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EXOPOLYGALACTURONASE IN TOMATO FRUIT

RUSSELL PRESSEY

USDA, Agricultural Research Service, Richard B. Russell Research Center, P.O. Box 5677, Athens, GA 30613, U.S.A.

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; polygalacturonase; ripening.

Abstract—A low level of polygalacturonase has been found in unripe tomato fruit. The enzyme was extracted with 0.5 M NaCl containing 0.05 M $CaCl_2$, concentrated by ultrafiltration and purified 150-fold by ion-exchange chromatography. The M_r of the enzyme was 47 000. It was optimally active at pH 5 and required Ca^{2+} for activity, with an optimum concentration of 0.42 mM Ca^{2+}. The enzyme has been characterized as an exopolygalacturonase that cleaves monomer units from the non-reducing ends of the substrate molecules. The optimum substrate size for the enzyme was that with a degree of polymerization of *ca* 13. The amount of exopolygalacturonase activity remained essentially constant during development and ripening of the fruit.

INTRODUCTION

Tomato ripening is accompanied by the appearance and increase of two forms of endopolygalacturonase (endo-PG) [1, 2]. The first traces of endo-PG have been detected *ca* 20 hr after ethylene evolution commenced [3]. This observation has been used as evidence against a role for endo-PG in the initiation of the ripening process, as has been previously suggested [4]. These studies assume that the first traces of endo-PG are actually detected and some problems in the extraction and assay of endo-PG in tomato fruit have been indicated [5]. Furthermore, unripe tomato fruits contain a low level of PG that is unrelated to the endo-PG. This paper describes the extraction, partial purification and characterization of this enzyme.

RESULTS

Extraction and purification of polygalacturonase

The ultrafiltered crude extracts of unripe tomato fruit contained low levels of PG activity. The activity was 0.07 unit/g fr. wt tomato tissue, which is considerably lower than that found in ripe fruit (*ca* 36 units/g). The enzyme in green fruit was readily extractable with water or low concentrations of salts. Extraction with 0.5 M NaCl containing 0.05 M $CaCl_2$ yielded maximal amounts of enzyme and low levels of soluble pectin which facilitated ultrafiltration. Purification of the enzyme was achieved by chromatography on DEAE-Sephadex A-50 (Fig. 1) and Mono S (Fig. 2). A summary of the purification procedure is given in Table 1. A 153-fold purification with 73% recovery was obtained by the procedure; the purified PG did not contain pectinesterase.

Characterization of polygalacturonase

The purified PG was readily soluble in water, with no loss of activity after dialysis against water. However, the activity was relatively low in the dialysed solution and it was completely abolished when assayed in 30 mM citrate or EDTA, suggesting a divalent cation requirement. Addition of Ca^{2+} to the reaction mixture stimulated the activity 360% (compared to a reaction mixture containing water) at an optimum concentration of 0.42 mM. Activation of the enzyme by Ca^{2+} was independent of pH. Concentrations of Ca^{2+} higher than 2 mM reduced the activity, presumably by precipitating the substrate. Sr^{2+} was much less effective as an activator, with a 140% increase in activity at 0.4 mM concentration. Mg^{2+} had no effect on the activity while Cd^{2+} and Hg^{2+} were slightly inhibitory. High concentrations of monovalent cations were inhibitory, with 40% inhibition by 50 mM Na^{+}.

The PG was optimally active at *ca* pH 5 with a rather broad peak of activity between pH 4.5 and 5.5. The pH optimum was independent of the degree of polymerization of the substrate. However, the substrate size had a large effect on the rate of hydrolysis by the enzyme. Digalacturonic acid was hydrolysed very slowly but the reaction rate increased with increasing galacturonan chain length to a maximum at a degree of polymerization of *ca* 13. The rate then decreased for larger substrates (Table 2).

The enzyme slowly solubilized uronic acid from isolated cell walls prepared from green tomato fruit. This study was conducted by suspending 10 mg of isolated cell walls in 2 ml of the PG assay reaction mixture containing 0–0.5 unit of PG. After incubation at 25° with shaking for 16 hr, the samples were centrifuged and the supernatant solutions were analysed for uronic acid. The highest level of PG (0.5 unit) solubilized 68 μg of uronic acid from the cell walls, and the relationship between PG concentration and uronic acid solubilized was nearly linear. The solubilized material was not characterized.

