An immunological study of the induction of polygalacturonases in *Botrytis cinerea*

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Received 12 June 1992
Revision received 20 June 1992
Accepted 1 July 1992

Key words: *Botrytis cinerea*; Polygalacturonase; Pectinase; ELISA; Enzyme induction

1. SUMMARY

There was no consistent increase with time in the total activity of extracellular polygalacturonase (PG), relative to mycelial dry weight, when *Botrytis cinerea* was grown on a modified Czapek Dox liquid medium containing galacturonic acid or glucose. However, preparative isoelectric focusing identified a peak of activity between pH 3.7 and 6.3 only when the fungus was grown in the presence of galacturonic acid. This activity was attributed to the induction of exo-PGs (exo-PGI and/or exo-PGII). Antiserum raised against one of two endo-PGs (endo-PGI) reacted positively and specifically with endo-PGI and endo-PG'I on Western blots, and both of these isozymes were present in Western blots when the fungus was grown in the presence of glucose or galacturonic acid after 48 h in vitro. Plate-trapped antigen-ELISA confirmed that the endo-PGs were constitutively expressed.

2. INTRODUCTION

*Botrytis cinerea* Pers.: Fr. attacks flowers, fruits and vegetables of a wide range of host plants in temperate regions, either as a necrotroph or through the establishment of latent infections which culminate in post-harvest grey mould [1–3].

Polygalacturonases (PGs) have been widely implicated in pathogenesis [4] but little attention has been directed towards the study of induction of these enzymes in *B. cinerea*, although in many plant pathogens the synthesis of PGs is regulated by galacturonic acid [5–8]. Endo-PGs are constitutively expressed in the ungerminated and germinated conidia of *B. cinerea* [9] and Leone and van den Heuvel found both constitutive and inducible PGs in this fungus [8]. We have described the production of two endo-PGs and two exo-PGs from *B. cinerea* grown on a pectin-enriched liquid medium [10] and the purification of all four

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enzymes to homogeneity. In this paper we describe the first use of immunological techniques to study the regulation of PGs of *B. cinerea* in vitro.

3. MATERIALS AND METHODS

3.1. Growth conditions

*B. cinerea* Pers.: Fr. isolate 347 (IMI 339491) [1,2] was maintained and grown in shake culture on a modified Czapek Dox liquid medium containing NaNO₃ 2.0 g, KCl 0.5 g, Mg glycerophosphate 0.5 g, FeSO₄ 0.001 g, K₂SO₄ 0.35 g, glucose 10 g per litre, as described elsewhere [10]. After 6 days, the mycelium was recovered, washed in sterile distilled water and transferred to fresh flasks containing either the identical medium or one in which the glucose was substituted with galacturonic acid at various concentrations, and these were incubated for a further 48 h.

3.2. Polygalacturonase assay and protein determination

PG activity was measured by a modified Nelson-Somogyi assay [10]. One enzyme unit was defined as the amount of enzyme which released 1.0 μmol of reducing-end groups from the polygalacturonic acid in 60 min. Protein concentration was measured by the method of Lowry et al. [11] using bovine serum albumin as a standard.

3.3. Purification of polygalacturonase

Four PGs, endo-PGI, endo-PGII, exo-PGI and exo-PGII, were purified [10]. These were used as standards at various dilutions for estimations of PG produced in culture media.

3.4. SDS-PAGE

Proteins were separated in a 5% stacking gel and a 10% resolving gel as described by Laemmli et al. [12] using a Biorad Mini Protean II system. The *M*₅₀ of the proteins were estimated against pre-stained molecular mass markers (Sigma) between 27 and 180 kDa.

3.5. Immunology

One New Zealand White rabbit was injected intramuscularly with 100 μg of homogeneous endo-PGI [10] dissolved in 1 ml of Freund's complete adjuvant. A subsequent injection was performed 15 days later and bleeding was done one month following this second injection. The gamma-globulin containing fraction of the antiserum, which was prepared as described by Clark and Adams [13], was used at a concentration of 1.0 μg/ml in all immunological studies.

For immunoblotting experiments following SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose [14]. Goat-antirabbit gamma-globulin-alkaline phosphatase conjugate (Sigma) diluted 1:5000 was used as the secondary antibody. The development of colour by alkaline phosphatase was performed using an alkaline phosphatase conjugate kit (BIORAD, Richmond, CA).

3.6. Isoelectric focusing (IEF)

This was performed using the Rotofor liquid-phase preparative isoelectric focusing column (BIORAD) with a pH range of 3.5–9.5 (LKB ampholines) [10].

3.7. Isozyme separation and concentration

In order to measure the concentration of endo-PGI and endo-PGII form culture filtrate, it was first necessary to separate and concentrate them. Crude filtrate from each test sample was prepared as previously described [10]. Endo-PGI was concentrated from 100 ml of prepared culture filtrate, dialysed previously against 20 mM acetate buffer at pH 5.2, on a S-Sepharose Fast Flow (Pharmacia) column (8.0 mm × 8 cm) equilibrated with the same buffer. Endo-PGI was eluted with 20 ml of the same buffer containing 1 M NaCl and 1 ml fractions were collected. The unbound material containing endo-PGII was applied to an identical column containing Q-Sepharose Fast Flow (Pharmacia) and eluted in the same way.

