properties and kinetic parameters

The purified polygalacturonase was highly specific to sodium polypectate. Pectin was also hydrolysed, but at a rate 7–8% of that for sodium polypectate. The enzyme was totally inactive on starch, microcrystalline cellulose, xylan, trehalose, sucrose and raffinose. The temperature

Fig. 2. Separation of pectolytic activity on CM-cellulose. Symbols: (●) A_{280} ; (○) polygalacturonase; (△) pectate lyase; (□) pectin lyase; (---) NaCl gradient. The arrows indicate fractions pooled for polygalacturonase characterization. Other details are described in the Methods and Results sections.

Table 2. *Purification of extracellular polygalacturonase activity*

These results correspond to the experiment shown in Fig. 2.

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification factor (-fold)	Yield (%)
Crude filtrate	600.00	144.37	768.00	5.32	—	100.00
70% Ethanol precipitate	10.50	45.78	328.09	7.17	1.35	42.72
Pooled BioGel P-60 eluates	127.00	19.81	530.16	26.76	5.03	69.03
Pooled DEAE-cellulose eluates	134.00	16.96	495.28	29.20	5.49	64.49
Pooled CM-cellulose eluates (Peak III)	15.00	0.72	215.03	298.65	56.14	28.00

Table 3. *Purification of pectate lyase and pectin lyase activities*

These results correspond to the experiment shown in Fig. 2.

Step	Pectate lyase			Pectin lyase		
	Total activity (U)	Yield (%)	Purification factor (-fold)	Total activity (U)	Yield (%)	Purification factor (-fold)
Crude filtrate	4.80	100.00	—	10169.00	100.00	—
Peak I (CMC)	0.14	2.92	13.03	115.60	1.14	4.97
Peak II (CMC)	0.76	15.83	33.33	539.20	5.30	11.03
Peak IV (CMC)	0.32	6.67	23.33	606.0	5.96	20.73

and pH optima of the purified polygalacturonase were 45 °C and 6.0, respectively. An Arrhenius plot showed that the purified enzyme had an E_a value of 4.0 Kcal (16.8 kJ) mol⁻¹ for the hydrolysis of sodium polypectate. At temperatures above 50 °C, and in the absence of its substrate, the purified polygalacturonase was rapidly denatured. The estimated half-life of the enzyme at 50 °C was about 4 min and, at 60 °C, about 1.2 min. The

purified enzyme showed good stability at -20 °C, losing less than 10% activity after a month. The activity of the purified polygalacturonase was not significantly affected by magnesium chloride, magnesium sulphate, zinc chloride, mercury chloride, barium chloride, EDTA or β -mercaptoethanol (all at 2.5 mM). On the other hand, 2.5 mM-calcium chloride or copper sulphate inhibited the enzyme by approximately 79% and 67% respectively.

Fig. 3. Viscosity change and sodium polypectate hydrolysis by the purified polygalacturonase as a function of time. Symbols: (●) relative viscosity (%); (○) hydrolysis of glycosidic bonds (% of nominal monomer concentration). Details are given in Methods.

Fig. 4. Silica gel thin-layer chromatogram of the reaction products of the purified polygalacturonase with sodium polypectate. The incubation mixture (1 ml) contained 1% sodium polypectate, 50 mM-sodium acetate buffer, pH 5.5, and 3.0 units of polygalacturonase. Samples (5 to 15 μ l) were withdrawn from the reaction mixture, boiled and spotted on the chromatogram at the indicated times (see Methods).

K_m and V_{max} were determined at 37 °C using sodium polypectate concentrations ranging from 0.5 to 20 mg ml⁻¹. Lineweaver-Burk plots gave an apparent K_m of 5.0 mg ml⁻¹ and an apparent V_{max} of 357 μ mol min⁻¹ (mg protein)⁻¹. The purified enzyme appeared to be inhibited by high substrate concentrations.

