Analysis of the Pectin-degrading Enzymes Secreted by Three Strains of *Erwinia chrysanthemi*

By FRÉDÉRIQUE VAN GIJSEGEM

Laboratoire de Génétique, Université Libre de Bruxelles, Rhode-Saint-Genèse, Belgium

(Received 17 June 1985; revised 3 September 1985)

The protein content of culture supernatants of three *Erwinia chrysanthemi* strains, B374, 3937j and 3665, grown on different carbon sources was compared. After growth in presence of polygalacturonate, four new polypeptides, identified as pectinases, were synthesized. These induced proteins, and the pattern of pectate lyase induction, differed among the strains. The proteins present in the supernatants of some mutants known or suspected to be affected in pectinase production (secretion-defective mutants and mutants in the degradative pathway of galacturonate and ketodeoxygluconate) were also analysed.

**INTRODUCTION**

Strains of *Erwinia chrysanthemi* are important phytopathogenic enterobacteria responsible for the soft rot disease which attacks fruits, vegetables and ornamental plants during growth, transit and storage. Their pathogenic properties are due in part to the ability to release a battery of extracellular enzymes including pectinases, cellulases and proteases (for a review see Chatterjee & Starr, 1980) to degrade pectin and cellulose, the major plant primary cell wall components.

Degradation of pectin involves demethylation to produce polygalacturonic acid (PGA) by pectin methylesterase (PME; EC 3.1.1.11) and breakdown of the PGA into 2-keto-3-deoxygluconate (KDG) through two enzymic processes: a transeliminative reaction catalysed by pectate lyase (PL), producing oligomers with an unsaturated uronic acid at the non-reducing end and a hydrolytic reaction catalysed by exopolygalacturonase (exo-PG), which produces digalacturonic acid. These two types of oligomers are then cleaved by an oligouronide lyase (OGL) producing uronic acid and galacturonate (GA) which are then transformed into KDG (Fig. 1). The PL and exo-PG enzymic activities can be detected in the supernatants of cultures of *E. chrysanthemi* and most are inducible by PGA and subject to catabolite repression (Garibaldi & Bateman, 1971; Collmer et al., 1982b).

Using slab polyacrylamide gel electrofocusing, Bertheau et al. (1984) separated five major PL activities from the supernatant of a culture of the *E. chrysanthemi* strain 3937j. One of these is acidic (PLa), two are neutral (PLb and c) and two are basic (PLd and e). The genes encoding most if not all of these pectate lyases have now been cloned into *Escherichia coli* from several *E. chrysanthemi* strains including B374, 3937j and 3665, the three strains studied in this work (Barras et al., 1984; Reverchon et al., 1985; Keen et al., 1984; Collmer et al., 1985; Kotojansky et al., 1985; van Gijsegem et al., 1985a). The PL genes were expressed in *Escherichia coli* but reports concerning the secretion and location of the proteins are so far contradictory. In addition Collmer et al. (1982a) have purified one hydrolytic enzyme (an exo-poly-α-D-galacturonosidase, exo-PG) as well as two PL species secreted by *E. chrysanthemi* strain 630. In the work described here I have further characterized the proteins present in culture supernatants of strains B374, 3937j and 3665, in order to identify those which correspond to PL species.

