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Production of pectolytic enzymes from *Erwinia* grown on different carbon sources

V.E. Shevchik,* A.N. Evtushenkov, H.V. Babitskaya and Y.K. Fomichev

A quantitative analysis of pectolytic enzymes (polygalacturonase (PG), pectin methyl esterase (PME) and six isoenzymes of pectate lyase (PL)) produced by *Erwinia* bacteria in the presence of diverse carbon sources was made by preparative electrophoresis. Synthesis of each of these enzymes was regulated independently; different induction and repression ratios (about 10- to 1000-fold) were observed for diverse PL isoenzymes, PG and PME. The possibility of using specially constructed media for the production of pectinase complexes with a specific spectra of pectolytic enzymes has been demonstrated.

Key words: Erwinia, *pectate lyase, pectin, polygalacturonase, methyl esterase.*

Bacteria of the genus *Erwinia* produce and secrete a variety of pectolytic enzymes effectively depolymerizing pectin substances and causing degradation of plant cell walls and maceration of plant tissues (Garibaldi & Bateman 1971; Basham & Bateman 1975). This property offers ample scope for a use of *Erwinia* pectinases as a processing aid for the food industry (fruit and juice technology) and for the treatment of plant raw material (fodder, flax-straw) (Rombouts & Pilnik 1986; Baran & Kmet 1987).

Erwinias synthesize several pectolytic enzymes characterized by different mechanisms of action and different substrate specificity: endopectate lyase (endoPL, EC 4.2.2.2) and endopolygalacturonase (endoPG, EC 3.2.1.67), which cleave α-1,4-glycoside bonds in the polypectate by β-elimination and by hydrolysis, respectively, pectin methyl esterase (PME, EC 3.1.1.11), which demethylates pectin and some other enzymes (Kotoujansky, 1987).

PL appears to be the principle enzyme causing rapid maceration of plant tissues. Other *Erwinia* pectolytic enzymes probably have an auxiliary significance in the pectinolysis; they support more effective and complete destruction of pectin polymers. Endo PG is characteristic for *E. carotovora* pectinase complex and PME is produced by *E. chrysanthemi* (Collmer & Keen 1986; Shevchik *et al.* 1988). A common feature of *Erwinia* is the production of several PL isoenzymes determined by different genes; recently a number of genes encoding PL isoenzymes have been cloned from *E. chrysanthemi* and *E. carotovora* into *Escherichia coli* cells and the genes were then expressed (Keen *et al.* 1984; Reverchon *et al.* 1985; Evtushenkov *et al.* 1986; Kotoujansky *et al.* 1985).

Several features of the regulation of *Erwinia* PL synthesis have been described at the physiological level. In *E. chrysanthemi*, PL synthesis is induced by growth in the presence of polygalacturonate or other pectin substances (Chatterjee *et al.* 1979; Collmer & Bateman 1981). PL synthesis in both *E. chrysanthemi* and *E. carotovora* is repressed in the presence of glucose (Moran & Starr 1969). Individual pel-genes have recently been shown to be expressed independently, but very little is known about the regulation of individual PL isoenzyme synthesis (Reverchon & Robert-Baudouy 1987). Regulation of synthesis of other *Erwinia* pectinases has not been extensively studied to date.

Synthetic media supplemented with sodium polypectate or polygalacturonic acid are usually used for the investigation of *Erwinia* pectinases. However, a necessary condition for the commercial production of *Erwinia* pectinases is the use of more readily available and cheaper media. The spectrum of *Erwinia* pectolytic activity may, however, be changed as a function of the chemical composition of medium (van Gijsegem 1986); this may be critical, as the balance of lytic enzyme complexes is of primary importance in the effective and complete destruction of polymers.

In this paper we report the results of quantitative analysis

V.E. Shevchik, A.N. Evtushenkov, H.V. Babitskaya and Y.K. Fomichev are with the Department of Microbiology, Byelorussian State University, Leninsky pr. 4, Minsk, 220080, Byelorussia. *Corresponding author.

of the pectinase complexes produced by bacteria of 14 strains of *Erwinia* in the presence of diverse carbon sources.

