Properties of a Polygalacturonase Produced by Geotrichum candidum

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ABSTRACT

A polygalacturonase was purified approximately 19-fold from culture filtrates by carboxymethyl cellulose column chromatography. Results obtained by ion exchange chromatography, disc electrophoresis, and temperature inactivation of the polygalacturonase suggested the presence of a single enzyme.

The enzyme cleaved pectic acid by a random mechanism of hydrolysis. Liberation of monogalacturonic acid was observed immediately at the onset of the reaction. The reaction proceeded linearly until it attained 40% hydrolysis of which galacturonic acid release accounted for 5% of the total galacturonic bonds split. After a 2-hr reaction period, the total hydrolysis of pectic acid was 55%, 15% of which was accounted for by galacturonic acid accumulation. The pH optimum, as measured by viscosity decrease, coincided with that required for monogalacturonic acid formation. The pH optimum for trigalacturonic acid as a substrate was 3.5 as compared to pH 4.5-5 for sodium polypectate. Trigalacturonic acid was slowly split to di- and monogalacturonic acid, but digalacturonic acid was not attacked. The enzyme was, therefore, classified as endopolygalacturonase. Phytopathology 60:27-30.

Endopolygalacturonase, an enzyme cleaving the α-1,4 glycosidic bonds of pectic substances by random mechanism of hydrolysis (2), has a stronger action on pectic acid than on pectin. In contrast, exopolygalacturonase catalyzes a terminal hydrolysis of pectic acid, causing the liberation of free galacturonic acid.

The fungus *Geotrichum candidum* Lk. ex Pers., which produces soft rot on citrus fruits, synthesizes polygalacturonase during germination and growth (1). Three peaks of polygalacturonase were obtained by column chromatography on carboxymethyl cellulose (CMC) when the enzyme was eluted by stepwise increase of pH (1). Peak 2 contained the majority of the polygalacturonase activity. Since all three enzyme fractions showed similar mechanism of hydrolysis and substrate specificity, it was suggested that they may not be different enzyme proteins (1). The lower activities detected in peaks 1 and 3 could be ascribed to recombination or dissociation of polygalacturonase into particles of varying sizes (20).

These three enzyme fractions all exhibit rapid liberation of reducing sugar and the appearance of monogalacturonic acid shortly after reaction with sodium polypectate (1). The former results are considered as being indicative of significant exopolygalacturonase activity. On the other hand, the rapid decrease in viscosity of polypectate and higher activity towards polypectate rather than pectin (1) is characteristic of endopolygalacturonase. It was, therefore, possible that each peak contained exo- and endopolygalacturonase activities. In view of the different types of enzyme activities, it was considered necessary to determine whether more than one polygalacturonase is produced in a culture of *Geotrichum candidum*.

MATERIALS AND METHODS.—*Geotrichum candidum* culture No. 205 (1) was grown in 1-liter flasks containing 200 ml of the following medium: 1 g of KH_2PO_4; 0.5 g of $MgSO_4 \cdot 7H_2O$; 0.001 g of $FeCl_3$; 2 g of asparagine; 2 g of glutamine; 10 g of glucose; 1 g of sodium polypectate; and 2 g of yeast extract in 1 liter of water. After 48-hr incubation on a shaker at 30 C, the cells were centrifuged from suspension at 6,000 *g* for 10 min, and the supernatant was dialyzed against distilled water at 4 C for 48 hr and saved for enzyme purification.

Average degree of polymerization (DP) was determined by the hypoiodite method (6) for reducing galacturonic acid at the chain end and by estimating the anhydrouronic acid (AUA) content (10). DP was calculated as follows (5); $DP = B/R$, where B is the total amount of AUA and R is the amount of the reducing galacturonic acid at the chain end. Monogalacturonic acid ($DP = 1$) was used as a standard for the above determinations. Di- and trigalacturonic acid were prepared according to Hasegawa & Nagel (4), and were further purified using the procedure of Phaff & Luh (17). The oligouronides produced single spots after paper chromatography (1), and had DP values of 2.04 and 2.97 for the dimer and trimer, respectively. Sodium polypectate, pectic acid (Nutritional Biochemical Corp., Cleveland, Ohio) and polygalacturonic acid (Schuchardt, Munchen) had an AUA of 62%, 84%, and 92%, respectively. All the above substrates were of an analytical grade.

