Journal of Industrial Microbiology & Biotechnology (1998) 20, 238–243

http://www.stockton-press.co.uk/jim

Characterization of galactose-induced extracellular and intracellular pectolytic activities from the *exo*-1 mutant strain of *Neurospora crassa*

LB Crotti, HF Terenzi, JA Jorge and MLTM Polizeli

Departamento de Biologia, Faculdade de Filosofia, Ciencias e Letras de Ribeirao Preto, Universidade de Sao Paulo, 14040-901 Ribeirao Preto, Sao Paulo, Brasil

Pectolytic enzymes from the hyperproducer *exo*-1 mutant of *Neurospora crassa* are induced either by pectin or galactose. Galactose-induced pectinases, in contrast with pectin-induced enzymes, are not affected by glucose repression. Here, the pectolytic enzymes induced by galactose were purified and characterized. Extracellular pectolytic activities were separated into two main fractions. Pool I contained lyases, and a polygalacturonase (PG) copurifying as a complex of about 80 kDa (gel filtration). Pool II contained PG only. Under urea-SDS-PAGE the lyases and polygalacturonase from pool I migrated with an apparent MW of 56.2 kDa, and 34.3 kDa, respectively. PG from pool II exhibited an apparent MW of 44.7 kDa. Cell extracts contained PG free of lyase activities. Purified intracellular PG migrated (SDS-PAGE) as a single band of apparent MW of 31.5 kDa. All pectinases were glycoproteins (18.5–39% carbohydrate), with stability and optimum pH at 5–6 and 9–10 for PG and lyases, respectively. Temperature optima were 40–50°C, respectively. All enzymes were inactivated at 60°C, with a half-life from 1.5 to 5 min. Activation energy (Ea) values for extracellular and intracellular PG varied between 0.45 and 2.0 Kcal mol^{-1}. Pool II and intracellular PG and lyases, exhibited a random mechanism of hydrolysis. Pool I PG exhibited an exo character.

Keywords: catabolic repression; *Neurospora crassa*; pectate/pectin lyase; pectic enzymes; polygalacturonase; inductor/induction

Introduction

Previous studies from our laboratory [21,22] demonstrated that the filamentous fungus *Neurospora crassa* produces pectic enzymes as efficiently as other hydrolases such as cellulases [8], amylases [25], or xylanases [17]. The *N. crassa* hypersecretory mutant strain *exo*-1 [9] produces five to six times more polygalacturonase than the wild-type, when cultured with pectin, or with galactose, as carbon sources. The pectin-induced enzyme was purified and classified as an endopolygalacturonase. Interestingly, the inducing effect of pectin, but not that of galactose, is repressed by glucose [22]. These results raised the question of whether the pectin- and galactose-induced enzymes are the same enzyme or two different enzymes. In this study of galactose-induced pectolytic activity, it was found that galactose- and pectin-induced enzymes differed in several biochemical properties.

Materials and methods

Strain and culture conditions

The *N. crassa* strain FGSC 2256 (*exo*-1) was obtained from the Fungal Genetics Stock Center (Kansas City, KS, USA). The organism was maintained on slants of solid Vogel's [28] medium supplemented with 2% (w/v) sucrose. Macroconidia from 10-day-old cultures were inoculated (5×10^5 ml^{-1} final concentration) in 250-ml Erlenmeyer flasks containing 50 ml Vogel's minimal medium supplemented with 2% (w/v) glucose. The cultures were incubated at 30°C with agitation for 24 h (step I), and then harvested, rinsed, and transferred to fresh medium supplemented with 2% (w/v) galactose plus 2% (w/v) glucose (induction medium) and incubated for 72 h (step II), or for the times indicated for each experiment.

