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ORGINAL PAPER

C. A. Clausen • F. Green lll

Characterization of polygalacturonase from the brown-rot fungus *Postia placenta*

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Abstract Two extracellular isoenzymes of polygalacturonase, isolated from the brown-rot fungus *Postia placenta,* were purified 342-fold by Mono S cation-exchange chromatography. The temperature optimum ranged from 25°C to 37°C. and the pH optimum ranged from 3.2 to 3.9. Apparent pI values of the isoenzymes (3.2 and 3.4) were lower than any previously reported. The estimated molecular mass from a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (PAGE) was 34 kDa. Isoenzymes of polygalacturonase in native PAGE and isoelectric focusing gels were identified by substrate/agar overlays (zymograms). Comparison of viscosity reduction rates with release of reducing sugars indicated that the enzyme from *P. placenta* is endo-acting. The objective of this study was to isolate polygalacturonase from the brown-rot fungus *P. placenta* and characterize the properties of the enzyme.

Introduction

Brown-rot fungi produce a variety of extracellular polysaccharidases, but their role in the initiation of the wood decay process remains elusive because their molecular masses and configurations do not permit them to penetrate wood pores (Evans et al. 1994). Pectic enzymes derived from fungal pathogens have been suggested as an important requirement for the colonization of plant tissue, because pectin is a significant constituent of the primary plant cell wall and middle lamellae (Bateman and Millar 1966; Collmer and Keen 1986). Pectin, a readily available and hydrolyzable source of nonlignified carbohydrate, is located in the pit membranes and compound middle lamellae of wood (Militz 1993; Daniel et al. 1994). Nicholus and Thomas (1968) have shown that pretreatment of pine with pectinases removed the pit membrane and increased the penetration of wood preservatives. The ability of commercial pectinases to improve penetration of wood preservatives has been investigated (Tschernitz 1973). although information regarding pectin-hydrolyzing enzymes from wood-decay fungi is scarce. Thus, pectin-hydrolyzing enzymes may provide access to wood polysaccharides early in the wood decay process by opening the pit membrane and permitting the entry of other fungal polysaccharidases.

Endopolygalacturonases (PG, EC 3.2.1.15) have been isolated from several fungal species. including the plant pathogen *Fusarium oxysporm,* molds such as *Trichoderma koningii, Aspergillus japonicus,* and *Aspergillus niger* (Kester and Visser 1990), and the white-rot fungus *Phanerochaete chrysosporium* (Shanley et al. 1993). Green et al. (1995a) confirmed the production on polygalacturonase when *Postia placenta* and other brown-rot fungi were grown on pectin-containing medium. This is the first reported isolation and characterization of PG from a brown-rot fungus. PG characterization was critical for the further investigation of the hypothesis that pectin hydrolysis in the pit membrane by PG is essential for hyphal colonization and permeability of fungal enzymes during incipient brown-rot decay of wood. Purified PG will also be used for polyclonal antibody production and immunocytochemical localization of PG during brown-rot decay of wood.

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C. A. Clausen (✉) • F. Green III
USDA Forest Service, Forest Products Laboratory.
One Gifford Pinchot Drive, Madison.
Wisconsin 53705-2398, USA

Materials and methods

Organism and growth conditions

Postia placenta (Fr.) M. Lars. et Lomb. MAD-698, was cultured on (Bailey's[1] minimal medium (Highley 1973) supplemented with 1% pectin. Nine, 1-1 flasks, each containing 300 ml medium, were inoculated with 10 ml 10-day-old liquid culture (Bailey's minimal medium inoculated with a malt agar plug of *P. placenta* and incubated at 25°C at 200 rpm.

Enzyme purification

culture filtrates were pooled and collected by filtration through Whatman filter-paper. Proteins in the crude filtrate were precipitated with ammonium sulfate (75% saturation), The precipitate was collected by cenfrifugation at 16000 *g* for 20 min. resuspended in 0.15 M NaCl and dialyzed overnight in 0.1 M acetate buffer. pH 4.0. containing 0.15 M NaC1, followed by ultrafiltration through a 10-kDa Amicon filter (Mikes and Revoxa-Benkova 1988). The retentate was purified by ion-exchange chromatography on a fast protein liquid chromatography (Pharmacia LKB Biotechnology) using a Mono S cation-exchange column (Bio Rad). PG eluted at 5%–7% NaCl in a linear salt gradient in 0.02 M citrate buffer. pH 4.2.