3.8. ELISA

Plate-trapped antigen ELISA was performed [15] following dialysis of samples against 0.05 M carbonate buffer at pH 9.8. Test samples of 50 μl were applied to each well. Standard curves using purified antigen were made for both endo-PGI
4. RESULTS

4.1. PG induction with galacturonic acid

Filtrate from cultures grown on a modified Czapek Dox liquid medium containing galacturonic acid at concentrations ranging from 0.01% to 1.0% revealed no consistent increase in total PG activity, relative to mycelial dry weight after 2 days (data not shown). A PG assay following preparative isoelectric focusing (IEF) of the filtrate revealed a novel isozyme peak with a pI value between 3.7 and 6.3, but only when the fungus was grown on a medium supplemented with galacturonic acid (Fig. 1). This acidic peak was not detected when B. cinerea was grown in the presence of 0.01–1.0% glucose.

4.2. Western-blot analysis

Antiserum raised against endo-PGI reacted positively with both endo-PGI and endo-PGII in Western blots, but no reaction was observed with either exo-enzyme (data not shown). Western-blot analysis of fractions from preparative IEF revealed the presence of endo-PGI in the medium when the fungus was grown with 1.0% glucose or with 0.01% galacturonic acid. Endo-PGII was not detected in either study (Fig. 2). Both isozymes were identified on blots from media supplemented with either 1% glucose or 0.01% galacturonic acid (Fig. 3) following separation and concentration of endo-PGII by ion-exchange chromatography.

4.3. Quantification of endo-PGs

A higher sensitivity towards endo-PGI was achieved than with endo-PGII, using ELISA for isozyme detection; the limit of sensitivity was 0.5
and 10 ng/well for endo-PGI and endo-PGII respectively (results not shown).

ELISA showed that *B. cinerea* produced similar levels of endo-PGI or endo-PGII when the medium was supplemented with glucose or galacturonic acid (Table 1).

5. DISCUSSION

Endo-PGs produced by the majority of phytopathogenic fungi are induced by the presence of low levels of galacturonic acid [5–7]. In the only recent study of PG production by *B. cinerea* [8], the increase in total PG activity, relative to mycelial mass, was maximal with 2 mM galacturonic acid as substrate after 2 days growth; maximal induction occurred at 10 mM galacturonic acid after a further 2 days growth. This was not observed in our study where there was no consistent increase in total PG activity relative to mycelial mass, which may be accounted for by the different inoculation procedures used in our work, or by the variability displayed in PG isozyme patterns between different strains of *B. cinerea* [16].

Although no overall increase of PG activity was observed, a new peak of activity was identified only when the fungus was grown in the presence of galacturonic acid. This could only be explained by the induction of either endo-PGII, exo-PGI or exo-PGII which have pIs within this pH range [10]. This new peak cannot be explained by glucose repression because at a glucose concentration identical to that used for galacturonic acid (0.01%) this peak was still absent. The anomaly between the appearance of a novel isozyme peak and absence of total PG induction could be explained by the fact that the IEF results were not expressed relative to mycelial dry weight and therefore were not directly comparable with total induction.

Western blots of the preparative IEF fractions, corresponding to endo-PGI, revealed a band in filtrates of cultures, grown on galacturonic acid and glucose, indicative of constitutive production of this isozyme. Although not detected after IEF, endo-PGII was detected in both media, following concentration by anion exchange chromatography, suggesting that endo-PGII is also produced constitutively under these conditions.

Leone et al. [8] reported constitutive expression of an isozyme designated PGI with an identical pI value to that of endo-PGI described here. However, Leone et al. used native PAGE to separate the isozymes and *B. cinerea* is known to display wide variation in PG isozyme patterns [16]. Therefore no comparison can be made between our endo-PGII and those described by Leone et al.

The higher sensitivity of ELISA against endo-PGI compared with endo-PGII is to be expected. The gamma-globulin was raised against endo-PGI and, when used against endo-PGII, will react only with those epitopes common with endo-PGI. This explains why endo-PGII was not detected on Western blots following IEF.

The results of using Western blots indicated that both endo-PGs were constitutive and the experiments with ELISA gave support to this

| Amounts of endo-PGs produced when *B. cinerea* was grown on media supplemented with either glucose (1.0%) or galacturonic acid (0.1%) |
|---|---|---|---|---|
|   | Mycelial dry weight (g) | Endo-PGI (ng/well)* | Endo-PGII (ng/well) | Endo-PGII (ng/g mycelial dry weight) |
|   | (ng/g mycelial dry weight) |   |   |   |
| Galacturonic acid | | | | |
| Rep 1 | 0.185 | 2.1 | 795 | 16 | 3459 |
| Rep 2 | 0.207 | 2.6 | 879 | 12 | 2319 |
| Glucose | | | | |
| Rep 1 | 0.506 | 2.5 | 692 | 18 | 2253 |
| Rep 2 | 0.712 | 5.5 | 1081 | 28 | 3146 |

* These values were derived from a mean of six optical density measurements.
interpretation. Both endo-PGs were produced in similar amounts when the fungus was grown in the presence of either glucose or galacturonic acid.

The constitutive production of endo-PGI under these growth conditions cannot account for the appearance of the novel PG activity, observed during preparative IEF. We suggest that this peak can only be explained by the induction of one or both exo-PG isozymes. The development of a specific antibody for the exo-PGs could resolve these questions, but this would involve purification of these isozymes in sufficient quantity, deglycosylation and re-purification before antisera could be obtained.

Endo-PGI is produced at lower levels than endo-PGI under either media but gives a significantly higher activity peak following preparative IEF of filtrate from cultures grown in glucose. This is due to endo-PGI having a markedly lower specific activity than endo-PGI towards polygalacturonic acid.

ACKNOWLEDGEMENTS

We thank Dr. G.D. Lyon for helpful criticism of the manuscript and the Scottish Office Agriculture and Fisheries Department for funding.

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