Mechanism of action

The viscosimetric assay and the identification of hydrolysis products were used to determine the mechanism of action of the purified polygalacturonase on sodium polypectate. Fig. 3 shows that the time required for 50% decrease in viscosity of a 2.0% (w/v) sodium polypectate solution at 37 °C was approximately 100 min, at which time about 7% of the total galacturonide bonds had been hydrolysed. These results suggested a random mechanism of hydrolysis of sodium polypectate by the purified polygalacturonase. Thin layer chromatography of the products of hydrolysis provided further evidence for this hypothesis. As shown in Fig. 4, oligogalacturonates accumulated initially, but the monomer was found in the hydrolysate only after reaction for 2 h. These results suggested that the *N. crassa* purified enzyme was an endo-polygalacturonase [poly(1,4- α -D-galacturonide)glycanohydrolase; EC 3.2.1.15].

Discussion

The occurrence of constitutive polygalacturonase activity at the outer layer of ungerminated conidia of *N. crassa* is not unique among fungi. Similar observations have been made on spores of some phytopathogenic fungi such as *Botrytis cinerea* (Leone & Van Den Heuvel, 1987) and *Geotrichum candidum* (Barash & Klein, 1969), in which the presence of the enzyme has been related with the process of invasion of the plant host. In *N. crassa*, the constitutive polygalacturonase activity of conidia provides the cells with an inherent capacity for using pectic substances as a source of carbon during germination. In addition, the constitutive polygalacturonase activity of conidia may contribute to the induction of pectinase synthesis, by degrading pectin to low-molecular-mass products which may act as inducers when taken up by the cells. The *N. crassa* cell wall seems to contribute to the regulation of polygalacturonase induction, because this enzyme is made constitutively and is not glucose-repressible in spheroplasts of a 'slime' strain (Polizeli *et al.*, 1990). The wall-less 'slime' cells also produce other hydrolases constitutively, including invertase, aryl- β -glucosidase, alkaline protease (Pietro *et al.*, 1989), alkaline phosphatase (Burton & Metzner, 1974), cellulase and xylanase (Polizeli *et al.*, 1990).

N. crassa grew well on organic matter rich in pectic substances, such as orange peel (data not shown), but grew poorly on minimal medium supplemented with pectin as the sole source of carbon. These observations have been reported by Roboz *et al.* (1952) and were reproduced in the present study. Glucose-grown cultures exhibited a relatively high basal activity of pectinase, but

this activity was increased by pectin, polygalacturonic acid and mono-galacturonic acid. Other polysaccharides such as microcrystalline cellulose, carboxymethyl cellulose, xylan or starch failed to induce pectinases. The inductive effect of pectin and related compounds was repressed by glucose. In these aspects, the carbon-source effects on pectinase production by *N. crassa* were essentially similar to those reported for other fungi (Rombouts & Pilnik, 1980).

N. crassa polygalacturonase was induced 4–5 times more efficiently by galactose than by pectin; however, galactose did not increase pectate lyase or pectin lyase activities. Galactose induction of polygalacturonase was not repressed by glucose. This selective induction of polygalacturonase by galactose, contrasting with the simultaneous induction of polygalacturonase, pectate lyase and pectin lyase by pectin, suggests that the pectinases of *N. crassa*, like the enzymes of *Rhizoctonia solani* (Lisker *et al.*, 1975) and *Verticillium albo-atrum* (Cooper & Wood, 1973) are not necessarily co-regulated. Alternatively, the galactose-induced and the pectin-induced polygalacturonase may be products of different, independently controlled genes. A detailed biochemical characterization of the galactose-induced polygalacturonase should clarify this question.

On average, about 90% of the pectolytic activity produced by induced mycelium of *N. crassa* was released into the culture medium. The remaining activity, recovered in the supernatants of crude cell extracts, may either be cytosolic enzyme, or extracellular enzyme trapped in the periplasm.

