Pectin methylesterase from *Botrytis cinerea*: physiological, biochemical and immunochemical studies

Philippe Reignault, Martine Mercier, Gilbert Bompeix and Martine Boccara

Pectin methylesterase (PME) was purified from the supernatant of *Botrytis cinerea* strain Bd90. SDS-PAGE showed a single band at 42 kDa, but this band corresponded to two distinct isoforms observed by IEF-PAGE at pl values of 7.0 and 7.4. PME was produced during the exponential phase of fungal growth and independently of the carbon source. Unlike other pectinases of *B. cinerea*, which are polymorphic, no differences were observed between the PME profiles of 25 strains of different origins. Polyclonal antibodies were raised against purified PME from *B. cinerea*, and immunochemical comparisons with PMEs from *Erwinia chrysanthemi*, *Vigna radiata* and *Glycine max* showed the presence of common epitopes between these different enzymes.

Keywords: *Botrytis cinerea*, pectin methylesterase, fungal pathogen

INTRODUCTION

The fungus *Botrytis cinerea* is responsible for important diseases involving maceration and rotting on a wide variety of crops in temperate regions. It produces a battery of enzymes that macerate plant cell walls, including pectinases such as polygalacturonases (EC 3.2.1.15) (PG), pectin lyases (EC 4.2.2.10) (PnL) and pectin methylesterase (EC 3.1.1.11) (PME) (Urbanek & Zalewska-Sobczak, 1975; Wasfy et al., 1978; Martinez et al., 1982; Hagerman et al., 1986; Movahedi & Heale, 1990). Pectin, the major component of plant middle lamella, is de-esterified to methanol and polygalacturonic acid (PGA) by the action of PME, thus allowing further hydrolysis of the glycosidic linkage of the resulting PGA by PGs. PnL, which catalyses a β-elimination of the linkage of methylated galacturonic residues, polymerizes native pectin. There is no evidence of pectate lyase (EC 4.2.2.2) (PL) production by *B. cinerea*.

Pectinases have been studied because of their possible involvement in microbial pathogenicity (Collmer & Keen, 1986). PG, PL, PME and PnL have been purified from many species of bacteria and fungi (Khanh et al., 1992), and in the case of bacterial pectinases, molecular cloning of the corresponding genes has allowed several research teams to study their role in pathogenicity by using the gene replacement method (Ried & Collmer, 1988; Boccara et al., 1988; Boccara & Châtain, 1989; Dow et al., 1989). Similar study with fungal pectinases has also been undertaken in the case of a PG produced by *Cochliobolus carbonum* (Scott-Craig et al., 1990).

The majority of the recent studies on pectinases produced by *B. cinerea* have focused on PGs (Johnston & Williamson, 1992a, b; Johnston et al., 1993). However, this fungus exhibits 6–10-fold more PME activity than other macerating fungi (G. Bompeix, unpublished) and the important role of PME in bacterial pathogenicity has been previously reported (Boccara & Châtain, 1989; Beaulieu et al., 1993). Here we report studies on PME production during growth of *B. cinerea*, in different growth conditions and by different strains. The two isozymes responsible for PME activity were purified from *B. cinerea* strain Bd90 and some of their characteristics were compared with previously reported data concerning *B. cinerea* PMEs (Marcus & Scheijer, 1983). Antibodies raised against PMEs allowed us to make immunochemical comparisons with PMEs from bacteria and plants.