Enzyme characterization

The single peak fractions from ion-exchange chromatography were characterized by several methods, Molecular mass was estimated on 10% continuous sodium dodecyl sulfate (SDS)/polyacrylamide Mini-Protean II ready gels (Bio Rad) (Wyckoff et al. 1977). using TRIS/glycine/SDS running buffer, pH 8.3, 200 V constant voltage for 30 min at 25°C. Silver standards (BioRad, 14.4--97.4 kDa range) served as molecular mass markers. Gels were stained using Bio Rad silver-stain reagents and protocol. Native polyacrylamide gel electrophoresis (PAGE) was conducted on TRIS/HCl ready gels (10% continuous) with TRIS/glycine running buffer, pH 8.3, under the same running conditions as the SDS-PAGE. Bands were visualized on native polyacrylamide gels by zymogram using an adaptation of the cup-plate assay (Dingle et al. 1953). Overlay medium consisting of 0.5% ammonium oxalate, 1% sodium-polypectate (Fluka), and 1.5% agar in 0.1 M acetate buffer, pH 5.0, was pipetted over the native PAGE gel and incubated for 18 h at 25°C. Precipitin zones were visualized by adding 50% HCl and developing for 3 min.

Isoelectric focusing on Ampholine/polyacrylamide gels. pH range 3.9–9.5, was performed on a LKB Multiphor II apparatus (Pharmacia LKB Biotechnology). A 30-µl sample of enzyme was applied to the gel surface with a filter-paper sample applicator. and focused at 5°C for 1 h at 25 W. Bands were visualized by silver stain and zymogram as previously described. The pH gradient was measured directly from the gel surface with a glass pH microelectrode.

The enzymatic activity of polygalacturonase was determined by a microadaptation of the Nelson-Somogyi reducing-sugar assay using 1% sodium polypectate in oxalate buffer (1% ammonium oxalate 1:1 with 1% oxalic acid). A unit of enzyme activity was defined as the amount needed to liberate reducing sugars equivalent to 1 µg glucose 24 h at 28°C (Green et al. 1989). Viscosity reduction was estimated with 1% sodium polypectate using a Cannon-Fenske viscometer by the method of Highley (1968, 1973). Viscometric data are expressed as 10000/ t_{50} ml enzyme $^{-1}$, where t_{50} equals the time (s) for the relative viscosity of the solution to be reduced by 50% at 28°C.

Protein was determined by the BCA microassay (Pierce) using bovine serum albumin as a standard.

Physical properties

The effect of temperature was determined by measuring reducing sugars betwecm 10°C and 65°C at pH 3.7. The effect of pH was established by measuring reducing sugars between pH 1.72 and 6.32 at 28°C.

[1] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service

Results

Polygalacturonase was isolated from *P. placenta* culture filtrate by ammonium sulfate precipitation and purified 343-fold by ion-exchange chromatography (Table 1). The enzyme had a specific activity of 182043 U/mg with a recovery of 1.1%. The temperature optimum of the isolated enzyme ranged from 25°C to 37°C (Fig. 1) and the pH optimum ranged from 3.2 to 3.9 (Fig. 2). SDS-PAGE of the purified enzyme preparation revealed one band with a molecular mass of 34 kDa. (Fig. 3), and native PAGE followed by zymogram demonstrated two separate pectic enzyme activities. A silver-stained isoelectric focusing gel followed by zymogram revealed two isoenzymes with apparent isoelectric point (pI) values of 3.2 and 3.4 (Fig. 4).

The rate of viscosity reduction was directly compared with the rate of reducing-sugar formation. At the time when the viscosity of 1% sodium polypectate was reduced by 50%, approximately 28% of the glycosidic linkages were broken, as demonstrated by the formation of reducing sugars (Fig. 5).

Discussion

Polygalacturonase isolated from *P. Placenta* eluted from an ion-exchange column as a single chromatographic protein peak. PG was characterized as two isoenzymes by pectic zymograms from native PAGE and by isoelectric focusing, although SDS-PAGE revealed only one protein subunit. Fungal polygalacturonases commonly occur in multiple forms (Rombouts and Pilnik 1980). Shanley et al. (1993) demonstrated that PG from *Phanerochaete chrysosporium* was a single-subunit glycoprotein composed of three isoenzymes. The extracellular endo-PG of *Penicillium capsulatum* was described as a single-subunit glycoprotein with a single PI of 3.78 (Gillespie et al. 1990). Rombouts and Pilnik (1980) and Whitaker (1990) noted striking differences in specific activities for PG enzymes from various fungi. Molecular masses range from 30 kDa with pI values from 3.8 to 7.6 (Rombouts

Table 1 Purification of polygalacturonase

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (-fold)
Crude enzyme	2600	709.80	377000	531	100.0	1.0
$(NH_4)_2SO_4$ precipitate	15	10.05	14415	1434	4.0	2.7
Cation exchange	4.2	0.023	4187	182043	1.1	342.8