Preparation of crude extracts

The cultures were harvested by filtration, and the mycelium and culture filtrates were saved. The culture filtrate was dialyzed overnight against 50 mM sodium acetate buffer, pH 5.5. The dialyzed culture filtrate was used as the source of extracellular enzymes. Mycelia were ground in a porcelain mortar with acid-washed sand at 4°C and extracted with 2 vol of sodium acetate buffer. The slurry was centrifuged at $16\,000 \times g$ for 20 min and the supernatant was used as the source of intracellular enzymes.

Enzymatic assays

Polygalacturonase activity was assayed by measuring the amount of reducing sugar released [16], as previously described [21]. An enzyme unit is the amount which releases reducing sugar at an initial rate of 1 μmol min^{-1} at 45°C, using monogalacturonic acid as the standard. Polygalacturonase activity was also determined by viscosity change using an Ostwald viscosimeter containing a 0.2% (w/v) pectin solution in 50 mM sodium acetate buffer pH 5.5, at 30°C [27]. One relative viscosimetric unit was the amount of enzyme which reduced the initial viscosity of the pectin solution by 50%, in 1 min. Pectate lyase activity

Correspondence: MLTM Polizeli, Departamento de Biologia, Faculdade de Filosofia Ciencias e Letras de Ribeirao Preto, Universidade de Sao Paulo, Avenida Bandeirantes, 3900, 14040-901 Ribeirao Preto, SP, Brasil
Received 20 October 1997; accepted 28 February 1998

was monitored spectrophotometrically by measuring the increase in A_{232} nm due to 4,5 unsaturated reaction products [18], according to Collmer *et al* [5]. 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 [2] according to the procedure described by Pitt [20]. One enzyme unit causes a change in absorbance of 0.01 under the conditions of the assay. Pectinesterase activity was assayed by continuous titration of the carboxyl groups liberated from methyl ester bonds. One unit is defined as the amount of enzyme which causes a decrease in pH of the reaction mixture of 0.1 in 30 min [23].

Separation of pectic enzymes

(A) Extracellular enzymes: The crude filtrate was precipitated with 2 vol of ethanol for 2 h at –20°C and then centrifuged at 16 000 × *g* for 10 min. The precipitate was dissolved in 10 ml of Tris-HCl buffer 10 mM, pH 7.5 (buffer A) and applied to a DEAE-cellulose column (1.6 × 20 cm) equilibrated with buffer A. The eluted protein was dialyzed against 10 mM sodium acetate buffer, pH 5.0 (buffer B) and applied to a CM-cellulose column (1.6 × 25 cm). The column was eluted with a NaCl gradient (0–500 mM) in buffer B. Fractions (10 ml) were collected at a flow rate of 33.5 ml h^{-1}.

(B) Intracellular enzymes: The crude extract was applied to a DEAE-cellulose column (1.6 × 20 cm), equilibrated with buffer A, and eluted with buffer A plus increasing NaCl concentrations (0–500 mM). Fractions (5 ml) were collected at a flow rate of 16 ml h^{-1}.

Determination of molecular mass

Molecular mass was determined by gel filtration in a Sepharose CL-6B column (1.8 × 88 cm) equilibrated and eluted with Tris-HCl buffer 50 mM, pH 7.5, plus 100 mM KCl. Fractions (3.3 ml) were collected at a flow rate of 10 ml h^{-1}. Molecular mass markers were: tyroglobulin (660 kDa); apoferritin (440 kDa); β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). Urea-SDS-PAGE (7%) was carried out according to Swank and Munkres [26], and SDS-PAGE (7%) according to Laemmli [12]. Molecular mass markers were: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase *b* (97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Proteins were silver-stained as recommended by Blum *et al* [4].

Isoelectric focusing

Isoelectric focusing using Pharmalyte pH 3–10 was carried out in 7% rod gels containing 5% (v/v) carrier ampholyte according to O'Farrell *et al* [19]. Twenty micrograms of purified polygalacturonase were loaded on the gel (0.6 × 13 cm). After focusing at 500 V for 6 h, the pH gradient was measured by cutting one gel into 5-mm thick slices and extracting each slice with 1 ml KCl 25 mM. Polygalacturonase activities were determined by reducing sugar release using slices submerged in sodium polypectate solution.