Polygalacturonase activity was determined by measuring reducing group liberation with dinitrosalycilic acid (DNS) reagent (13) using galacturonic acid as a standard. The colorimetric method of Somogyi (19) as modified by Patel & Phaff (15) was used for measuring the rate of hydrolysis of oligogalacturonides. The appearance of free galacturonic acid was determined by a modified naphthoresorcinol procedure (18) previously shown to be specific to monogalacturonic acid. Hydrolysis per cent of pectic acid was calculated according to Luh & Phaff (9). Viscometric measurements for polygalacturonase activity have been previously described (1). Unless otherwise stated, the reaction mixture contained 0.1-0.5 ml enzyme preparation, 50 µmoles acetate buffer, pH 5, 0.25% sodium polypectate, and water to make a total volume of 1 ml. A reaction mixture of heat-denatured enzyme served as a control. The reaction was carried out at 30 C. One unit of polygalacturonase activity was defined as being the amount of enzyme which catalyzes the release of 1 mg galacturonic acid/hr under these conditions.

CMC column was prepared as elsewhere described (1) and equilibrated with 0.05 M acetate buffer of pH 4.5. Enzyme elution was performed by gradient increase in NaCl concentration from 0 to 0.5 M. Amount of

proteins was determined by the method of Lowry et al. (7), using bovine serum albumin fraction V as a standard.

The procedure of Ornstein & Davis (14) was used for disc electrophoresis on polyacrylamide gel; however, Tris[tris (hydroxymethyl) amino methane]-EDTA-boric acid buffer (16), pH 8.3, showed better resolution than the commonly used Tris-glycine buffer. The current was regulated to 4 ma/tube. All electrophoretic procedures were carried out at 4 C. Corresponding gels were cut into sections of 0.125 cm each. Each section was placed in a test tube containing 1 ml of reaction mixture for polygalacturonase activity and incubated with continuous shaking for 3 hr at 30 C. DNS reagent was used for determination of enzyme activity. Staining of gels with 1% amido black in 7% acetic acid was employed for detection of protein bands.

RESULTS.—*Purification of polygalacturonase.*—Following dialysis, the crude enzyme preparation containing 1,200 enzyme units, 62 units/mg protein, was placed on the column and washed with 100 ml of 0.05 M acetate buffer of pH 4.5. Elution of the enzyme was performed when the concentration of the NaCl approached 0.1 M. Under these conditions, all the original polygalacturonase activity was recovered in a single peak (Fig. 1). Protein determination, as measured by absorbance at 280 mμ, was found to be lower than 0.05 absorbance units in all fractions. The activity of polygalacturonase obtained from the column in the peak fraction after dialysis was 1,210 units/mg protein, a purification of about 19-fold.

The partially purified enzyme was further concentrated by placing the dialyzing tube on polyvinylpyrrolidone of a molecular weight of 40.000 at 4 C. Disc electrophoresis was employed to ascertain whether the partially purified polygalacturonase is a single enzyme. A fraction containing approximately 14 units/100 μg protein in 200 μliter distilled water was placed on a single acrylamide tube. The results presented in Fig. 2 indicate the presence of a single enzyme peak which migrated 0.5 cm from the origin after 1 hr of running period. Staining of gels with amido black reagent revealed no stainable protein bands.

Temperature inactivation.—The stability of the purified polygalacturonase at various temperatures is given in Fig. 3. A linear inactivation curve was obtained at temperatures between 30 and 65 C. After 10-min incubation at 65 C, the enzyme was completely inactivated. Such a linear pattern of temperature inactivation is indicative of a single polygalacturonase.

Hydrolysis of pectic acid and oligogalacturonides.—It has been previously observed (1) that in addition to a rapid viscosity decrease of sodium polypectate, monogalacturonic acid appeared as the first and principal breakdown product detected by paper chromatography. Similar results were also observed with pectic acid. The foregoing results, therefore, suggest that a single polygalacturonase is involved in either random or end group splitting of pectic acid.