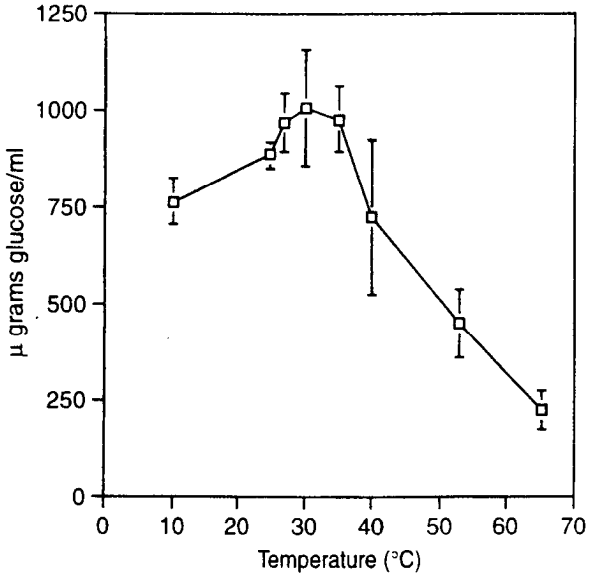

Fig. 1 Optimal thermal activity for polygalacturonase from *Postia placenta*. *Vertical bars* SD for $n = 3$

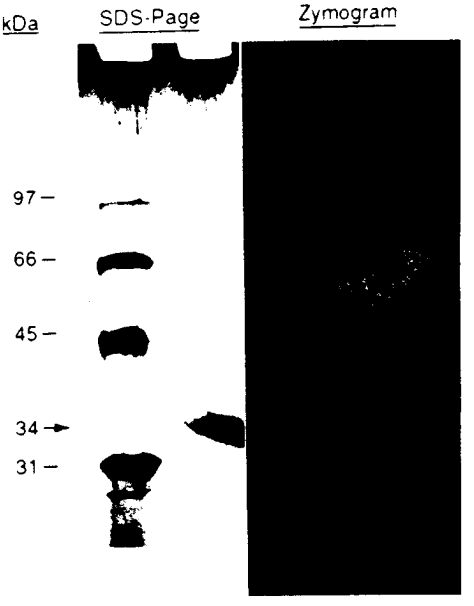

Fig. 3 Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) profile of polygalacturonase (*left*). Molecular mass markers are labeled. Zymogram overlay from native polyacrylamide gel electrophoresis showing zones of clearing indicating polygalacturonase activity from *P. placenta* (*right*)

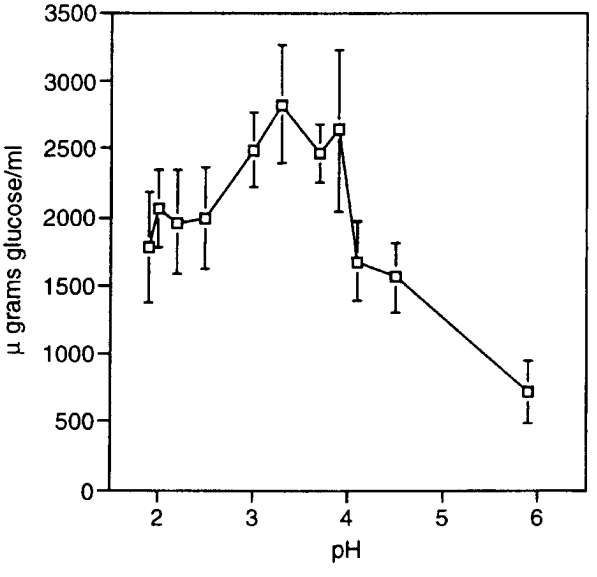

Fig. 2 Optimal pH for polygalacturonase activity from *P. placenta*. *Vertical bars* SD for $n = 3$

and Pilnik 1980; Whitaker 1990). Isoelectric points for the two PGs of *P. placenta* (3.2 and 3.4) are lower than the typical values reported in the literature. These low pI values are not surprising, because brown-rot decay of wood is facilitated by oxalic acid production and accumulation during the decay process. pH readings as low as 1.7 have been estimated by direct pH microprobe measurements. during incipient brown-rot decay by *P. placenta* (Green et al. 1991).

Typical recoveries for PG from other fungi. 7.3% for *Rhizopus* (Elegado and Fujio 1994) or 9.5% for PG from *Colletotrichum gloeosporioides* (Prusky et al. 1989), exceed the 1.1% recovery of *P. placenta* PG, but are still relatively low. Isolation procedures may account for the low recoveries of this enzyme. because it is quite stable and most active at room temperature (optimum 25°–37°C).