Materials and Methods

Bacterial Strains, Growth Conditions, Media and Reagents

The following bacteria were studied: *E. chrysanthemi* EC 16, obtained from M. Starr's laboratory (Department of Bacteriology, University of California, Davis, USA); *E. chrysanthemi* EP-3, *E. chrysanthemi* ENA 49, *E. chrysanthemi* g252, *E. carotovora* var. *atroseptica* 75V, *E. carotovora* var. *atroseptica* 26/1, *E. carotovora* var. *atroseptica* g125 obtained from Dr C. Quinn (Department of Agriculture and Fisheries for Scotland, Agricultural Scientific Services, Edinburgh, UK); *E. chrysanthemi* 910, *E. chrysanthemi* 8451, *E. carotovora* var. *carotovora* 550 provided by Dr I. Lazar (University of Bucharest, Romania) and the strains *E. carotovora* var. *atroseptica* 36A, *E. carotovora* var. *carotovora* 9B, *E. carotovora* var. *carotovora* 17A from the culture collection of Dr A. N. Evtushenkov (Byelorussian State University, Minsk, USSR).

Bacterial cultures were grown on a rotory shaker (200 rev/min) at 28°C for 20 h in 250 ml Erlenmeyer flasks containing 50 ml of minimal salts medium 1A (Miller 1972), supplemented with either glucose (0.5% w/v), glycerol (0.5% w/v) or peptone (1% w/v), alone or used in combination with sodium polypectate (0.5% w/v). 'Flax-medium' was prepared by placing 2.5 g chopped flax-straws (4 to 5 cm in length) in 250 ml Erlenmeyer flasks containing 50 ml of 1A medium and sterilized by autoclaving (20 min at 120°C). 'Potato-medium' was prepared from peeled potato tubers which were surface-sterilized by swabbing them with alcohol and flaming, then washed with sterile water, cut into 1 cm slices, and washed again with sterile water. About 180 g of the potato slices were added to 1 l of 1A medium.

Citrus polypectate (Sigma Chemical Co.), peptone (Serva Finebiochemica), DEAE cellulose and reagents for electrophoresis (Reanal, Hungary) and sodium polypectate, prepared from beet pectin (Rombouts, 1972) were used in the study.

Pectolytic Enzymes Purification

Cells were removed from cultures by centrifugation (15,000 × ***g***, 15 min) and then 1.5 volume of cold acetone (−20°C) was added to each supernatant. After 1 h at −20°C supernatants were centrifuged (10,000 × ***g***, 4°C, 15 min) and the precipitate dried at 4°C for 1 h, resuspended in a minimal volume of 1 mM $CaCl_2$ and dialysed overnight at 4°C against several litres of the same solution. This crude enzyme preparation (its PL activity was about 90% of the initial) was centrifuged (15000 × ***g***, 4°C, 15 min) and the supernatant applied to a DEAE cellulose column equilibrated with 0.1 M Tris/HCl buffer (pH 8.7). Fractions with PL, PG or PME activity were eluted with the same buffer in the void volume and then pooled, precipitated with cold acetone and dialysed as above. Preparative disc electrophoresis of such enzyme preparations was carried out according to the method of Reisfeld *et al.* (1962) with some modifications. The separation gel contained 7% acrylamide, 0.184% bis-acrylamide, 0.5% N,N,N',N'-tetramethylethylenediamine (TEMED), 0.07% ammonium persulphate, 0.2 M potassium acetate (pH 5.0). The stacking gel contained 3% acrylamide, 0.8% bis-acrylamide, 0.27% TEMED, 0.07% ammonium persulphate, 0.04 M potassium acetate (pH 5.0). The electrode buffer (pH 4.5) contained 0.032 M acetic acid, 0.035 M histidine. Electrophoresis was carried out at 13°C with a current of 5 mA per gel rod (0.6 × 7.0 cm) for 5 h. One sample of a pectinase preparation was applied to several gel rods (about 70 μg of protein to each rod). After electrophoresis, one of the gel rods was stained with 0.04% Coomassie brilliant blue G 250 in 3.5% perchloric acid at 30°C for 20 min, then the rest of the gels were cut apart and proteins eluted from the gel portions corresponding to the protein bands of the stained gel rod, with 0.05 M Tris/HCl buffer (pH 8.5) for 48 h. These eluates were tested for pectate lyase (PL), polygalacturonase (PG), pectin methylesterase (PME) activities and for homogeneity by Laemmli electrophoresis.