**Abbreviations:** DK1, 5-keto-4-deoxy-D-uronate; DKII, 2,5-diketo-3-deoxy-D-gluconate; GA, D-galacturonic acid; KDG, 2-keto-3-deoxy-D-gluconate; KDGp, 6-phospho-2-keto-3-deoxygluconate; OGL, oligouronide lyase; PG, polygalacturonase; PGA, polygalacturonic acid; PL, pectate lyase.
Fig. 1. Degradative pathway of pectin in *E. chrysanthemi*. PME, pectin methylesterase; PL, pectate lyase; PG, polygalacturonase; OGL, oligouronide lyase; ISO, 4-deoxy-L-three-o-5-hexosulose-uronate ketol-isomerase; DH, 2-keto-3-deoxy-3-glucuronate dehydrogenase. PGA, polygalacturonate; diGA, digalacturonate; GA, galacturonate; DKI, 5-keto-4-deoxyuronate; DKII, 2,5-diketo-3-deoxyglucuronate; KDG, 2-keto-3-deoxygluconate; KDGP, 6-phospho-2-keto-3-deoxygluconate. I, uronate isomerase; II, altronate oxidoreductase; III, altronate hydrolyase; IV, 2-keto-3-deoxygluconate kinase; V, 2-keto-3-deoxy-6-phosphogluconate aldotase. The symbols in parentheses are those of the genes coding for the enzymes.

Table 1. *Strains of E. chrysanthemi*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3665</td>
<td>Wild-type, isolated from <em>Dijfenbachia</em></td>
<td>M. Lemattre, INRA collection; Barras et al. (1984)</td>
</tr>
<tr>
<td>B374</td>
<td>Wild-type, isolated from <em>Pelargonium rosa</em></td>
<td>Hamon &amp; Peron (1961)</td>
</tr>
<tr>
<td>ERH231</td>
<td>B374 GurA+ Rif' kdgK3</td>
<td>His+ derivative of ERH205; van Gijsegem et al. (1985b)</td>
</tr>
<tr>
<td>ERH207</td>
<td>B374 his-5 GurA+ Rif' uxaAl'::Tn9</td>
<td>van Gijsegem et al. (1985b)</td>
</tr>
<tr>
<td>ERH208</td>
<td>B374 his-5 GurA+ Rif' uxaB1'::Tn9</td>
<td></td>
</tr>
<tr>
<td>ERH214</td>
<td>B374 his-5 GurA+ Rif' uxaCB1</td>
<td></td>
</tr>
<tr>
<td>3937j</td>
<td>Wild-type, isolated from <em>Saintpaulia ionantha</em></td>
<td>Kotoujansky et al. (1982)</td>
</tr>
<tr>
<td>HfrQ</td>
<td>3937j Hfr Lac- Te' Tra+ Nal'</td>
<td>Mutagenesis of HfrQ with Mu13.4A5; Andro et al. (1984)</td>
</tr>
<tr>
<td>24</td>
<td>HfrQ Cm' out</td>
<td>Mutagenesis of HfrQ with D108-1; T. Andro</td>
</tr>
<tr>
<td>34</td>
<td>HfrQ Cm' out</td>
<td>Mutagenesis of HfrQ with Mu18A-1; T. Andro</td>
</tr>
<tr>
<td>48</td>
<td>HfrQ Ap' out</td>
<td></td>
</tr>
</tbody>
</table>

**METHODS**

**Bacterial strains.** These are listed in Table 1. The out mutants of 3937j were isolated after mini-Mu insertion mutagenesis (Andro et al., 1984).

**Media.** Bacteria were grown in liquid minimal 132 medium (Glansdorff, 1965) (7 g K2HPO4, 3H2O, 3 g KH2PO4, 1 g (NH4)2SO4, 0.1 g MgSO4.7H2O and 0.5 g sodium citrate per litre) supplemented with 0.2% of the desired carbon source, glycerol, glucose, GA, PGA or a mixture, and 40 μg amino acids ml−1 when required.
Sample preparation. Cultures in the late exponential growth phase \((2 \times 10^6\ \text{bacteria ml}^{-1})\) were centrifuged twice and frozen at \(-20^\circ\text{C}\). When necessary, the supernatants were concentrated by ultrafiltration through a PM10 Amicon filter.

Gel filtration. The samples were passed through a 15 ml Sephacryl S-200 column equilibrated with a buffer containing 50 mm-Tris/HCl pH 8.6, 1 mm-CaCl\(_2\), 50 mm-KCl, 5 mm-MgCl\(_2\), and fractions of 500 \(\mu\text{l}\) were collected. The void volume of the column was determined by using blue dextran and the included volume by using pyronin Y.