The extent of terminal hydrolysis was further examined by following the formation of free galacturonic

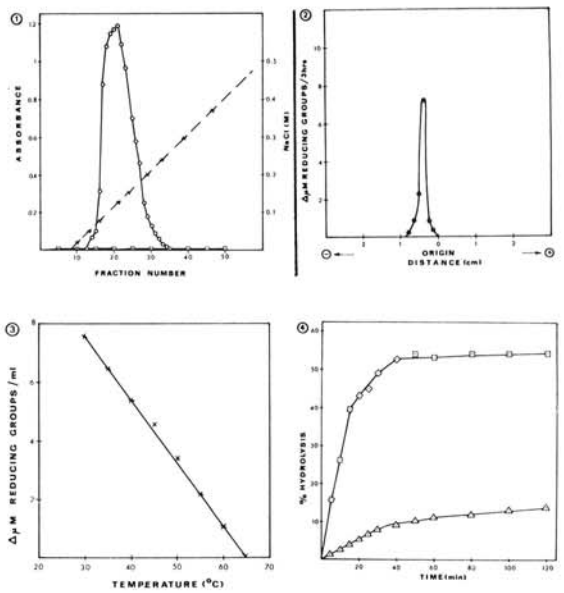

Fig. 1-4. **1)** Purification of polygalacturonase from *Geotrichum candidum* on carboxymethyl cellulose. Elution of enzyme was achieved by gradient increase in NaCl concentration from 0 to 0.5 M. Polygalacturonase activity was measured by incubating 0.5 ml of eluate from each fraction with an equal volume of 0.5% sodium polypectate at 30 C for 15 min. Absorbance was recorded at 570 mμ after reaction with DNS. Protein was estimated by extinction at 280 mμ. Polygalacturonase (– ○ –); Protein (– □ –); NaCl (– X –). **2)** Electrophoretic separation pattern of purified polygalacturonase from *Geotrichum candidum*. **3)** Temperature inactivation of purified polygalacturonase from *Geotrichum candidum*. One-ml portions of enzyme (specific activity 924) were heated at indicated temperatures for 10 min. Preparations were immediately cooled and assayed for polygalacturonase. **4)** Hydrolysis of pectic acid and formation of free galacturonic acid as a function of time. Reaction mixture consists of 0.1 M acetate buffer pH 5, 0.25% pectic acid, and 7.2 units polygalacturonase from *Geotrichum candidum*/ml. Total hydrolysis (– □ –) was measured with DNS, and formation of free galacturonic acid (– △ –) with naphthoresorcinol reagent.

acid with the naphthoresorcinol reagent. Pectic acid was selected as a substrate rather than sodium polypectate because of its higher AUA content. However, the initial hydrolysis rate of the two substrates by *G. candidum* polygalacturonase was similar. Immediately after the onset of reaction, total hydrolysis proceeds linearly until it attains 40%, 5% of which was accounted for by galacturonic acid formation (Fig. 4). After a reaction period of 2 hr, galacturonic acid accumulation accounted for 14% of the total galacturonide bonds split, while total hydrolysis reached approximately 55%. At 10-hr incubation, 70% and 18% were recorded for total hydrolysis and galacturonic acid formation, respectively. The optimum pH for terminal hydrolysis coincides with that of random hydrolysis (Fig. 5).