The extracellular pectinases produced by pectin-induced mycelium were separated into several components by ion exchange chromatography on CMC. The enzyme sample applied to the CMC column had been previously subjected to gel filtration in BioGel P 60 and DEAE-cellulose chromatography to remove contaminating activities. It is noteworthy that polygalacturonase, pectate lyase and pectin lyase co-eluted in the void volume of the BioGel P 60 column, because the apparent molecular mass of polygalacturonase (37 kDa) and that of pectate and pectin lyase (approximately 37 kDa and 50 kDa, respectively; not shown) was within the fractionation range of the gel. Perhaps the enzymes in the crude medium formed aggregates, either through protein–protein or protein–carbohydrate–protein interactions, as reported for the pectinases of *Fusarium oxysporum* (Swinburne & Corden, 1967) and *Geotrichum candidum* (Barash *et al.*, 1984). Titrimetric assays applied to samples of crude filtrates of induced cultures failed to demonstrate any methyl esterase activity. We have no explanation for that, because this activity should be a prerequisite for effective action of polygalacturonase on pectin. Pectate lyase and pectin lyase activities co-eluted

in three main peaks from the CMC column and their characterization was not pursued further. Polygalacturonase eluted as a single, symmetrical peak.

Polygalacturonase activity was purified 56-fold and the enzyme preparation was apparently homogeneous. The enzyme was a monomeric glycoprotein with a molecular mass of about 37 kDa and 38% carbohydrate. Its catalytic properties were very close to those of the partially purified (11.7-fold) extracellular ‘pectin depolymerase’ of *N. crassa* described by Roboz *et al.* (1952). ‘Pectin depolymerase’ has as pH optimum of 5.5–6.0, is 50% inactivated by 1 h incubation at 40 °C and fully inactivated at 70 °C, and decreases the viscosity of a polypectate solution by 50% with a release of reducing sugar groups of only 2%. Roboz *et al.* (1952) considered at the time that the *N. crassa* enzyme was a new enzyme, different from other polygalacturonases. Indeed, these authors compared the activity of ‘pectin depolymerase’ with Pectinol, a commercial *Aspergillus niger* preparation containing a mixture of polygalacturonase activities (Cooke *et al.*, 1976), which produced mainly monomer as the final reaction product. In contrast, ‘pectin depolymerase’ accumulated low-molecular-mass polyuronides. It seems likely that the ‘pectin depolymerase’ described by Roboz *et al.* (1952) and the endopolygalacturonase reported in the present study are the same enzyme. A noted difference between the two enzymes, however, was that ‘pectin depolymerase’ attacks pectin and sodium polypectate with the same efficiency (Roboz *et al.*, 1952), while the purified polygalacturonase hydrolysed pectin at a rate 7–8% of sodium polypectate, which is typical of endopolygalacturonases. This discrepancy could be attributed to contaminating activities in the ‘pectin depolymerase’ preparation used by Roboz *et al.* (1952).

Some properties of the *N. crassa* purified polygalacturonase such as glycoproteic nature, molecular mass, specific activity and ability to hydrolyse polypectate rather than pectin resemble those of other fungal polygalacturonases (Rombouts & Pilnik, 1980). However, the pH optimum of the *N. crassa* enzyme (6.0) was somewhat higher than most other similar enzymes (pH 5.0 or below) but similar to that of the endopolygalacturonase of *Verticillium albo-atrum* (pH 6.5; Wang & Keen, 1970) and *Botrytis cinerea* (pH 6.0; Martínez *et al.*, 1988). The K_m for hydrolysis of polypectate of most fungal polygalacturonases is $<1 \text{ mg ml}^{-1}$ (Rombouts & Pilnik, 1980). Thus, the *N. crassa* enzyme K_m (5.0 mg ml^{-1}) was unusually high, as is that of a polygalacturonase produced by *Tricladium splendens* (K_m 4.3 mg ml^{-1} ; Chamier & Dixon, 1982).

The molecular characterization of fungal pectolytic enzymes and their genes is not as advanced as, for instance, that of the cellulolytic system. Reports have

recently appeared in the literature concerning the molecular characterization of pectolytic genes of *Aspergillus niger* (Gysler *et al.*, 1990; Khanh *et al.*, 1990; Rutkowski *et al.*, 1990) but, as far as we are aware, no similar studies in other fungi have been reported. Thus, we would like to suggest that the apparent biochemical simplicity of *N. crassa* polygalacturonase (e.g. single polypeptide; no apparent isoforms) and its straightforward purification procedure, allied with the recent development of molecular cloning techniques in this fungus, may constitute a useful model system for investigating this important family of biomass-degrading hydrolases.

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