The M_r of the PG was determined by gel filtration on a 2.5 × 95 cm column of Sephadex G-100 in 0.15 M NaCl. From the elution volumes of the protein standards BSA, ovalbumin, carbonic anhydrase and cytochrome *c*, the M_r of the PG was calculated to be 47 000 ± 1500.

The pH optimum at 5 and the activation of Ca^{2+} suggested that the enzyme may be an exo-PG which is common in other plant tissues [6]. The following experi-

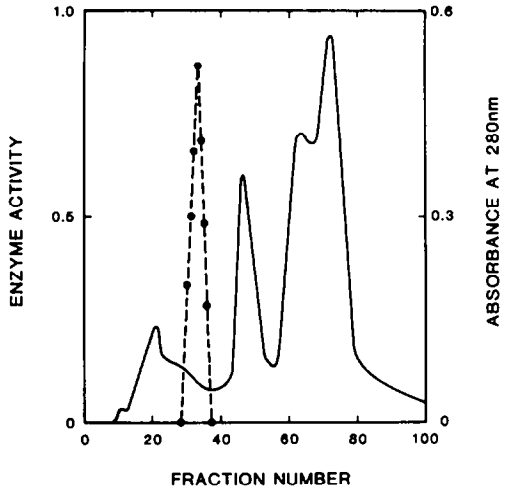

Fig. 1. Chromatography of polygalacturonase from unripe tomato fruit on DEAE-Sephadex A-50. O---O, PG activity; ——, absorbance at 280 nm.

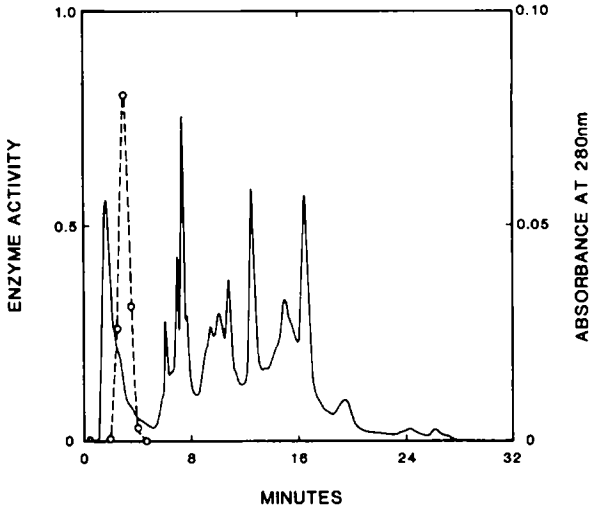

Fig. 2. Chromatography of the DEAE-Sephadex A-50 fraction of polygalacturonase on Mono S. O---O, PG activity; ———, absorbance at 280 nm.

ments were conducted to elucidate the mode of action of the tomato PG. First, the effectiveness of the enzyme in decreasing the viscosity of pectate was determined to distinguish between endo- and exo-mechanisms of cleavage. The conditions of the assay were standard except that pectate was the substrate and the concentration of Ca^{2+} was decreased to 0.1 mM to prevent gelling of the solution. After given times of reaction, aliquots of the mixture were analysed for reducing groups and the viscosity was measured with an Ostwald viscometer. The relative viscosity of the solution decreased only 6% after 1 hr while 1.9% of the glycosidic linkages in the pectate were hydrolysed. Such a small effect on the viscosity of pectate is characteristic of an exo-PG [7]. The product of exo-PG action is usually galacturonic acid [6, 7]. Analysis of a reaction mixture of the PG acting on PGA III by HPLC on Polyanion SI revealed a single peak of uronic acid which coincided with that for a galacturonic acid standard.

Further details of the mode of action of the enzyme were obtained by monitoring the reaction products from oligogalacturonides. Reaction mixtures containing the enzyme and 0.5% oligogalacturonides were incubated for various periods and heated at 100° for 5 min to stop the reactions. The products in the solutions were identified and measured by separation on a Polyanion SI column. Data are shown for the enzyme hydrolysing pentagalacturonic acid in Table 3. The initial products were tetragalacturonic and galacturonic acids; longer reaction periods yielded progressively more of the tetramer and monomer. Eventually the trimer appeared as a product of tetragalacturonic acid hydrolysis, but a considerably longer reaction time was needed before the dimer could be detected. This is consistent with a decreased rate of hydrolysis as a galacturonan chain decreases. Similar results were obtained with tetragalacturonic acid as the substrate, with the trimer and monomer as the primary products of hydrolysis (data not shown). The results confirm that the PG removes monomer units from the substrate in a stepwise fashion.