Determination of neutral carbohydrate

Total neutral carbohydrate in protein samples was estimated by the phenol/sulfuric acid method of Dubois [6] using mannose as standard.

Amino acid composition analysis

The purified intracellular polygalacturonase was subjected to acid hydrolysis with 6 N HCl in vacuum-sealed glass tubes at 110°C for 24 h. Amino acid analysis was carried out using phenylthiocarbamyl-derivatives which are separated by reverse phase HPLC, detected and analyzed by their absorbance at 254 nm [1,3,7]. The homology was calculated using the Propsearch Database computer program [11].

Chemicals

Pectin (8.9% methoxy content), polygalacturonic acid sodium salt, d-galacturonic acid sodium salt and other carbohydrates were from Sigma (USA). All other reagents were analytical grade.

Results and discussion

Time-course development of pectolytic activities

We reported earlier that pectin and galactose induce different amounts of extracellular polygalacturonase in the *exo*-1 mutant of *N. crassa*. Galactose induces approximately four times more activity than does pectin. The galactose-induced activity increased when glucose was added to the induction medium; in contrast the pectin-induced activity is glucose-repressible [22]. We decided to study more closely the influence of galactose on the development of pectolytic activities of *N. crassa*. Figure 1 shows the time-course of production of pectolytic enzymes by the *exo*-1 mutant incubated with 2% (w/v) glucose plus 2% (w/v) galactose. With the two-stage culture procedure (see Methods), the activity levels were about 8-fold higher than those obtained inoculating spores directly into the induction medium (not shown). A consistent sequence for the release of pectolytic enzymes was observed. Polygalacturonase activity was the first to be detected. It reached the highest activity at 72 h, but most of the activity was present at 24 h. Pectate/pectin lyases appeared later (maximum at 72 h). Pectinesterase activity was not detectable. During the incubation in the induction medium, the mycelial mass did not change significantly (not shown). A comparable sequential production of pectolytic enzymes was previously reported for *Botrytis cinerea* [13].

Purification of pectolytic activities

Pectolytic enzymes were extracted and prepared from cultures incubated for 72 h in induction medium (see Figure 1). Extracellular and intracellular activities behaved differently during chromatographic separation. Extracellular activities were not retained in DEAE-cellulose and were eluted in the void volume of the column (not shown). The active fractions (Figure 2), containing polygalacturonase and pectate/pectin lyase activities, were pooled and applied

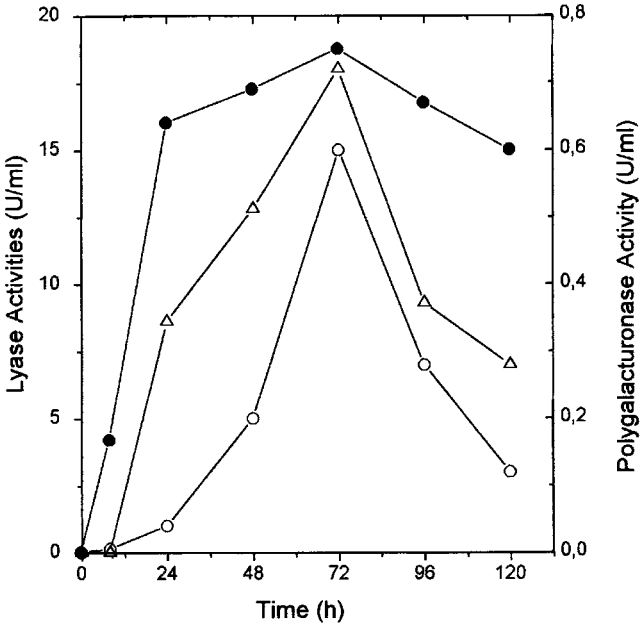

Figure 1 Time-course of pectolytic enzymes production by the *exo*-1 mutant of *Neurospora crassa* in two-stage cultures. The organism was cultivated with 2% (w/v) glucose for 24 h and then transferred to fresh medium supplemented with 2% (w/v) glucose and 2% (w/v) galactose for different times. Symbols: (●) polygalacturonase activity; (○) pectate lyase activity; (△) pectin lyase activity.