Enzyme Assays

PL activity was determined by spectrophotometric measurements of the increase in absorbance at 235 nm of a reaction mixture containing sodium polypectate (Shevchik *et al.* 1988).

The PG product was determined by iodometric titration of the aldehyde groups of the sugars formed in the breakdown of sodium polypectate (Moran *et al.* 1968).

PME activity was assayed by titration of carboxyl groups formed in the de-esterification of pectin (Rombouts 1972).

Protein was determined in all samples by the method of Lowry.

Results and Discussion

Thirty eight strains of *Erwinia* were tested for PL production from which 14 strains, showing a wide variation of PL activities, were selected as a representative sample. The total extracellular activity of PL, the main pectolytic enzyme of *Erwinia*, of each of the tested strains was studied as the first stage of the work. Bacteria were cultivated in various synthetic media containing different carbon sources (Table 1). The results revealed that 'non-induced' cells of all three *Erwinia* subspecies (cultivated in the media without sodium polypectate) possessed approximately equal PL activities. In contrast, when bacteria were grown in the presence of polypectate and in the absence of glucose, *E. chrysanthemi* produced about five times as much PL activity as *E. carotovora* var. *carotovora* and about ten times more than *E. carotovora* var. *atroseptica*. The range of specific PL activities (Table 1) show a large strain-variation in PL production in the different media among the bacteria of each species and subspecies, making it difficult to draw any conclusion about the effect of each carbon source on PL synthesis. However 'relative PL activities' took on very constant values for the bacteria belonging to one species (subspecies), indicating that each *Erwinia* subspecies has common features of PL synthesis regulation. Comparison between the relative PL activities indicates that PL production was increased in the presence of polypectate about 15 times for *E. crysanthemi* and four to five times for *E. carotovora*. This suggests the different inducing ability of sodium polypectate for the various *Erwinia* species.

Clearly, total PL productions only give a partial indication of total *Erwinia* pectolytic activity. The amount of other pectolytic enzymes and PL isoenzymes, which display very different substrate specificities and other properties, was determined by preparative electrophoresis, permitting each

Table 1. Production of pectate lyase (PL) by *Erwinia* with different carbon sources

Carbon source*	Total extracellular PL activity for:								
	***E. carotovora* var. *atroseptica* (four strains)**			***E. carotovora* var. *carotovora* (four strains)**			***E. chrysanthemi* (six strains)**		
	Specific PL activity†		**Relative PL activity‡ Mean ± error**	**Specific PL activity**		**Relative PL activity Mean ± error**	**Specific PL activity**		**Relative PL activity Mean ± error**
	Mean	**Range**		**Mean**	**Range**		**Mean**	**Range**	
Glucose	0.76	0.25–1.30	1.0	0.79	0.30–1.75	1.0	0.61	0.18–1.15	1.0
Glucose + Sodium polypectate	0.71	0.45–1.10	1.0 ± 0.17	2.74	0.60–7.15	2.9 ± 0.61	1.82	0.60–4.65	2.3 ± 0.53
Glycerol	0.72	0.34–1.20	0.9 ± 0.08	0.97	0.70–1.40	1.4 ± 0.38	1.38	0.70–2.75	2.4 ± 0.57
Glycerol + Sodium polypectate	2.83	1.34–5.05	3.7 ± 0.16	5.03	2.67–10.1	6.3 ± 0.71	26.7	15.1–61.5	33.3 ± 1.8
Peptone	0.57	0.18–1.05	0.7 ± 0.07	0.82	0.32–1.54	1.1 ± 0.27	1.30	0.77–1.93	2.3 ± 0.40
Peptone + Sodium polypectate	2.52	1.26–4.30	3.4 ± 0.09	4.76	2.78–9.65	6.0 ± 0.25	28.4	12.4–70.5	33.0 ± 2.2
Sodium polypectate	1.41	0.38–2.07	1.9 ± 0.31	3.66	1.43–7.32	5.1 ± 0.96	19.2	13.2–21.6	28.7 ± 4.1

