Comparative Properties of Pectate Lyases Secreted by *Erwinia chrysanthemi*

The purpose of this experiment is to compare the pectate lyases secreted by the plant pathogenic bacterium, *Erwinia chrysanthemi* EC16, with respect to the mechanism of depolymerization of polygalacturonate. The pectate lyases secreted by *Erwinia chrysanthemi* provide a battery of enzymes that catalyze different depolymerization processes that collectively contribute to the maceration of plant tissues associated with their phytopathogenic response (1,2,11). The structural genes coding for the PLs secreted by EC-16 have been cloned and individually expressed in *E. coli*, and each structural gene has been sequenced (3,4). Mechanism comparisons have indicated similarities and differences among different enzymes secreted by the two strains (2,5,6). The complete structures of PLc and PLe enzymes have been solved by x-ray crystallography, leading to the discovery of the parallel beta helix as a new motif in protein structure (12-14).

Specific objectives include:

1. The expression of pPEL410 and pPEL748, plasmids encoding the pectate lyases PLc and PLe formed by *E. chrysanthemi* EC16, in *E. coli*.

2. The purification of the pectate lyases PLc and PLe expressed in *E. coli*.

3. The comparison of each enzyme with respect to the kinetic depolymerization of polygalacturonate and the formation of unsaturated oligogalacturonates.

4. The structural comparisons of the PLc and PLe.

**PROCEDURES**

Preparation and purification of recombinant PLc and PLe in *E. coli*. Recombinant enzymes PLc and PLe, naturally secreted by *Erwinia chrysanthemi* EC16, will be “overexpressed” in *E. coli* HB101 according to refs 3 and 4. The presence of sec genes in *E. coli* that are similar to those in *Erwinia chrysanthemi* allows the transfer of the pectate lyases, containing appropriate signal sequences, to be moved into the periplasmic space. Cleavage of the signal sequence as part of the transfer process contributes to folding and formation of a mature and active enzyme. Plasmids pPEL410 (carrying pelB as the structural gene for PLc) and pPEL748 (carrying pelE as the structural gene for PLe) have been used as transforming vectors. Cultures induced for overexpression with isopropyl-β-D-galactopyranoside (IPTG) will be grown to stationary phase. Enzyme preparations will be obtained from the periplasm of *E. coli* upon the formation of spheroplasts followed by osmotic shock (3,4,8).

Following concentration (Amicon, polysulfone membrane, 3 MW cut-off) and dialysis against 5 mM Tris-HCl, pH 6.5, the enzymes may be purified (ref 17) on a carboxymethyl cellulose column.
(Whatman CM-52) with a gradient from 0 to 0.5 M NaCl. The dialyzed preparation is added to a cation exchange (CM52) column (0.9 x 20 cm) that has been pre-equilibrated with start buffer (see hand out for specific protocol). Wash the column with 50 ml of start buffer, followed by eluting buffer with a linear gradient ranging from 0 to 0.5 M NaCl, collecting 4 ml fractions. Collect fractions on the Gilson microfractionator, controlling the flow rate at 1.6 ml/min (Gilson Minipuls pump setting at 185). Assay for PL by the formation of unsaturated oligogalacturonates, detected spectrophotometrically at 235 nm. Prepare a plot of the activity (units per ml) versus fraction number. Determine the NaCl concentration in every 5th fraction by determining conductivity on a 0.01 dilution in water and comparing with a standard curve of conductivity vs NaCl concentration. Combine the tube contents comprising each resolved enzyme and store in the cold.

The final purification can be accomplished by FPLC (Fast Protein Liquid Chromatography) using a BioRad UnoS1 Cation Exchange column operated with BioLogic Work Station and eluted with a gradient as used for the CM52 column. Prior to this second purification, fractions containing PL activity derived from the CM52 column should be dialyzed to lower the concentration of NaCl to less than 0.02 M to allow the PLs to bind to the UnoS1 cation exchange column.

PL activities are determined by measuring the increase in absorbance at 235 nm as a measure of the unsaturated residues formed during the lyase mediated depolymerization of sodium polygalacturonate. The molar absorptivity of the unsaturated residue formed during bond cleavage is 4.6 mM⁻¹cm⁻¹. A unit of enzyme activity is estimated as the activity that will form one umol of unsaturated residue per min at room temperature in Buffer A. Total protein will be determined by the micro BCA assay.

Assay the crude and purified fractions for PL by the formation of unsaturated oligogalacturonates, detected spectrophotometrically at 235 nm. Prepare a plot of the activity (units per ml), and of corrected (for A₃₅₀) A₂₈₀, and corrected A₂₈₀/A₂₆₀ ratio versus fraction number. Combine the tube contents comprising each resolved enzyme (including the peak fraction) and store in the cold for later study. Assay each enzyme preparation for total PL activity (spectrophotometric assay at 235 nm) and for protein (BCA assay). PL proteins will be identified by SDS-PAGE and immunoblots (Western blots, using chicken IgY antibodies formed against highly purified PLc and Ple). These preparations will be analyzed for their activities in catalyzing individual product formation by the HPLC assay described below.