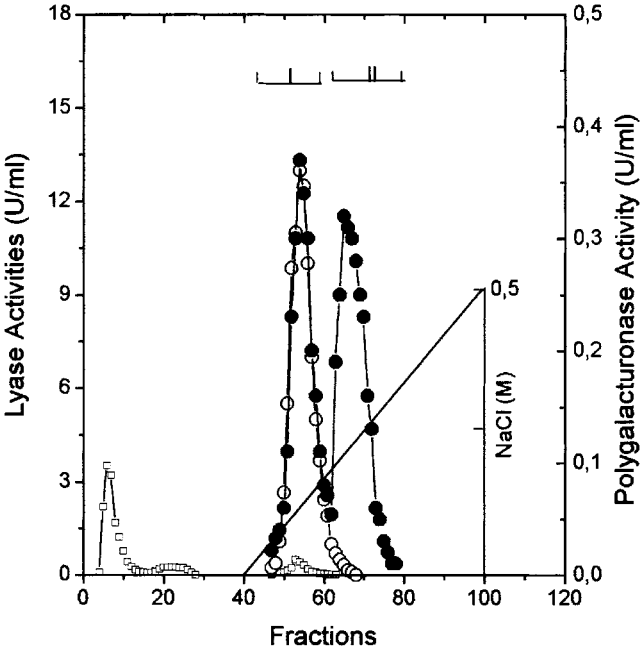

Figure 2 Separation of extracellular pectolytic activities by CM-cellulose chromatography. The column was eluted with 10 mM Tris-HCl, pH 7.5 and a continuous gradient (0–0.5 M) of NaCl, at a flow rate of 34 ml h^{-1}. Ten-milliliter fractions were collected and assayed for pectolytic activities. I and II represent pool I and pool II activities. Symbols: (□) Absorbance 280 nm (left axis); (●) polygalacturonase activities; (○) pectate and pectin lyase activities; (—) NaCl gradient. Bars over the activity peaks represent the limits of the pooled fractions. Other details as described under Methods.

to a CMC-cellulose column. Polygalacturonase activity was resolved into two sharp fractions (Pool I, 125 mM NaCl, and Pool II, 215 mM NaCl). Pool I contained polygalacturonase and pectate/pectin lyase activities, and Pool II contained polygalacturonase activity only. Lyases (pectin/pectate) and polygalacturonase activities were purified, respectively, 22-, 33- and 39-fold, with 3–4% recovery. Pool I was rechromatographed under the same conditions, but polygalacturonase and lyase activities co-eluted at 125 mM NaCl, as before. The pectolytic activities in pool I also co-eluted as a single peak, with apparent molecular mass of 79.4 kDa, by Sepharose CL-6B column chromatography (not shown).

However, in some preparations, lyases and polygalacturonase activities were totally separated into pool I (lyases only), and pool II (polygalacturonase only). In this case, when a pool I preparation was submitted to urea-SDS-PAGE electrophoresis (Figure 3), pectate/pectin lyase activities migrated as a single protein band of 56.2 kDa apparent molecular mass (Figure 3A). Pool II containing only polygalacturonase activity, also migrated as a single protein band, but of 44.7 kDa apparent molecular mass (Figure 3C). For preparations of pool I which contained both lyases and polygalacturonase activities, the apparent molecular mass (urea-SDS-PAGE) for polygalacturonase was 34.3 kDa (Figure 3B). This suggested that lyases and polygalacturonase activities might form a complex under some circumstances.