METHODS

Fungal isolates and cultural techniques. The *B. cinerea* strains used in this study are listed in Table 1. All the strains were maintained on 2% (w/v) malt agar medium. Liquid cultures were performed in a modified Czapek liquid medium containing, per litre: NaNO₃ 2.5 g, KCl 0.5 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 10 mg, K₂HPO₄ 1 g, and the carbon source. For
**Table 1. B. cinerea strains used in this study**

<table>
<thead>
<tr>
<th><strong>B. cinerea strain</strong></th>
<th><strong>Origin</strong></th>
<th><strong>Host</strong></th>
<th><strong>Year of isolation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bd90</td>
<td>Bordeaux (France)*</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>Bd6</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1984</td>
</tr>
<tr>
<td>Bd7</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>Bd10</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>Bd16</td>
<td>Bordeaux (France)*</td>
<td>Vine</td>
<td>1990</td>
</tr>
<tr>
<td>Bd20</td>
<td>Bordeaux (France)*</td>
<td>Vine</td>
<td>1991</td>
</tr>
<tr>
<td>Bd22</td>
<td>Bordeaux (France)*</td>
<td>Vine</td>
<td>1992</td>
</tr>
<tr>
<td>Bd37</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1989</td>
</tr>
<tr>
<td>Bd55</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1989</td>
</tr>
<tr>
<td>3.171.a,c</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>SV 17 H</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>6.173.E</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>8.147.1</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>9.16.T</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>0.2 U 3</td>
<td>Champagne (France)</td>
<td>Vine</td>
<td>1988</td>
</tr>
<tr>
<td>190</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1990</td>
</tr>
<tr>
<td>Bot. II 2</td>
<td>Bordeaux (France)</td>
<td>Vine</td>
<td>1990</td>
</tr>
<tr>
<td>Ag 4</td>
<td>Angers (France)</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>Ag11</td>
<td>Angers (France)</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>M.II 11</td>
<td>Moissac (France)</td>
<td>Vine</td>
<td>1986</td>
</tr>
<tr>
<td>4</td>
<td>Yugoslavia*</td>
<td>Rose</td>
<td>1976</td>
</tr>
<tr>
<td>5</td>
<td>Yugoslavia*</td>
<td>Apricot</td>
<td>1978</td>
</tr>
<tr>
<td>6</td>
<td>Yugoslavia*</td>
<td>Apple</td>
<td>1978</td>
</tr>
<tr>
<td>D80</td>
<td>Homokaryotic</td>
<td>Laboratory strain</td>
<td>1990</td>
</tr>
<tr>
<td>SAS 405</td>
<td>Laboratory strain</td>
<td>Laboratory strain</td>
<td>1991</td>
</tr>
</tbody>
</table>

* Sources of strains: a, INRA Bordeaux; b, P. Leroux, INRA Versailles; c, Faretra & Pollastro (1991); d, our personal collection; e, INRA Angers.

**Preparation of concentrated supernatant.** Concentrated supernatant was obtained by a modification of the method described by Schejter & Marcus (1988). Cold acetone was added to 3:2 v/v of the Czapek-pectin medium to a final concentration of 60% (v/v) and left to stand for 3 d at 4 °C to allow maximum separation of proteins. After precipitation, the supernatant was discarded and the remaining suspension was centrifuged at 27000 g for 10 min and the pellet resuspended in 10 mM sodium acetate buffer, pH 4.5. It was reduced to about 60 ml by PEG concentration in dialysis bags and extensive dialysis against the same buffer. Concentrated supernatant was stored frozen at −20 °C and thawed for enzyme purification.

**Purification of PME.**

**Preparative cation-exchange chromatography.** Concentrated supernatant was applied directly to a column (1.2×12 cm) of CM-Sephrose CL-6B, Pharmacia) and, after washing with 10 mM sodium acetate buffer, pH 4.5, eluted with the same buffer containing 0.5 M NaCl (flow rate 0.4 ml min⁻¹). The eluted fractions (1.5 ml) containing PME activity were pooled and extensively dialysed against the same buffer without NaCl and maintained at 4 °C.

**HPLC cation-exchange chromatography.** The fractions containing PME activity were loaded onto an HPLC column (CM-Sephrose SP-5PW, Waters) equilibrated with 10 mM acetate buffer, pH 4.5. Elution was performed with an increasing linear gradient of NaCl in the same buffer (flow rate 1 ml min⁻¹). Fractions (1 ml) were collected and PME-active fractions were stored at 4 °C.