SDS-PAGE. A vertical gel apparatus \((15 \times 20\ \text{cm})\) was used. The gels were poured as described by Laemmli \((1970)\) and run for 16 h at 70 mA before staining with AgNO\(_3\)/NH\(_4\)OH as described by Oakley et al. \((1980)\). The molecular mass standard proteins were bovine albumin \((66\ \text{kDa})\), ovalbumin \((45\ \text{kDa})\), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle \((36\ \text{kDa})\) and carbonic anhydrase from bovine erythrocytes \((29\ \text{kDa})\).

PL activity assay. Two assays were used: the thiobarbituric acid method \((Sherwood, 1966)\) or the absorption at 235 nm in a reaction buffer containing 0.1 M-Tris/HCl pH 8.6, 1 mM-CaCl\(_2\) and 0.125% PGA. In the second assay, 1.73 absorbance units min\(^{-1}\) represents the formation of 1 pmol unsaturated uronide min\(^{-1}\) \((Keen et al., 1984)\), and one unit \((\text{U})\) of activity was defined as the liberation of 1 pmol product min\(^{-1}\).

Electrofocusing. This was done on an LKB2117 Multiphor apparatus, using a 10\%(w/v) polyacrylamide gel containing 5\%(w/v) LKB pH 3.5-10.0 ampholines. The pectinase activity was revealed by the sandwich technique devised by Bertheau et al. \((1984)\), in which the proteins were blotted onto an agarose-PGA gel, which is soaked in 1\% (v/v) cetyl trimethyl ammonium bromide (CTAB), which precipitates the undigested PGA and leaves clear zones where there was pectinase activity.

**RESULTS**

**Analysis of the proteins secreted by strain 3937j after induction of the pectinase activities**

The proteins contained in culture supernatants of strain 3937j grown on either glycerol or PGA were analysed by SDS-PAGE. After growth on glycerol, 10 to 20 bands could be seen after silver staining of the gel; after growth on PGA, four additional major polypeptides of 44, 42, 40 and 38 kDa were found (see Fig. 3a). To determine whether the PGA-induced polypeptides exhibited PL activity, the supernatant was passed through a Sephacryl S-200 column and fractions were tested for PL activity. Activity resided in one peak (Fig. 2a) and portions of the active fractions (12 to 17) were run on an SDS-PAGE gel (Fig. 2b). All the active fractions contained the four PGA-induced polypeptides and there was a good correlation between the intensity of the protein bands on the SDS-PAGE gel and the level of activity measured (Fig. 2b).

The five major PL activities detected in the fractions by electrofocusing were the same as those found in the culture supernatant of 3937j (see Introduction) (data not shown). Similar results were obtained after fractionation using a Sephadex G-75 column rather than Sephacryl S-200 (data not shown).

