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Note

Detection of polygalacturonase and pectin lyase isoenzymes in polyacrylamide gels

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Pectic enzymes are widespread among bacteria, fungi, insects and higher plants[1]. They play an important role as the agents of tissue maceration, fruit juice clarification, and citrus cloud destabilization[1]. Among these enzymes, endo-polygalacturonase (endo-PG, poly-α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) and pectin lyase (PL, poly-α-1,4-D-galacturonide lyase, EC 4.2.99.3), which cleave the α (1→4) linkages of pectic substances, are particularly important in processes that lead to plant cell maceration and death of plant tissue[2,3]. PG and PL isoenzymes have been found after diethylaminoethylcellulose column chromatography, electrofocusing and gel electrophoretic procedures[4–6]. Several PG and PL isoenzymes could be detected by the above methods, which require fractionation or cutting of the gels, elution and recovery of the enzymes. These methods, however, are time consuming and require much work.

The purpose of this study was to develop a simple, rapid and accurate method for the differential identification of PG and PL activity directly in polyacrylamide gels.

MATERIALS AND METHODS

Sources of enzymes

Endo-PG preparations were obtained from a suspension of commercial pectinase (N.B.C., Cleveland, Ohio, U.S.A.) in distilled water.

Green lemon peels were inoculated with *Penicillium digitatum* and used as a source for exo-PG after the following treatment[7]. Four days after inoculation, soft areas around the inoculation sites were removed, weighed and frozen and then macerated in distilled water (1:1, w/v) for 3 min at 4° in a Sorvall Omni-mixer. The resulting homogenate was filtered through several layers of cheesecloth and the filtrate was centrifuged at 16,000 *g* for 15 min at 4°. The supernatant was used as crude enzyme preparation.

Alternaria solani was used as the source of PL. The fungus was grown on potato–dextrose–agar (Oxoid, London, Great Britain) for 1 week at 27°. A heavy suspension of the fungus was prepared by shaking the culture with distilled water. One millilitre of this suspension was used to inoculate 20 ml of a modified Czapek medium that contained, per litre of water: pectin grade I (Sigma, St. Louis, Mo.,

U.S.A.), 10 g; $NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; and chloramphenicol (Abic, Tel Aviv, Israel), 250 mg. The medium was adjusted to pH 8.0 with 0.2 *M* NaOH. The inoculated 250-ml erlenmayer flasks were shaken (90 strokes/min) for 1 week at 27 in the dark. The culture was then centrifuged at 16,000 *g* for 15 min at 4 . The resulting supernatant was lyophylized and the remaining residue was suspended in a 10-fold volume of water. This suspension was used as crude enzyme.

Enzyme assay

Exo-PG, endo-PG and PL activities were assayed viscometrically with an Ostwald viscometer. PL activity was also determined by measuring the increase in absorbance of the reaction mixture at 230 nm (ref. 8), using a Varian Techtron Model 635 spectrophotometer. The reaction mixture consisted of 2.5 ml of enzyme preparation plus either 6 ml of 1.2% sodium polypectate (Sigma) in 0.02 *M* citrate buffer (pH 4.8) for endo- and exo-PG or 6 ml of 0.9% pectin grade 1 in 0.1 *M* Tris–HCl buffer (pH 8.3) containing 0.002 *M* $CaCl_2$ for PL. All reactions were carried out at 30 .

Enzyme activities were expressed in terms of relative activity per millilitre of enzyme preparation (RA/ml), calculated as the reciprocal of 1000 × the time (in minutes) required for 1 ml of enzyme preparation to reduce the substrate viscosity by 50% (ref. 9).

Enzyme characterization

Endo- and exo-PG were characterized by thin-layer chromatography according to Ayers *et al.*[10] using plates coated with microcrystalline cellulose (Sigmacell Type 19, Sigma). The chromatograms were loaded with 15-μl aliquots that had been drawn from the reaction mixture at intervals from 15 min to 6 h. They were developed by the ascending technique to 17 cm from the starting line using ethyl acetate–acetic acid–water (2:1:2, v/v) as the solvent system[10]. After drying the chromatograms under a fan at room temperature, the compounds were located by spraying with 0.5% $AgNO_3$ in water[11]. α-D-Monogalacturonic acid (Sigma) was used as a standard (R_F 0.57). The enzyme preparation from green lemon peels was defined as exo-PG as only one spot, with an R_F value equal to that of α-D-monogalacturonic acid, was detected. On the other hand, the commercial pectinase preparation contained endo-PG, as a series of four reducing components was detected in addition to monogalacturonic acid. As these products had R_F values that differed from each other by constant values (0.05–0.07) and were lower than those of monogalacturonic acid it was assumed that they were oligomers of galacturonic acid, ranging from the dimer to the pentamer[10,12].

PL was characterized by comparing the result of breakdown of the pectin molecule, as determined by viscometric measurement, with the increase in absorbance at 230 nm resulting from release of unsaturated bonds. The enzyme was defined as endo-PL, as the viscosity decreased rapidly (50% after 5 min) while simultaneously the increase in absorbance was slow and linear for 4 h.