**Protein determination and PME assays.** Protein concentration was measured according to Bradford (1976), using bovine serum albumin as a standard, and by measurement of absorbance at 280 nm. Glycoprotein analysis was performed as described by Dubois et al. (1956). The semi-quantitative PME cup-plate assay was performed as described by Bertheau et al. (1984). For assays at different pH values, activity was measured by titration of the carboxyl groups from liberated pectin. The reaction mixture consisted of 3 ml 0.5% (w/v) Sigma P 9436 pectin solution, 0.5 ml 2 M NaCl, in a total volume of 6 ml. The pH was maintained at constant value by adding 10 mM NaOH to the reaction mixture. Quantitative PME assay at pH 5.5 was performed by a modification of the method of Hagerman & Austin (1986). In our assay, the pH indicator was bromocresol green and PME activity was monitored spectrophotometrically at 620 nm. The reaction mixture consisted of 1 ml 0.5% (w/v) Sigma pectin (P-9436) solution, 75 μl 0.01% bromocresol green in 3 mM phosphate buffer, various amounts of sample, and distilled water to bring the final volume to 1.5 ml. All reagents were adjusted to pH 5.5 and the assay was calibrated with 1 mM acetic acid. One PME unit (U) is the amount of enzyme that releases 1 μmol H⁺ ions s⁻¹ at pH 5.5 and 25 °C.

**Isoelectric focusing (IEF).** Twenty microlitres of each sample were layered on a thin polyacrylamide gel and electrophoresis was performed in a pH gradient from 3 to 10 (Pharmacia). PME activity was assessed directly on the gel by an overlay technique according to Bertheau et al. (1984). The pH values on the separation gel were assessed with a surface electrode (Multiphore electrode; LKB).

**SDS-PAGE.** Electrophoresis under denaturing conditions (SDS-PAGE) was performed according to Laemmli (1970), with a 2% (w/v) stacking gel and a 12% (w/v) running gel, using the Mini-Protean II dual slab cell system (Bio-Rad). Bio-Rad low molecular mass markers were used. Gels were fixed and proteins silver-stained according to Merrill et al. (1981).

**Origin and production of antibodies.** Polyclonal antibodies were produced in two New Zealand White rabbits. The first immunization of one intradermal injection of 500 μg purified protein with complete Freund's adjuvant. Then, two subcutaneous injections of 500 μg of purified protein with incomplete Freund's adjuvant were performed at 21 d intervals. The rabbits were bled 14 d after the last immunization.
Immunoblotting (see below) with purified protein revealed a reacting band up to a 3200-fold serum dilution.

**Immunoblotting.** Western blotting was performed according to the method of Burnette (1981). Immunodetection was performed by the alkaline-phosphatase-conjugated anti-antibody method of Blake et al. (1984).

**RESULTS**

**Mycelial growth, culture media pH and PME activity**

*B. cinerea* strain Bd90 grew rapidly *in vitro* on Czapek medium containing 1% pectin as the sole carbon source, reaching maximum yield on day 5. There was no significant increase in mycelial dry weight from day 5 to day 10. The initial pH of the cultures declined rapidly from 4.6 to 3.7 during the beginning of fungal growth for the first 2 d, then increased to pH 7.1 simultaneously with the maximal phase of growth from day 2 to day 5. This pH value remained constant until the end of the culture (Fig. 1a). PME activity was detected by cup-plate assay in the culture filtrate of *B. cinerea* by day 2; it had a maximum specific activity on day 2 and maximum total activity on day 4. Further stabilization of the mycelial dry weight and the pH value resulted in a slight decrease in PME specific activity (Fig. 1b).