The intracellular pectolytic activities, different from the extracellular ones, were retained on DEAE-cellulose. Polygalacturonase eluted in a single sharp symmetrical peak with 110 mM NaCl (Figure 4). Lyase activities were below detection levels. The intracellular polygalacturonase was purified 35-fold, with 20% recovery. Under SDS-PAGE,

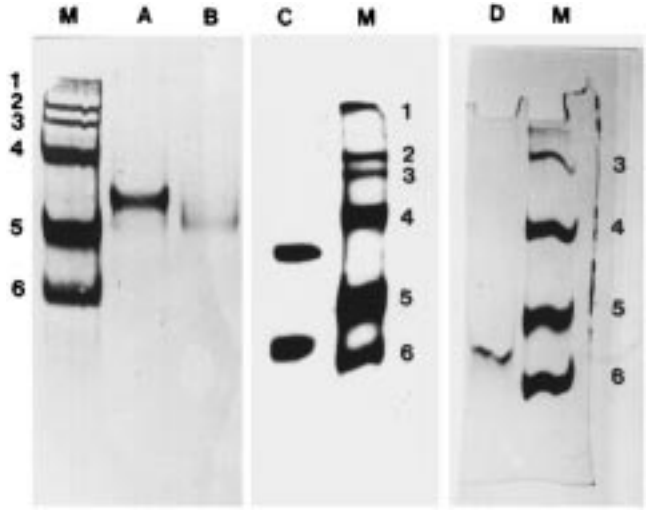

Figure 3 Gel electrophoresis of purified pectolytic activities. (A) Urea-SDS-PAGE 7% of lyases activities; (B) Urea-SDS-PAGE 7% of polygalacturonase activity, pool II; (C) Urea-SDS-PAGE 7% of polygalacturonase activity, pool I. Molecular mass markers (M) were: (1) myosin (205 kDa); (2) β-galactosidase (116 kDa); (3) phosphorylase *b* (97 kDa); (4) bovine serum albumin (66 kDa); (5) ovalbumin (45 kDa); and (6) carbonic anhydrase (29 kDa). (D) SDS-PAGE 7% of intracellular polygalacturonase activity. Molecular mass markers were the same.

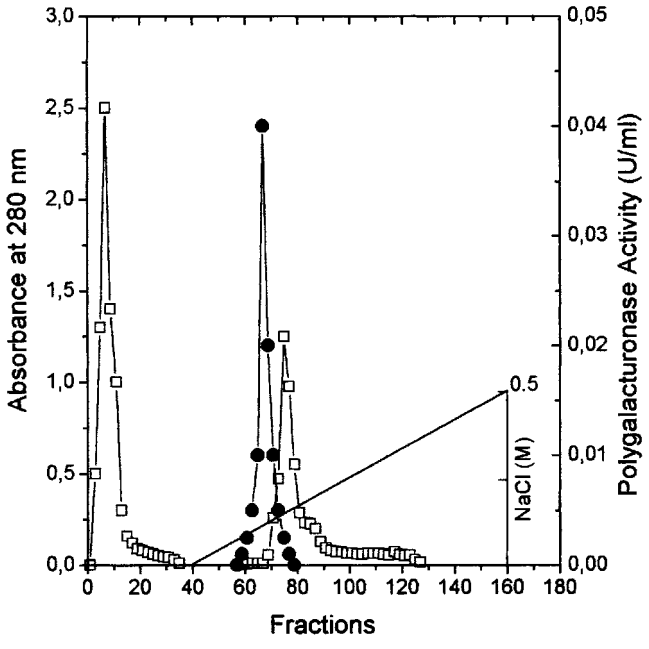

Figure 4 Separation of intracellular polygalacturonase activity by DEAE-cellulose chromatography. The column was eluted with 10 mM acetate sodium buffer, pH 5.5 and a continuous gradient (0–05 M) of NaCl, at a flow rate of 16 ml h^{-1}. Five-milliliter fractions were collected and assayed. Symbols: (□) Absorbance 280 nm; (●) polygalacturonase; (—) NaCl gradient. Other details as described under Methods.

the intracellular polygalacturonase migrated as a single band of 31.5 kDa (Figure 3D).