**Comparison of the pectinases produced by different E. chrysanthemi strains in different growth conditions**

Strains B374, 3937j and 3665 exhibit a very similar pattern of major PL activity upon slab gel electrofocusing of supernatants of cultures induced by PGA (Y. Bertheau, personal communication). The five major bands of activity have a pl of about 9.5 (PLd and e), 8.5 (PLb and c) and 3.8 (PLa). PLa has a higher p1 value in the case of 3665 (data not shown). I therefore compared by SDS-PAGE the proteins secreted by the three strains, grown either with glycerol or with the PL inducer PGA as a carbon source (Fig. 3a). Growth with PGA resulted in the appearance of four (or five) additional polypeptides of distinguishable molecular mass which were absent in the supernatants of cultures grown with glycerol. However, the apparent molecular mass, and the relative intensities, of these induced polypeptides varied among the strains. Strain 3937j displayed four bands of 44, 42, 40 and 38 kDa, strain 3665 showed four bands of 45, 42, 41 and 40 kDa (and possibly a faint band of 37 kDa), and strain B374 showed five bands of 45, 42, 40, 38 and 28 kDa (see also Fig. 4b). Thus although the PL isoenzymes secreted by the three strains had the same pl values (with the exception of PLa from 3665), some of them had slightly different molecular masses.
Fig. 2. Correlation between the PGA-induced polypeptide bands revealed by SDS-PAGE and PL activities. Analysis by gel filtration of the supernatant of a culture of strain 3937j grown on PGA. (a) Gel permeation chromatography. A sample (500 μl) of the supernatant of strain 3937j grown in minimal medium supplemented with PGA was layered on a column of Sephacryl S-200 equilibrated as described in Methods. Bovine serum albumin (66 kDa) and lysozyme (14.3 kDa) were also added as size markers. The two peaks corresponding to these two markers were located by measuring the of the fractions. The PL activity of the fractions was measured by the thiobarbituric acid method (see Methods). The void volume was located in fraction 9 and the included volume in fraction 48. (b) SDS-PAGE of the active permeation fractions. A sample (50 μl) of each active fraction (12 to 17) was run on a 10% (w/v) polyacrylamide gel, which was stained with AgNO₃. The arrows indicate the PGA-induced polypeptides. A, supernatant of 3937j grown on PGA.

The influence of the different sugars used for growth on the production of secreted pectate lyases was also investigated. The three strains were grown to late exponential phase on either glycerol, GA, PGA or glucose (to assay the basal level of PL, possible induction by GA or PGA and catabolite repression by glucose, respectively), and on a mixture of glycerol and GA or PGA, or glucose and GA or PGA, to ascertain the effect of either glycerol or glucose on the induced production of PL. The supernatants of the cultures were assayed quantitatively for PL activity, at 37 °C, by following the production of unsaturated sugar derivatives (increase in $A_{235}$) resulting from transeliminative degradation of PGA by PL (Table 2). The three strains behaved differently both in the amounts of PL they produced and in their responses to the different sugars supplied for growth.

The basal level of PL measured after growth on glycerol was 20 to 40 times lower in strains 3937j and B374 than in strain 3665. The analysis by electrofocusing of a supernatant of 3665 (concentrated sevenfold) grown in such conditions showed that the PLd to PLe ratio was lower than when the enzymes were induced by PGA (Fig. 3b).
Pectinases secreted by Erwinia chrysanthemi

Fig. 3. Comparison of the proteins contained in culture supernatants of strains B374, 3937j and 3665. (a) SDS-PAGE gel of the supernatants of the three strains grown in the absence or in presence of the PL inducer PGA. Conditions were the same as for Fig. 2. A, Supernatants of cultures grown on glycerol; B, supernatants of cultures grown on PGA. * The sample loaded was concentrated fivefold. † The sample loaded was concentrated twofold. (b) Electrofocusing of supernatants of strain 3665 grown on glycerol (1) or PGA (2). Lane 3 contained supernatant 1 concentrated sevenfold.

Table 2. Assay of PL activity of the culture supernatants of E. chrysanthemi strains grown on different carbon sources