**Variability of PME production with different growth substrates**

Although *B. cinerea* strain Bd90 grew very well with all the pectin preparations tested (see Methods) as the sole carbon source, its growth was much slower on the Czapek-PGA medium: after 6 d of culture, mycelial dry weight reached only 28% of the amount obtained with 1% pectin. Moreover, growth with 1% glucose as the sole carbon source resulted in a dramatically reduced mycelial dry weight (4-5% of the amount obtained when grown with 1% pectin). We therefore used 3% glucose in order to obtain, after 6 d culture, a comparable amount of mycelial dry weight.

![Fig. 2. PME activity on IEF polyacrylamide gels (pH range 3-10).](image)

Concentrated supernatants (20 μg protein loaded) from: lane 1, monokaryotic strain D80 cultured for 4 d in 1% pectin; lane 2, strain Bd90; lane 3, strain Bot. II 2; lane 4, strain Ag 4; lane 5, strain M.II 11; lane 6, strain 8.147.1; lane 7, strain 4; lane 8, strain 5; lane 9, strain 6.

**Table 2. Recovery of PME activity and protein during the purification procedure**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification (fold)</th>
<th>PME activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>800</td>
<td>376.0</td>
<td>16/88</td>
<td>0.04</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Concentrate crude extract</td>
<td>16</td>
<td>72.0</td>
<td>5/24</td>
<td>0.07</td>
<td>1.6</td>
<td>31</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>24</td>
<td>1.9</td>
<td>3.24</td>
<td>1.69</td>
<td>37.5</td>
<td>19</td>
</tr>
<tr>
<td>HPLC CM-Sepharose</td>
<td>40</td>
<td>1.5</td>
<td>1.84</td>
<td>1.27</td>
<td>28.2</td>
<td>11</td>
</tr>
</tbody>
</table>
dry weight (111% of the amount obtained when grown with 1% pectin).

Despite these differences in fungal growth, PME specific activity (U per 100 mg mycelial dry weight) was similar whatever carbon source was used (pectin 1%, PGA 1%, glucose 3%). IEF-PAGE analysis of the supernatant of B. cinerea strain Bd90 grown with 1% pectin showed two PME isozymes at PI values of 7.0 and 7.4 (Fig. 2, lane 2). The same profile was obtained irrespective of the carbon source used (data not shown).

## Variability of PME production in different B. cinerea strains

All strains tested, from different geographical origins, different hosts and/or different years of isolation (see Table 1), as well as the monokaryotic laboratory strain D80, produced PME activity in the culture filtrate. The IEF profiles of the 25 different strains exhibited the same typical pattern with two isozymes (Fig. 2, results for nine strains presented). The detection in some cases of a small additional band by IEF-PAGE may be due to oxidative or proteolytic modification of the two major species of PME, resulting in a charge modification. This hypothesis is supported by the fact that such an additional band appeared after 20 d culture and in samples stored for a long period at 4°C (data not shown).

Ruthenium red extensive staining of pectin overlay revealed that strains 6173.E and 916.T from the Champagne area and Bd6, Bd7 and Bd22 from the Bordeaux region produced a distinguishable PnL activity at PI 7.8 (data not shown).

## Purification of PME

PME activity from the concentrated supernatant of strain Bd90 was purified using cation-exchange chromatography with 11% recovery (Table 2). The PME resulting from this purification procedure was homogeneous as judged by SDS-PAGE after silver nitrate staining and exhibited a molecular mass of 42 kDa (Fig. 3a, lane 3), but native electrophoresis (data not shown) and IEF (Fig. 3b, lane 3) showed two isozymes of the enzyme of quite similar PIs, 7.0 and 7.4, respectively, as previously observed for concentrated supernatant. No other pectinolytic activity was co-purified with PME. The titration curve showed that these two isoforms of PMEs had a weak charge variation around their PI value (data not shown). We made several unsuccessful attempts to separate the two PME species: long linear NaCl gradients at very low flow rate (0-1 ml min⁻¹), pH gradient between 6.0 and 8.0, or linear NaCl gradient on pectin with different degrees of cross-linking according to Rombouts et al. (1979).