Pectinases were the first enzymes secreted by certain fungal pathogens grown upon isolated cell walls

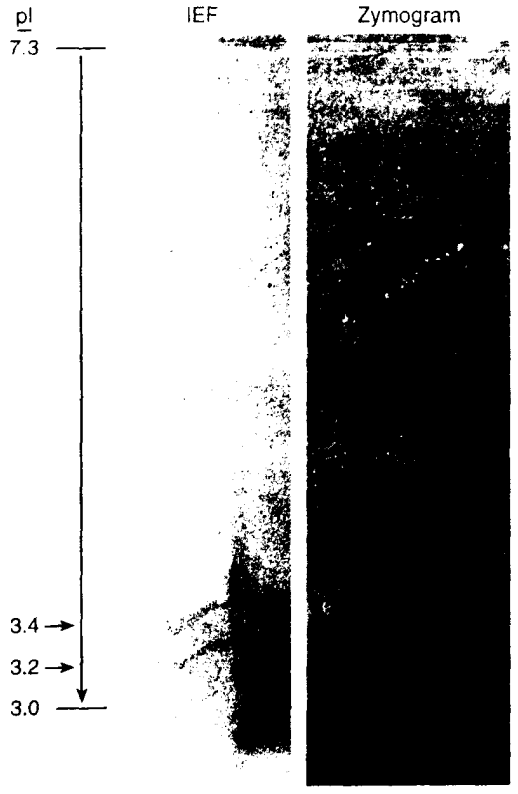

Fig. 4 Silver-stained isoelectric focusing (*IEF*) gel (*left*) and matching zymogram overlay (*right*) of polygalacturonase from *P. placenta*. *Arrows* mark pI of polygalacturonase isozymes

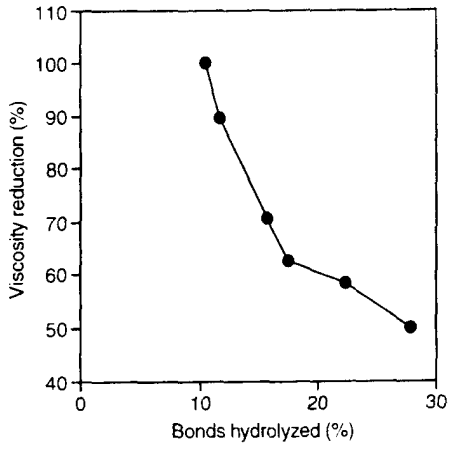

Fig. 5 Rate of viscosity reduction compared with bonds hydrolyzed by *P. placenta* polygalacturonase in 1% sodium polypectate at 28°C

(Collmer and Keen 1986). Early *in vitro* formation of PG was also noted in *P. placenta* MAD-698 with peak activity at 9 days, which exceeded the production of xylanase and endoglucanase (Green et al. 1995a).

The purified PG preparation tested negative for other polysaccharidases commonly found in culture filtrates of *P. placenta*, i.e., xylanase, endoglucanase, or laminarinase. Purified PGs were also negative for pectin methylesterase activity (unpublished results) assayed by the method of Zimmerman (1978).

Polygalacturonase activity was characterized as being of the endo type, as shown by 28% of the glycosidic bonds being cleaved during the time required to reduce the viscosity of 1% sodium polypectate by 50% (Gillespie et al. 1990; Gao and Shain 1994; Elegado and Fujio 1994). To be classified as an endoenzyme, the rate of decrease in viscosity must exceed the rate of release of reducing sugars (Gillespie et al. 1990; Shanley et al. 1993). Typically, as a result of random splitting of glycosidic bonds (i.e. reducing sugar release), endo-PGs cause pronounced reduction in viscosity of pectic substrates (much more rapidly than an exo-PG), and reducing-sugar activity will be less than 10% at viscosity t_{50} (Whitaker 1990). However, for different enzymes, this percentage may vary (Rombouts and Pilnik 1980).

PGs are thought to participate early in the wood decay process because of their hydrolytic effects on pit membranes resulting in increased wood permeability (Tschernitz 1973). The production of PG during incipient brown-rot may be crucial to rapid and efficient colonization of the wood. Pectin is readily available in the pit membranes and compound middle lamellae and is a source of nonlignified carbohydrate for pectin-hydrolyzing enzymes (Militz 1993; Daniel et al. 1994). PG and oxalic acid are thought to function synergistically for successful colonization (Bateman and Beer 1965; Tanaka and Nonaka 1981). Green et al. (1995a) have shown that PG is produced by brown-rot fungi when pectin is the carbohydrate source. Green has also shown that hydrolysis of pit membranes provides rapid access to adjacent tracheids and increases wood permeability in 7–14 days. ME-20, an atypical strain of *P. placenta* that is unable to cause wood weight loss, produces 78% less PG activity than MAD-698 as determined by reducing sugars. ME-20 is also not capable of hydrolyzing pit membranes or increasing wood permeability (Green et al. 1995a, b).

In conclusion, this is the first reported isolation and characterization of polygalacturonase from the brown-rot fungus *P. placenta* MAD-698. Polygalacturonase will be used to produce antibodies for immunocytochemical studies to determine if bordered pit-membrane hydrolysis initiates the spread of fungal enzymes during incipient brown-rot decay.

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