Electrophoresis

Isoelectric focusing in 7.5% acrylamide gels was carried out according to Wrigly[13]. LKB (Stockholm, Sweden) Ampholine carrying 40% ampholytes of pH 3–10

was used. The gels (8 cm long) were chemically polymerized with potassium persulphate. Enzyme samples were applied in 10% sucrose, covered with a solution of 5% sucrose and 2.5% Ampholine. The upper anodic reservoir was filled with 0.2% sulphuric acid and the cathodic reservoir with 0.4% ethanolamine. Electrophoresis was carried out for 4 h at 5°, applying 1.5 mA per gel, gradually raising the voltage to 300–350 V, which was then maintained constant until the end of the run.

The pH gradient in the gels was determined by cutting the gels without protein into 10-mm slices. The slices were soaked in 2 ml of distilled water for 1 h and the pH was measured with a Beckman Zeromatic pH meter.

Isoenzyme identification

Following electrophoresis, the gels were placed in 0.02 *M* citrate buffer (pH 4.8) for exo- and endo-PG and in 0.02 *M* Tris–HCl buffer (pH 8.3) for PL isoenzyme determination. After 10 min, the buffers were replaced with 1.2% sodium polypectate in 0.02 *M* citrate buffer (pH 4.8) for endo- and exo-PG or 0.9% pectin grade 1 in Tris–HCl buffer (pH 8.3) containing 0.002 *M* $CaCl_2$ for PL. The gels were then incubated for 15 min at 30°, washed with tap water and immersed in 0.05% ruthenium red solution[14] for staining. After the appearance of the isoenzyme bands, the staining solution was replaced with distilled water.

RESULTS AND DISCUSSION

Gels loaded with commercial pectinase produced colourless PG isoenzyme bands 60 min after immersion in the ruthenium red solution. The bands became sharp-

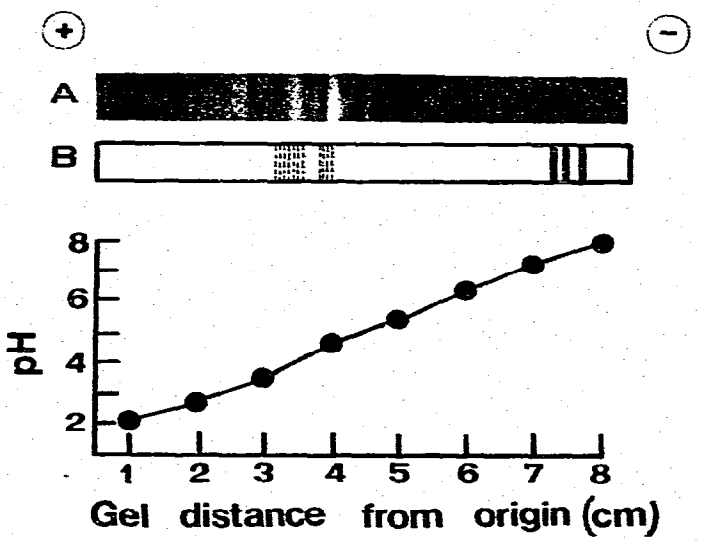

Fig. 1. Electrophoretic patterns obtained by isoelectric focusing (pH range 3–10) of (A) PG (obtained from commercial pectinase) and (B) PL (obtained from *Alternaria solani*). Solid lines: high enzyme activity; broken lines: weak enzyme activity. The gels were loaded with 150 RA/ml of each enzyme. Experimental conditions are given under Materials and Methods.

er after 1 day (Fig. 1A). At least seven bands were observed, all of them in the acidic half of gel (pH 3–5). The stained gels could be stored in water for a few weeks. As commercial pectinase might contain some exo-PG, the possibility that the PG bands observed are a result of both exo- and endo-PG activity was tested. The PG preparation from lemon peels, which had previously been shown to be pure exo-PG, was used. In this case, although the enzyme activity was very high, *i.e.*, the viscosity was reduced by 50% in 126 min, no bands were detected even after 4 h of incubation. This result may be due to the fact that this enzyme acts on the pectic molecule in a terminal mechanism of hydrolysis[2], resulting in slow breakdown of the molecule. This indicates that the bands observed on pectinase-loaded gels are due to endo-PG activity.

PL isoenzyme bands were detected 1 day after staining (Fig. 1B). Although these bands were not as clear as the PG bands, satisfactory scanning of the stained gels was usually achieved, using a linear transport unit built on a Perkin-Elmer Model 137 UV spectrophotometer. The PL isoenzymes were mostly found at the basic end of the gel (pH 7–8) and only very weak activity was detected in the acidic half. A dark red area was observed around the PL bands, but not near the PG bands. This area may be used as an additional means of differentiation between the two enzymes.

Sharp, well defined bands of PG were usually obtained with highly active enzyme solutions (1000 RA/ml). Both PG and PL, however, gave satisfactory results when the enzyme activity was at a minimum level of 125–150 RA/ml.

Direct identification of pectinase isoenzymes in the gels was also obtained by Stegman[15]. His procedure, however, does not enable PG and PL isoenzymes to be differentiated. Our procedure, by using different substrates for each enzyme, makes possible both the identification and differentiation between PG and PL isoenzymes.

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