Intrinsic properties of the purified activities

Table 1 summarizes some properties of the pectolytic enzymes from the *exo*-1 mutant. Apparent K_m and V_{max} values, calculated from Lineweaver–Burk plots, were determined at the temperature optimum for each activity, using sodium polypectate concentrations ranging from 0.5 to 20 mg ml^{-1}. Higher substrate concentrations were inhibitory. Pool II extracellular polygalacturonase, and intracellular polygalacturonase activities, exhibited approximately 55-fold more affinity for sodium polypectate than pool I extracellular polygalacturonase. It should be noted that polygalacturonase activity in pool I was measured in the presence of lyase activities. All pectolytic activities were glycoproteins (18.5–39% carbohydrate), with stability and optimum pH at 5–6 and 9–10 for polygalacturonase and lyase activities, respectively. Temperature optima ranged between 40–50°C, but all activities were inactivated at 60°C, with a half-life varying from 1.5 to 5 min. Activation energy values (Ea) for the hydrolysis of sodium polypectate, calculated from Arrhenius plots, showed that extracellular (pool I and II) and intracellular polygalacturonase values varied between 0.45 and 2 Kcal mol^{-1}.

The endo (random cleavage) or exo (cleavage at the extremities) character of the pectolytic activities was determined using sodium polypectate and pectin as substrate. The mechanism of action was determined both by viscosimetry and by release of reducing sugar. The time required for a 50% decrease in viscosity of a 2% (w/v) substrate solution, at the temperature optimum of each activity, was 250 min for pool II extracellular polygalacturonase, 24 h for intracellular polygalacturonase, and 120 min for pectate/pectin lyases, while about 1%, 9%, and 6.5% of total galacturonide bonds, respectively, had been hydrolyzed. These results suggested a random mechanism of hydrolysis for pool II and intracellular polygalacturonase activities, and for pectin/pectate lyases. On the other hand pool I polygalacturonase required 30 min to decrease by 50% the viscosity of the solution of sodium polypectate, and hydrolyzed 18% of total galacturonide bonds, indicating that this enzyme had an exo character. Extracellular (pool I and II) and intracellular polygalacturonase activities hydrolyzed principally sodium polypectate and pectin to a minor extent (19%, 22%, and 51%, respectively). Pectate lyase was more active against sodium polypectate, but also hydrolyzed pectin 25%. Pectin lyase hydrolyzed only pectin. Other substrates, such as starch, microcrystalline cellulose, carboxymethyl cellulose, *p*-nitrophenyl-*β*-d-galactopyranoside (PNP-Gal), raffinose, sucrose, trehalose and xylan were ineffective.

Table 1 Kinetic constants and other intrinsic properties of *N. crassa* pectolytic activities

Properties	Polygalacturonases				Lyases	
	Pool I	Pool II	Intra	Pectin-induced[a]	Pectate	Pectin
K_m (mg ml^{-1})	1.161	0.023	0.019	5.0	0.50	0.076
V_{max} (U mg^{-1} protein)	177.94	2.08	0.03	357	273.20	363.40
Isoelectric point	6.60	6.24	7.14	nd	6.80	6.80
Carbohydrate content (%)	18.5	38.8	35.0	38.0	38.0	
Optimum pH	5.5	5.5	5.5	6.0	9.0	10.0
pH stability	5.5–6.0	5.0	5.5–6.0	nd	10.0	9.5
Optimum temperature (°C)	40	45	40	45	50	
Stability 60°C (T50 – min)	1.0	5.0	5.0	1.2	3.0	1.5
Act. energy (Ea Kcal mol^{-1})	1.0	0.5	2.0	4.0	0.45	0.5
Molecular weight (kDa)	34.3	44.7	31.5	37	56.2	
Character	exo	endo	endo	endo	endo	endo

[a]Data from Reference [22].
nd, not determined.
exo = exopolygalacturonase; endo = endopolygalacturonase.