Cultures in the late exponential phase (about 10^9 bacteria ml^-1) were centrifuged; samples (50 μl) of the supernatant were mixed with 950 μl reaction buffer and the change in A_{235} was monitored for 10 min at 37 °C.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>B374</th>
<th>Relative activity</th>
<th>3937j</th>
<th>Relative activity</th>
<th>3665</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U ml^-1</td>
<td></td>
<td>U ml^-1</td>
<td></td>
<td>U ml^-1</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>9.7 x 10^-3</td>
<td>1</td>
<td>4.6 x 10^-3</td>
<td>1</td>
<td>1.8 x 10^-1</td>
<td>1</td>
</tr>
<tr>
<td>Glycerol + GA</td>
<td>2.5 x 10^-2</td>
<td>2.5</td>
<td>4.0 x 10^-2</td>
<td>9</td>
<td>4.5 x 10^-1</td>
<td>2.5</td>
</tr>
<tr>
<td>GA</td>
<td>7.6 x 10^-2</td>
<td>8</td>
<td>2.7 x 10^-1</td>
<td>59</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>Glycerol + PGA</td>
<td>2.9 x 10^-1</td>
<td>30</td>
<td>3.0 x 10^-1</td>
<td>65</td>
<td>6.9</td>
<td>38</td>
</tr>
<tr>
<td>PGA</td>
<td>5.0 x 10^-1</td>
<td>50</td>
<td>8.1</td>
<td>1760</td>
<td>8.1</td>
<td>45</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.3 x 10^-3</td>
<td>0.25</td>
<td>1.2 x 10^-2</td>
<td>2.5</td>
<td>1.6 x 10^-1</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose + GA</td>
<td>5.3 x 10^-2</td>
<td>5</td>
<td>2.5 x 10^-1</td>
<td>54</td>
<td>3.3 x 10^-1</td>
<td>2</td>
</tr>
<tr>
<td>Glucose + PGA</td>
<td>8.0 x 10^-2</td>
<td>8</td>
<td>7.2 x 10^-1</td>
<td>156</td>
<td>1.1</td>
<td>6</td>
</tr>
</tbody>
</table>
Apart from the amount of PL produced, the PL induction patterns of strains B374 and 3665 were very similar. Relative to glycerol, GA induced the production of PL six- to eightfold and PGA about 50-fold. In the presence of glycerol, PL induction by GA was repressed 2 to 3-fold while glycerol only slightly affected PGA induction. For strain 3665, PL production after growth on glucose was similar to that after growth on glycerol, while in strain B374, there was a fourfold decrease in PL production after growth on glucose. Strain 3937j showed a very different pattern of PL induction. In glycerol, PL production was very low, even lower than in glucose and the repression of GA and PGA induction by glycerol was more severe (7- and 30-fold respectively) than in the two other strains. Glucose did not repress the induction of PL by GA, but did repress its induction by PGA about 10-fold. Finally, in strain 3937j, the ratio of PL induction by PGA to that by GA was higher (30 compared to 6) than in strains B374 and 3665.

Portions of the different supernatants were also analysed by SDS-PAGE. The polypeptides induced by PGA were also observed after induction by GA and the differences in band intensities correlated with the differences in pectinase activities (data not shown).

**PGA-induced polypeptides in mutants affected in pectinase production**

The supernatants of cultures of mutants derived from strains B374 or 3937j were also analysed by SDS-PAGE and electrofocusing. Mutants of strain 3937j unable to secrete PL (out) have been isolated and characterized (Andro et al., 1984). They still produce pectinases but these accumulate in the periplasm and almost no activity is detected in the medium. SDS-PAGE of the supernatants of cultures grown in the presence of PGA showed that the four polypeptide bands induced by PGA in the parental strain were not seen in the out mutants (except for one intense band present in the parent that was faint in the mutants) (Fig. 4a). These results provide
Pectinases secreted by Erwinia chrysanthemi

further evidence that these polypeptides are isoenzymes of PL. Strain ERH231, a $kdgK$ mutant of strain B374, was also analysed. This mutant is unable to metabolize KDG (a degradation product of PGA) into KDGP because it lacks KDG kinase. When grown in the presence of either GA or PGA, it produces about fivefold more pectinase than the parental strain grown in the presence of PGA (2.7 U ml$^{-1}$ compared to 0.5 U ml$^{-1}$ for strain B374 grown in the same conditions). Electrophoresis revealed that in this mutant only PLb and PLc were overproduced (Fig. 4c). SDS-PAGE showed the appearance in the supernatant of two intense polypeptide bands of 45 and 28 kDa and a minor one of 40 kDa after growth in the presence of GA or PGA. The 41 kDa already present in the supernatant of the glycerol-grown culture was also more intense in induced conditions (Fig. 4b).