* Strains were grown in minimal medium with glucose (0.5%), glycerol (0.5%) or peptone (1%), with or without sodium polypectate (0.5%).

† Specific activities are expressed in U per mg of cell protein.

‡ Relative activity is the ratio of specific activity to that observed in medium with glucose. It was first determined for each strain and then the averages were calculated.

isoenzyme to be quantified in the test samples (Figure 1). Two 'typical' strains of both *E. carotovora* subspecies and two *E. chrysanthemi* strains, belonging to different pathovars, were analysed. The ratios of activities of pectolytic isoenzymes varied and depended on the chemical composition of the growth media. Moreover, although total PL activities were similar in some media, dramatic differences in the relative amount of each isoenzyme were noticed.

The production of each PL isoenzyme, PG and PME by bacteria cultivated in all the test media was calculated on the basis of the preparative electrophoresis results and the data on total extracellular PL activity (Table 2). A

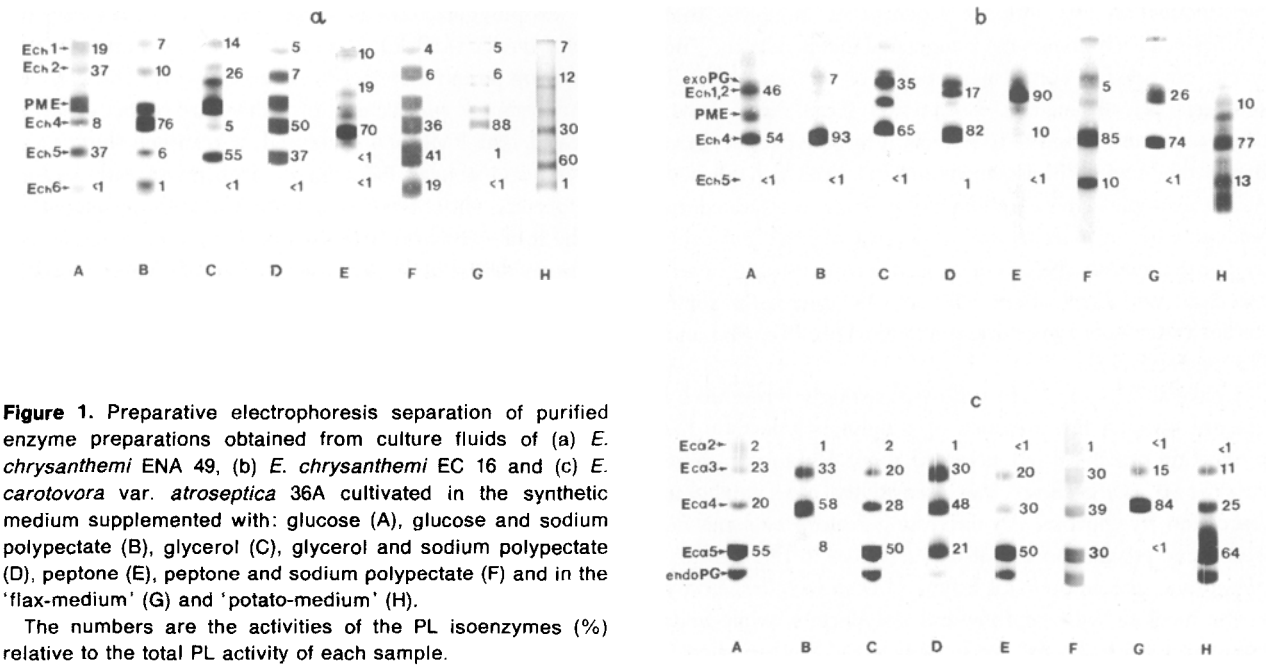

Figure 1. Preparative electrophoresis separation of purified enzyme preparations obtained from culture fluids of (a) *E. chrysanthemi* ENA 49, (b) *E. chrysanthemi* EC 16 and (c) *E. carotovora* var. *atroseptica* 36A cultivated in the synthetic medium supplemented with: glucose (A), glucose and sodium polypectate (B), glycerol (C), glycerol and sodium polypectate (D), peptone (E), peptone and sodium polypectate (F) and in the 'flax-medium' (G) and 'potato-medium' (H).

The numbers are the activities of the PL isoenzymes (%) relative to the total PL activity of each sample.

Table 2. Production of pectolytic enzymes by *Erwinia* with different carbon sources