Table 2 Amino acid composition of intracellular polygalacturonase

Amino acid	mol mol^{-1}
Aspartic acid	27.2
Glutamic acid	25.6
Serine	24.2
Glycine	42.3
Histidine	13.6
Arginine	6.9
Threonine	26.3
Alanine	32.0
Proline	18.8
Tyrosine	5.9
Valine	23.3
Methionine	3.2
Cysteine	2.4
Isoleucine	12.2
Leucine	19.3
Phenylalanine	8.3
Lysine	16.4
Σ	307.9

The effects of some metal ions on the purified enzymes were studied. Polygalacturonase activities were drastically inhibited by copper sulfate and mercuric chloride, with the intracellular polygalacturonase being more sensitive. Lyase activities were stimulated by low concentrations of most of the ions tested, but higher concentrations sometimes inhibited these activities (not shown). This was in agreement with results reported for other fungal pectolytic activities [10,15,24].

The estimated amino acid residue composition of the purified intracellular polygalacturonase is shown in Table 2. It should be noted that about 17.1% of the residues were acid, such as aspartic acid and glutamic acid, 12% of the residues were basic and 71% were neutral. These values were compatible with the isoelectric point (7.14, Table 1). The amino acid composition resembled the polygalacturonase of *Prunus persica* (peach) [14].

Previous studies on the pectolytic enzymes of *N. crassa* induced by pectin [22], showed that these activities separate into four main fractions containing pectate/pectin lyase activity, and a fourth fraction containing polygalacturonase only. The properties of the pectin-induced polygalacturonase (Table 1) were close to those of pool I galactose-induced polygalacturonase, except its endo character. On the other hand, galactose-induced Pool II extracellular and intracellular polygalacturonases, differed from the Pool I and pectin-induced polygalacturonases by comparatively lower K_m and V_{max} values, and higher stability at 60°C. Altogether, these results suggest a versatile pectolytic system for *N. crassa*. This is the first report of an intracellular polygalacturonase activity in this fungus. We cannot say at this time to what extent the differences reflect post-translation modifications or the products of different genes.

Acknowledgements

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) and Conselho de Desenvolvimento Científico e Tecnologico (CNPq). HFT, JAJ and MLTMP are Research Fellows of CNPq. LBC is a recipient of a CAPES Fellowship. This work is part of a Doctor Thesis submitted by LBC to the Department of Biochemistry of the School of Medicine of Ribeirao Preto (University of Sao Paulo). We thank Dr Joel Lewis Greene and Jose Cesar Rosa, CIQP-FMRP-USP for amino acids analysis and Maurício de Oliveira for technical assistance.