The last detail of the mode of action is whether the enzyme hydrolyses the substrate molecule at the reducing or non-reducing end. It was confirmed first that reducing the substrate with $NaBH_4$ did not affect the rate of hydrolysis. This observation in itself suggests that the substrate is attacked at the non-reducing end. To prove this, pentagalacturonic acid was reduced with NaB^3H_4 to label the reducing end of the molecule. The labelled pentamer was then treated with the PG and the products were separated by HPLC. The pattern of product formation was identical to that for the original pentagalacturonic acid. The fractions corresponding to each peak of uronic acid were pooled, evaporated and assayed for radioactivity. Radioactivity was found in the unhydrolysed pentamer and in the tetramer but not in the galacturonic acid. The results established that the enzyme

Table 1. Summary of purification of polygalacturonase from green tomato fruit

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude	25	310	72	0.23	—
DEAE-Sephadex A-50	5	13.3	65	4.89	90
Mono S	2	1.5	53	35.3	73

Table 2. Effect of substrate size on the rate of hydrolysis by the polygalacturonase

Substrate	Degree of polymerization	Relative rate
Digalacturonic acid	2	0.3
Trigalacturonic acid	3	0.7
Tetragalacturonic acid	4	1.1
Pentagalacturonic acid	5	1.6
Polygalacturonic acid III	13	3.3
Polygalacturonic acid II	20	2.6
Polygalacturonic acid I	79	1.4
Pectate	201	1.0

Table 3. The products of pentagalacturonic acid hydrolysis by the polygalacturonase

Product	Reaction time 1 hr	2 hr
	mg	mg
Galacturonic acid	0.3	0.7
Digalacturonic acid	0	0
Trigalacturonic acid	0.1	0.4
Tetragalacturonic acid	1.0	1.4
Pentagalacturonic acid	3.7	2.6

Table 4. Changes in exo-polygalacturonase in tomatoes during development and ripening

Stage	Weight (g)	Polygalacturonase (units/g)
Green	59	0.082
Green	109	0.052
Green	129	0.068
Green	177	0.070
Mature green	230	0.065
Turning	247	0.073
Ripe	262	0.076

removes monomer units from the non-reducing ends of the substrate.

Changes during development and ripening

Tomato fruits were harvested and sorted into green, turning and ripe categories. The green fruit were sorted into five groups according to size ranging from 59 to 230 g (mature green stage). The samples were stored at −20° until they could be analysed. One kg of each sample was extracted with 0.5 M NaCl and 0.05 M $CaCl_2$ according to the standard procedure. The ultrafiltered extracts were dialysed against 0.15 M NaCl. The extracts obtained from green tomatoes could be assayed directly for exo-PG because of the absence of endo-PGs in unripe fruit [2]. The level of exo-PG varied somewhat but appeared to remain relatively constant during fruit development (Table 4).

The endo-PG activity which appears at the turning stage and increases markedly during tomato ripening [2, 3] made it impossible to measure exo-PG in crude extracts of ripening fruit. It was necessary to separate the exo-PG from the endo-PGs and this could be accomplished by the purification procedure. Chromatography on DEAE-Sephadex A-50 separated PG I but not PG II from exo-PG. However, PG II did not elute from the Mono S column until fraction 12, well removed from the exo-PG peak (Fig. 2). The two-step purification procedure thus allowed analyses for exo-PG in extracts containing even much higher levels of endo-PGs. The results indicate that exo-PG persists during tomato ripening without changing significantly (Table 4).

DISCUSSION

The level of PG activity in unripe tomato fruit is much lower than that in ripe fruit. Whereas the activity in ripe tomatoes is due primarily to endo-PGs [1, 2], the enzyme in green fruit is an exo-PG. Exo-PGs have been reported in other fruits including peaches [7], pears [8] and apples [9] and appear to be widespread in vegetative tissues [6]. The exo-PG in tomatoes persists in the fruit during ripening but it represents only a small fraction of the total PG activity in ripe fruit. Therefore, it is unlikely that this enzyme has an important role in pectin degradation and softening of tomatoes. However, the presence of exo-PG in green tomatoes is of considerable interest in relation to its possible role in fruit growth and development.