Strain	Pectolytic enzyme	Specific activity (U per mg of cell protein) in minimal medium with:					
		Glucose	Glucose + Sodium polypectate	Glycerol	Glycerol + Sodium polypectate	Peptone	Peptone + Sodium polypectate
E. chrysanthemi ENA 49	Pectate lyase (PL) isoenzymes:						
	Ech1	0.16	0.32	0.16	3.1	0.13	3.1
	Ech2	0.30	0.51	0.30	4.3	0.21	4.7
	Ech4	0.06	3.69	0.06	30.7	0.78	25.4
	Ech5	0.30	0.30	0.62	22.8	0.01	29.3
	Ech6	NA*	NA	NA	0.2	NA	9.2
	Pectin methyl esterase	0.02	0.04	0.03	0.17	0.02	0.16
E. carotovora var. *carotovora* 550	PL isoenzymes:						
	Eca1	0.01	0.01	0.01	0.02	NA	0.01
	Eca2	0.01	0.01	0.01	0.02	0.01	0.02
	Eca3	NA	NA	NA	0.87	0.01	0.85
	Eca4	0.18	0.54	0.28	0.94	0.01	0.71
	Eca5	NA	NA	NA	0.57	0.25	0.53
	Eca6	NA	NA	NA	0.03	NA	0.01
	Polygalacturonase	NA	NA	NA	0.40	NA	0.35
E. carotovora var. *atroseptica* 36A	PL isoenzymes:						
	Eca2	0.01	0.01	0.01	0.02	NA	0.01
	Eca3	0.12	0.27	0.12	0.75	0.10	0.56
	Eca4	0.10	0.49	0.17	1.20	0.16	0.73
	Eca5	0.27	0.07	0.30	0.53	0.25	0.58
	Polygalacturonase	0.10	NA	0.10	NA	0.10	0.30

* NA—No activity detected.

comparison of the induction ratios for polypectate, the repression ratios for glucose and influence of peptone on the production of different isoenzymes suggests that synthesis of each isoenzyme is regulated independently. The results revealed a common property of all *Erwinia*: that synthesis of 'alkaline' PL isoenzymes (Eca3, Eca4, Ech4, Ech5) was more sensitive to induction by polypectate, than that of the 'neutral' PL isoenzymes (Eca1, Eca2, Ech1, Ech2). As an example, when sodium polypectate was added to medium without glucose, the production of Ech1 and Ech2 by *E. chrysanthemi* ENA 49 increased 'ony' 10 to 20 times, and Ech4 and Ech5 about 500 to 1000 times. (In some studies these isoenzymes were denoted PLb, PLc, PLd and PLe, respectively.)