## Biochemical characteristics

The PME-active fraction obtained from the HPLC cation-exchange chromatography was shown to contain glycoproteins with 30% carbohydrates. It was active over a broad range of pH (2-8) with the cup-plate assay but exhibited an apparent optimum at pH 5.5 when using titration of the liberated carboxyl groups. Using the colorimetric assay described in Methods, monovalent cations (Na⁺ and K⁺, 20 and 120 mM) and divalent cations (Ca²⁺ and Mg²⁺, 20 and 120 mM) increased the PME activity twofold and three- to four-fold, respectively. The optimal temperature was 60°C but PME was active at more than 10% of the optimal activity at 10°C and 80°C.
Purified PME fraction, with an 800-fold serum dilution. Here we found an identical molecular mass of 42 kDa for both isozymes; their different PI values, of 7.0 and 7.4, allowed us to distinguish them. However, we were not able to separate our PME isozymes. Since the two isoforms have identical molecular masses, and their pI values seem to be closely linked to the beginning of the stationary phase, indicating that production of each PME isoform is modulated by the available carbon source. The strong band observed at 42 kDa in electrophoretic profiles indicates that PME is a major protein in the supernatant of strain Bd90 (Fig. 4a, lane 1). PME production seemed to be closely linked to the beginning of the exponential phase of growth and to the neutralization of the culture medium; it reached a maximal value at the beginning of the stationary phase. The enzyme activity was found, in similar amount, in culture filtrates irrespective of the carbon source used: pectin, PGA or glucose. This result suggested that PME activity was not induced by pectic derivatives and not subject to catabolic repression. These results indicate the crucial role of PME activity in fungal metabolism; the constitutive production of PME could be related to the importance of this activity for pectin degradation during the early steps of plant invasion by the fungus.

In accordance with our observations, there are several reports of production of pectinolytic enzymes as several isoforms of distinct molecular mass and/or pI by B. cinerea (Drawert & Kreft, 1978; Wasfy et al., 1978; Magro et al., 1980; Johnston & Williamson, 1992a). The profile observed with the monokaryotic strain D80 showed that the presence of the two PME isoforms could not be explained by the heterokaryotic nature of Botrytis. Both isoforms of PME have been found under different conditions, indicating that production of each PME isoform is not modulated by the available carbon source.

A wide variation in PG isoform patterns between strains has been observed in B. cinerea (Magro et al., 1980). In this respect, the PMEs of B. cinerea are somewhat unusual: PME production is not qualitatively affected by the difference in the characteristics of the 25 tested strains: geographical origin, host or year of isolation. The lack of variability seems to be typical of PMEs. Indeed, IEF-PAGE revealed that a diversity exists for PnL production, even between strains of the same geographical origin (strains from Champagne) and host and year of isolation (strains from Bordeaux).

PME is produced by many phytopathogenic organisms responsible for rotting (Collmer & Keen, 1986). In addition, it is present in the plant cell wall and it is supposed to be involved in cell division and pectin modification (Goldberg, 1984; Tieman et al., 1992) and in plant ripening (Fisher & Bennett, 1991). For these reasons, PME activity has been purified from many organisms. Several common points have been established...
between enzymes of different origins: common biochemical properties, interaction with plant cell wall, and some highly conserved amino acid sequences (Lin et al., 1989; Albani et al., 1991). Using a polyclonal antiserum raised against PMEs from B. cinerea, serological cross-reactivities were obtained with PME from Erwinia chrysanthemi, Vigna radiata and Glycine max, indicating the presence of common epitopes to these different enzymes. Although a purified basic molecular form of PME called PME (Bordenave & Goldberg, 1993) was not detected with our antiserum, our results strengthen and extend previous results about the conservation of motifs among PME from different taxa (Albani et al., 1991).

The physiological, biochemical and immunological information presented in this paper should help us to isolate the corresponding gene(s) of B. cinerea and study the role of this enzyme in pathogenicity.

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