Cell walls expand in growing plant tissue and the structural components must be modified to allow expansion and to accommodate newly synthesized components. It has been suggested that endohydrolases functioning as transferases may be involved in these cell wall changes [10]. The endoenzymes could rearrange large segments of cell wall polysaccharides through transglycoslation. In contrast, a role for exo-PG in pectin modification would involve only the monomers at the non-reducing ends of the galacturonan chains. Evidence has not been presented showing that such terminal residues have critical functions in cell wall structure, but this cannot be ruled unlikely. A possibility is that the terminal residues may be involved in calcium cross-links between adjacent chains. Such cross-links could be broken by removal of Ca^{2+} but also by hydrolysis of the glycosidic linkage of one of the residues by exo-PG. Presumably other mechanisms involving exo-PG could lead to cell wall alteration. Nevertheless, exo-PG exhibits preference for moderately long galacturonan chains and cleaves short oligogalacturonides very slowly, suggesting that its function may be other than complete hydrolysis of cell wall galacturonans.

EXPERIMENTAL

Extraction. Green tomato fruit tissue (100 g) of the cultivar Better Boy was blended in 100 ml of 1 M NaCl containing 0.1 M $CaCl_2$ using a VirTis homogenizer. 10 extracts were combined and homogenized for 2 min in a Polytron. The homogenate was adjusted to pH 6 with 0.1 N NaOH, stirred for 1 hr at 3° and then centrifuged at 8000 *g* for 30 min. The supernatant soln was ultrafiltered to 25 ml using a PM-10 membrane in an Amicon model 2000 system. The extract was then dialysed against 4 l. of 0.15 M NaCl for 16 hr at 3°.

Purification. The dialysed crude extract was clarified by centrifugation and applied to a 5 × 70 cm column of DEAE-

Sephadex A-50 equilibrated with 0.01 M NaOAc, pH 5.5, containing 0.14 M NaCl. The column was eluted with 0.01 M NaOAc, pH 5.5, containing 0.14 M NaCl. The fractions collected were assayed for PG. Fractions containing PG were pooled, cond to 5 ml by ultrafiltration and dialysed against 0.02 M MES, pH 6. Aliquots (1 ml) were then applied to a Mono S HR 5/5 column (Pharmacia Fine Chemicals) in an HPLC system. Buffer A consisted of 0.02 M MES, pH 6, and Buffer B consisted of 0.02 M MES, pH 6, containing 0.5 M NaCl. The system was programmed for linear segments of 0 to 2 min (0% B) and 2 to 30 min (0–100% B) at a flow rate of 1 ml/min. Fractions (1 ml) were assayed for PG and fractions containing PG activity were pooled, ultrafiltered to 2 ml and dialysed against 0.02 M NaCl.

Assay. The reaction mixture contained 0.1 ml of 0.1 M NaOAc, pH 5, 0.2 ml of H_2O, 0.1 ml of 5 mM $CaCl_2$ and 0.1 ml of enzyme soln. Blanks were prepared by heating duplicate solns in boiling H_2O for 5 min. The solns were heated for 10 min at 37°, and 0.5 ml of 1% PGA I was then added to initiate the reaction. After 1 hr at 37°, the solns were analysed for reducing groups by the arsenomolybdate method [11]. A unit of PG is defined as that amount which catalyses the hydrolysis of 1 μmol of galacturonic acid glycosidic linkages under these conditions.

Substrates. Pectate, polygalacturonic acids and oligogalacturonides were prepared and purified as described earlier [6]. Reduced substrates were prepared by treating with $NaHB_4$ [6]. Pentagalacturonic acid was labelled by reducing with NaB^3H_4 [6]. The substrates were dissolved in H_2O and adjusted to the desired pH with HCl or NaOH.

Isolated cell walls. Pericarp tissue (100 g) from green tomato fruit was homogenized in 100 ml of cold H_2O. The homogenate was adjusted to pH 5 and centrifuged. The pellet was washed twice with cold H_2O by homogenizing briefly followed by centrifugation. The insoluble material was then washed with EtOH and Me_2CO and dried under vacuum; the yield was 2.25 g.

Oligogalacturonide analysis. Oligogalacturonides were separated on a 7.8 × 300 mm column of Polyanion SI-17 (Pharmacia Fine Chemicals) at 65°. The mixture of oligogalacturonides adjusted to pH 5 was applied to the column and eluted with a linear gradient of 0–0.2 M Na_2SO_4 at a flow rate of 1 ml/min. The fractions collected were analysed for uronic acid by the hydroxydiphenyl reagent [12]. Standard oligogalacturonides were isolated as described earlier [6]. Details of the HPLC method used for separation of the oligogalacturonides will be published elsewhere.

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