Mutants defective in the other steps of the degradation of GA ($uxaA$, $uxaB$ and $uxaC$ mutants, see Fig. 1) do not exhibit altered pectinase production.

**DISCUSSION**

The analysis of the proteins in the supernatants of different *E. chrysanthemi* cultures grown in the absence or presence of PGA has revealed that, in the presence of PGA, four (or five) new polypeptides are synthesized. Several observations suggest that these are PL species: (1) they were present in all growth conditions which allowed PL production; (2) there was a good correlation between the intensity of these protein bands and the level of PL activity; (3) when the supernatant of strain 3937j grown on PGA was passed through a permeation filtration column, these four proteins represented the major components in the active fractions and the intensity of the protein bands corresponded to the activity of the fractions; (4) in *out* mutants, which are defective for PL secretion and retain most of the proteins in the periplasm (Andro et al., 1984), the four inducible proteins were virtually absent from the supernatant.

Both the PGA-induced protein bands and the pattern of PL induction differed among the three *E. chrysanthemi* strains studied. Strains 3937j and 3665 had the same PL activities after induction with PGA while strain B374 produced 10- to 20-fold less PL activity. The non-induced level of PL activity in strain 3937j was about 50-fold lower than that in strain 3665. This high basal level in strain 3937j was not subject to glucose repression, and electrophoresis showed that four of the five peptides (PLa, b, c and e) were produced constitutively to a different extent, PLd being almost completely absent. This suggests that the different PL genes are not necessarily always co-regulated.

The very low basal PL activity produced by strain 3937j does not hinder its pathogenicity. Therefore it seems likely that enzymes other than PL could act *in planta* to produce the PL inducer(s). In this context an exo-PG of the type characterized by Collmer et al. (1982a) may play an important role.

A $kdgK$ mutation which affects the catabolism of GA and KDG, the two degradation products of PGA, had a strong effect on PL production. The $kdgK$ mutant grown on PGA or GA produced about fivefold more pectinase than its parental strain grown in the same conditions. The degradation pathway of PGA outlined in Fig. 1 shows that the hydrolytic or transeliminative degradation of PGA produces oligouronides which are cleaved by the oligo-transeliminase (OGL), generating 5-keto-4-deoxyuronate (DKI) which is then dehydrogenated into 2,5-diketo-3-deoxygluconate (DKII) which in turn is isomerized into KDG. GA is also catabolized into KDG. The $kdgK$ mutation blocks the transformation of KDG into KDGP, leading to the accumulation of KDG. Since the transformation of DKII into KDG is reversible (Condemine et al., 1984), accumulation of KDG leads to the accumulation of DKII, which is most probably the cause of the very high inducibility in the $kdgK$ mutant. Indeed, Collmer & Bateman (1981) have also proposed DKI or DKII as the most probable inducer of pectinase production. In $kdgK$ mutants, mannionate and altronate, two metabolites of GA degradation upstream of KDG, should also accumulate. These intermediates do not seem to induce PL synthesis, since an $uxaA$ mutant which also accumulates these metabolites does not overproduce the enzymes (data not shown).
I have been unable to establish a precise correlation between the active proteins detected by electrofocusing and the inducible polypeptides characterized by electrophoresis. There are five active bands upon electrofocusing but, for some strains, only four polypeptide bands upon electrophoresis. This problem should be solved by analysing the proteins produced by the PL genes cloned on plasmid pBR322 in an active system; this work is in progress.

I thank A. Toussaint for very fruitful discussion and help in writing this paper, and M. Chandler for the critical reading of the manuscript. This work was carried out under research contract GBI-3-013-B from the Biomolecular Engineering Program of the EEC and the Action Thématique Programmée ‘Microbiologie’ from the Centre National de la Recherche Scientifique.

REFERENCES