Additional information on the characteristics of *G. candidum* polygalacturonase was provided by its action on oligogalacturonides. The optimum pH hydrolysis of trigalacturonic acid was 3.5 as compared to 4.5-5 for sodium polypectate (Fig. 5). The initial breakdown

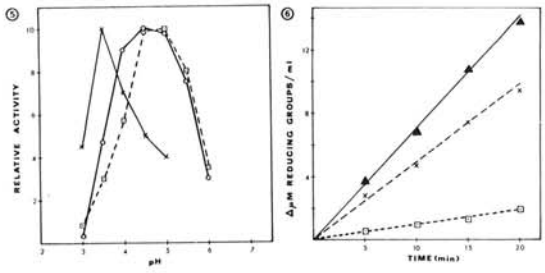

Fig. 5-6. 5) Optimal pH for the activity of polygalacturonase from *Geotrichum candidum* as measured in terms of (i) the diminution in viscosity of 0.5% solution of sodium polypectate (−□−); (ii) the release of free galacturonic acid from 0.25% solution of sodium polypectate (−○−) as measured by naphthoresorcinol reagent; and (iii) the release of reducing groups from 0.25% solution of trigalacturonic acid (−X−). **6)** Rate of hydrolysis for 0.25% solutions of pectic acid (−▲−) and polygalacturonic acid (−X−) at pH 5 and trigalacturonic acid (−□−) at pH 3.5. Enzyme concentration from *Geotrichum candidum* was 7.1 units/1 ml of reaction mixture.

rates of pectic acid (DP = 112) and polygalacturonic acid (DP = 12.7) were, respectively, 8 and 5.5 times higher than that of trigalacturonic acid (Fig. 6). Tri-, di- and monogalacturonic acid were detected by paper chromatography during hydrolysis of trigalacturonic acid. Attempts to detect hydrolysis of digalacturonic acid at various pH after an 8-hr incubation period were unsuccessful.

DISCUSSION.—These results indicate that *G. candidum* produced a single polygalacturonase in culture under the above specified conditions (Fig. 1, 2, 3). Since this enzyme has been shown to rapidly reduce the viscosity of sodium polypectate solution, and has a higher activity towards polypectate than pectin NF (1), it can be classified as endopolygalacturonase.

The appearance of monogalacturonic acid during the initial stages of pectic acid hydrolysis (Fig. 4) is not unique to the *G. candidum* enzyme. Detection of monogalacturonic acid after sodium polypectate was incubated for 2 min with the enzyme (12), or before pectic acid hydrolysis reached 20% (18), was reported for purified fungal endopolygalacturonases. In contrast to our enzyme (Fig. 4), purified yeast endopolygalacturonase failed to release monogalacturonic acid until 25% of the glycoside bonds of pectic acid were hydrolyzed (9). Detection of oligogalacturonides appearance by paper chromatography showed the formation of penta-, tetra-, tri-, and di-, but not monogalacturonic acid during the initial phase of pectic acid cleavage by the yeast enzyme (3). On the other hand, galacturonic acid was detected as being the principal product during the initial phase of polypectate cleavage by *G. candidum* endopolygalacturonase (1). Traces of penta-, tetra-, and trigalacturonic acid were also recovered in chromatogram soon after reaction started with the former enzyme, but digalacturonic acid appeared as a faint spot only at a later stage (1). The appearance of mono- and oligogalacturonides during polypectate digestion with purified endopolygalacturonase of *Aspergillus niger* (12) has shown similar patterns to that of the *G. candidum* enzyme, except that the relative concentration of monogalacturonic acid was apparently lower. The above observations suggest that although oligogalacturonides are utilized as substrates for terminal liberation of free galacturonic acid by the yeast enzyme (3), polygalacturonides serve as substrates for direct formation of monogalacturonic acid by *G. candidum* endopolygalacturonase.

The fall in the optimum pH of trigalacturonic acid cleavage (Fig. 5), the lower activity towards trigalacturonic acid (Fig. 6), and the inability to attack digalacturonic acid were also reported for purified endopolygalacturonases of yeast (3) and *A. niger* (12). Digalacturonic acid is generally attacked by exopolygalacturonase (11). The inability of the *G. candidum* enzyme to hydrolyze digalacturonic acid excludes the possibility that the enzyme preparation was contaminated with exopolygalacturonase. The endopolygalacturonase of *G. candidum,* therefore, liberates monogalacturonic acid from polygalacturonides by the random mechanism type of hydrolysis. This is also true of pentagalacturonic acid, which, with the yeast enzyme, has been reported (3) to undergo random hydrolysis to a (3 + 2) and a (4 + 1) cleavage.

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