Unlike all PL isoenzyme synthesis, strongly repressed by glucose even in the presence of sodium polypectate, the production of Ech4 (PLd) increased about 60 times in these conditions, suggesting low sensitivity to catabolite repression by glucose. An interesting feature of some PL isoenzyme production is that it is sensitive to the presence of peptone; the amounts of Ech6 and Eca6 were large only in the medium with peptone and polypectate, while very low production levels were detected in other media. Although the strain *E. chrysanthemi* EC 16 is distinct from *E. chrysanthemi* ENA 49 in its pectinase complex (the first has exopolygalacturonase and only one 'alkaline' PL isoenzyme, Ech4 (PelE); Keen *et al.* 1984), the induction and repression patterns of the PL isoenzymes were similar.

Analysis of production of other pectolytic enzymes revealed that PME was produced at relatively high basal activity and a weak induction was observed with sodium polypectate (about ten times). PME accounts for 30 to 40% of the total extracellular protein of *E. chrysanthemi* ENA 49 in the media without inductor. The specific activity of PG produced by *E. carotovora* was positively correlated with that of PL Eca5.

The next experiments were aimed at determining the effect of more complex media on pectolytic enzyme production. Two such media 'flax-medium' and 'potato-medium' were used. Both media contained approximately equal amounts of dried materials (about 45 g/l) and pectin substances (about 1 g/l). The soluble sugar content of the 'flax-medium' (about 2 g/l) was about 10 times higher than that of the 'potato-medium', while the latter was rich in protein (about 1 g/l). Thus, the 'flax-medium' was similar in its chemical composition to the synthetic medium with

Table 3. Production of pectolytic enzymes by *Erwinia* in 'flax-medium' and 'potato-medium'

Strain	Pectolytic enzyme	Specific activity (U per mg of cell protein) in:	
		Flax-medium	Potato-medium
E. chrysanthemi ENA 49	Total pectate lyase (PL)	2.9	33.0
	PL isoenzymes:		
	Ech1	0.12	2.1
	Ech2	0.15	3.6
	Ech4	2.64	9.1
	Ech5	0.03	18.2
	Ech6	NA*	0.3
	Pectin methyl esterase	0.03	0.15
E. carotovora var. *carotovora* 550	Total PL	0.9	3.0
	PL isoenzymes:		
	Eca1	0.03	0.09
	Eca2	0.06	0.24
	Eca3	NA	1.80
	Eca4	0.82	0.38
	Eca5	NA	0.45
	Eca6	NA	0.03
	Polygalacturonase	NA	0.50
E. carotovora var. *atroseptica* 36A	Total PL	1.0	3.2
	PL isoenzymes:		
	Eca1	NA	NA
	Eca2	0.01	0.01
	Eca3	0.16	0.38
	Eca4	0.85	0.84
	Eca5	NA	1.92
	Polygalacturonase	NA	0.50

* NA—No activity detected.

glucose and sodium polypectate, and the 'potato-medium' was similar to the synthetic medium with peptone and sodium polypectate.

Four tested strains of *Erwinia* were cultivated in these 'plant-media' for 20 h, causing complete maceration and disintegration of the plant tissues. Analysis of these cultures by preparative electrophoresis (Table 3, Figure 1) revealed, as expected, a similarity in the levels of total PL activity and the patterns of pectolytic enzymes detected in the 'flax-medium' and in the synthetic medium with glucose and sodium polypectate. The pectinase spectra, produced in the 'potato-medium' and in the synthetic medium with peptone and sodium polypectate were also very much alike.

The results presented here suggest that the regulatory pattern of pectolytic enzyme synthesis, which was established by experiments with synthetic media, may be extended to include media with a more complicated chemical composition. A concentration of pectin substances and soluble sugars in the media is the decisive factor determining total PL production level and the pectolytic enzyme spectrum; other features of medium composition and growth conditions are probably of minor importance. This conclusion was confirmed by cultivating *Erwinia* on the potato slices placed on glass, which caused production of a spectrum of pectolytic enzymes identical to that in the 'potato-medium'. Thus, on the basis of knowledge of the chemical composition of media one can predict the production levels and pectolytic system composition of *Erwinia*. It should also be possible to use specially selected media for the production of a specific spectrum of pectolytic enzymes.

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