References

1 Atherton D. 1989. Successful PTC amino acid analysis at the picomole level. In: Techniques in Protein Chemistry (TE Hugli, ed), pp 273–283, Academic Press, New York.
2 Ayers WA, GC Papavizas and AF Diem. 1966. Polygalacturonase trans-eliminase and polygalacturonase production by *Rhizoctonia solani*. Phytopathology 56: 1006–1011.
3 Bidlingmeyer BA, SA Cohen and TL Tarvin. 1984. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 336: 93–104.
4 Blum H, H Beier and HJ Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–97.
5 Collmer A, JL Ried and MS Mount. 1988. Assay methods for pectic enzymes. In: Methods in Enzymology (Wood WA and ST Kelloggy, eds), Vol 161, pp 326–335, Academic Press, New York.
6 Dubois M, KA Gilles, JK Hamilton, PA Rebers and F Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.
7 Dupont DR, PS Keim, AH Chui, R Bello, MB Bozzini and KJ Wilson. 1989. A comprehensive approach to amino acid analysis. In: Techniques in Protein Chemistry (TE Hugli, ed), pp 284–294, Academic Press, New York.
8 Eberhart BM, RS Beck and KM Goolsby. 1977. Cellulase of *Neurospora crassa*. J Bacteriol 130: 181–186.
9 Gratzner HG and DN Sheehan. 1969. *Neurospora* mutant exhibiting hyperproduction of amylase and invertase. J Microbiol 97: 544–549.
10 Guo W, L Gonzalez-Candelas and PE Kolattukudy. 1995. Cloning of a new pectate lyase gene *pelC* from *Fusarium solani* f sp *pisi* (*Nectria haematococca*, Mating Type VI) and characterization of the gene product expressed in *Pichia pastoris*. Arch Biochem Biophys 323: 352–360.
11 Hobohm U, T Houthaeve and C Sander. 1994. Amino acid analysis and protein database compositional search as a rapid and inexpensive method to identify proteins. Anal Biochem 222: 202–209.
12 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
13 Leone G and J Van Den Heuvel. 1987. Regulation by carbohydrates of the sequential 'in vitro' production of pectic enzymes by *Botrytis cinerea*. J Can Bot 65: 2133–2141.
14 Lester DR, J Speirs, G Orr and CJ Brady. 1994. Peach (*Prunus persica*) endopolygalacturonase cDNA isolation and mRNA analysis in melting and nonmelting peach cultivars. Plant Physiol 105: 225–231.
15 Manachini PL, MG Fortina and C Parini. 1987. Purification and properties of an endopolygalacturonase produced by *Rhizopus stolonifer*. Biotechnol Lett 9: 219–224.
16 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31: 426–428.
17 Mishra C, S Keskar and M Rao. 1984. Production and properties of extracellular endoxylanase from *Neurospora crassa*. Appl Environ Microbiol 48: 224–228.
18 Nagel CW and MM Anderson. 1965. Action of a bacterial transeliminase on normal and unsaturated oligogalacturonic acids. Arch Biochem Biophys 112: 322–330.
19 O'Farrel PZ, HM Goodman and PH O'Farrel. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133–1142.
20 Pitt D. 1988. Pectin lyase from *Phoma medicaginis* var *pinodella*. In: Methods in Enzymology (Wood WA and ST Kelloggy, eds), Vol 161, pp 350–354, Academic Press, New York.
21 Polizeli ML TM, RCLR Pietro, JA Jorge and HF Terenzi. 1990. Effects of cell wall deficiency on the synthesis of polysaccharide-

degrading exoenzymes. A study on mycelial and wall-less phenotypes of the *fz, sg, os-1* ('slime') triple mutant of *Neurospora crassa*. J Gen Microbiol 136: 1463–1468.

22 Polizeli MLTM, JA Jorge and HF Terenzi. 1991. Pectinase production by *Neurospora crassa*: purification and biochemical characterization of extracellular polygalacturonase activity. J Gen Microbiol 137: 1815–1823.

23 Rexova-Benkova L and O Markovic. 1976. Pectic enzymes. Adv Carbohydr Chem Biochem 33: 323–385.

24 Rombouts FM and W Pilnik. 1980. Pectic enzymes. In: Economic Microbiology (Rose AH, ed), Vol 5, pp 227–282, Academic Press, New York.

25 Sigmund RD, MT McNally, DB Lee and SJ Free. 1985. *Neurospora* glucamylase and a mutant affected in its regulation. Biochem Genet 23: 89–103.

26 Swank RW and KD Munkres. 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulphate. Anal Biochem 39: 462–477.

27 Tuttobello R and PJ Mill. 1961. The pectic enzymes of *Aspergillus niger*. I. The production of active mixtures of pectic enzymes. Biochem J 79: 51–57.

28 Vogel HJ. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am Nat 98: 435–446.