Kinetic comparisons of enzymes. Concentrate the samples comprising the different peaks from the CM52 and assay for PL activity on a recording spectrophotometer. Reaction mixtures should contain 0.1% polygalacturonate (PGA) in Buffer A. Initiate the reactions with the addition of enzyme. Prepare plots of product versus time. Determine initial velocities as estimates of maximum velocities, and determine units per ml. Assay each enzyme for the formation of individual products as a function of time, using the kinetic HPLC assay (ref 7). Prepare kinetic plots comparing each enzyme with respect to the formation of unsaturated oligomers ranging from DP 2 to 6. Analyze 24 h reactions by reverse-phase ion-pair HPLC to determine the limit products formed in the presence of each enzyme.
Evaluation of purity of pectate lyases. The pectate lyases as recombinant enzymes derived from *E.chrysanthemi* EC16 will be evaluated for purity and relative size by SDS-PAGE. Resolved proteins may be detected by staining gels and on blots to which resolved proteins will be electrophoretically transferred. Detection of proteins on the blots will be made with a sensitive colloidal gold stain (Aurodye) and the pectate lyases will be identified with a polyclonal antibody raised against pectate lyase from *E. chrysanthemi* EC16.

Comparisons of PLc and PLe structures on the basis of selective proteolysis. The treatment of PLe and PLc with specific proteases under conditions in which the native structures are retained will be followed by mass spectrometry to determine the molecular masses of the resulting peptides (19). Incubation with trypsin will allow cleavage on the amino-terminal side of lysine and arginine residues; incubation with endoproteinase AspN will allow cleavage on the amino-terminal side of aspartic acid residues (15). Each PL will be dissolved as 2 mg/ml in 0.1M Tris-HCl, pH 8.1. To a sample of 0.02 ml in a microfuge tube, add 5 ul of either trypsin or AspN protease, each at 0.1 ug/ml. Remove 0.5 ul and add to 0.5 ml of Buffer A. From this dilution, remove 0.01 ml and assay for PL activity in 1.0 ml reaction mix containing 0.1 % PGA. Remove another 0.5 ul sample and add the 4.5 ul of 0.2% trifloroacetic acid (TFA) for the MS analysis. Samples should be removed at 0,2,6, and 24 h for analysis by MALDI-TOF-MS. The interpretation of the data will be made on the basis of the known sequences of the enzymes and the allowed cleavage sites for the proteases used. The sites cleaved will then be related to the three-dimensional structures established by x-ray crystallography to identify specific sites that are exposed in solution. In order to assure that the sites of cleavage reflect those that are susceptible in the native enzyme, digestions should be carried out following denaturation. For this comparison, mix 10 uL of each PL (2 mg/ml) with 30 uL of 8 M guanidine HCl, heat to 50 C for 30 min and dilute 10-fold in 0.1M Tris-HCl, pH 8.1. To 10 uL of this diluted sample, add 1.0 uL trypsin or AspN (0.1 ug/ml) and incubate over night at 37 C, add 10 uL 0.2% TFA in 40% acetonitrile, and analyze a sample by MALDI MS.

**PRESENTATION OF RESULTS**

Present the following:

FIGURE 1. Fractionation of recombinant pectate lyases from *E. chrysanthemi* EC16 by CM-52 chromatography. Plot the PL activities, as the initial (and maximal) delta absorbance at 235 nm, corrected A\textsuperscript{280}, and corrected A\textsuperscript{280}/A\textsuperscript{260} against the fraction number. Also plot the conductivity values for every other tube.

TABLE 1. Purification of pectate lyases from recombinant PL’s. Report data showing the total activities, total protein, specific enzyme activities, yields, and fold purification for the different fractions.

FIGURE 2. Kinetics of the pectate lyase activities secreted by *Erwinia chrysanthemi* EC16. Present plots of the absorbance at 235 nm (as a measure of bond cleavage) versus time (for 30 min at room
temperature), using 0.05 unit of activity for each enzyme.

FIGURE 3. Comparisons of pectate lyases with respect to their catalytic depolymerization of polygalacturonate and the formation of unsaturated oligogalacturonates. Present HPLC chromatograms for early reactions (e.g. 30-60 min reactions) obtained during the kinetic HPLC analyses of each enzyme.

FIGURE 4. SDS-PAGE and immunoblot comparisons of PLc and PLe in the transformed E. coli periplasmic fraction.

TABLE 2. Comparison of the PLs with respect to limit product formation and percent alpha helix.

FIGURE 5. Comparison of trypsin and AspN proteases on the specific cleavage and inactivation of PLc and PLe. a) Cleavage sites in the primary sequences in a MACAW alignment of PLc and PLe. b) Cleavage sites in the quaternary structure of PLc depicted by WebLab Viewer. c) Cleavage sites in the quaternary structure of PLe depicted by WebLab Viewer.

REFERENCES


