Properties of a Polygalacturonase Produced by *Acrocyldrium*

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SUMMARY

A polygalacturonase has been purified from the culture fluid of a strain of *Acrocyldrium*. The purified preparation was homogeneous when examined by ultracentrifugation. The hydrolysis of di- and trigalacturonic acids by the enzyme was undetectable. Tetragalacturonic acid was hydrolysed to tri- and monogalacturonic acids, while pentagalacturonic acid was hydrolysed to either tri- and digalacturonic acids or tetra- and monogalacturonic acids. The rate of hydrolysis of pentagalacturonic acid to tri- and digalacturonic acids was about four times as fast as that to tetra- and monogalacturonic acids. It was concluded that the enzyme can hydrolyse any glycosidic bond between the reducing end and the third galacturonic-acid unit from the non-reducing end of polygalacturonic acid. The manner of hydrolysis of polygalacturonic acid by the enzyme is different from that of any other polygalacturonase reported so far.

INTRODUCTION

One plant and many micro-organisms are known to produce enzymes that hydrolyse polygalacturonic acid. Such polygalacturonases have been classified into two groups. One group which hydrolyses internal bonds of polygalacturonic acid has been found from fungi (Mill & Tuttobello, 1961; Endo, 1964*a*; Uchino, Kurono & Doi, 1966; Koller & Neukom, 1969), from a bacterium (Nasuno & Starr, 1966), from a yeast (Patel & Phaff, 1959) and from tomato (Patel & Phaff, 1960). According to Demain & Phaff's (1957) definition, these enzymes are called endo-polygalacturonases. Another group, the exo-polygalacturonases, hydrolyse only a terminal bond. Enzymes of this kind were studied by Endo (1964*b*) and (Mill 1966*a*, 1966*b*).

In our laboratory, Uchino *et al.* (1966) found that a strain of *Acrocyldrium* produced a significant amount of polygalacturonase. The enzyme was crystallized and called endo-polygalacturonase because it was not able to hydrolyse more than 50 % of the glycosidic bonds in polygalacturonic acid. Recently we have studied the action of the enzyme on oligogalacturonic acids. The present paper reports the properties of the enzyme with special reference to substrate specificity.

METHODS

Organism and cultivation. The fungus used was a strain of *Acrocyldrium*, which was kindly supplied by the National Institute of Agricultural Sciences, Tokyo, Japan. The growth medium contained (g/100 ml): pectin (Katayama Kagaku Co., Tokyo), 5; yeast extract (Oriental Yeast Co., Tokyo), 0.2; NH_4NO_3 , 1; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; pH 5.6. Cultures were grown in 500 ml flasks containing 100 ml medium using a reciprocal shaker (136 strokes/min) at 30 °C for 72 h.

Enzymatic assay. Polygalacturonase activity was measured either by determining aldehyde groups formed or by measuring the decrease in viscosity of polygalacturonic acid (PGA), a product from Sigma Chemical Co., St Louis, Missouri, U.S.A.

For measuring the rate of formation of aldehyde groups, 1 ml of suitably diluted enzyme solution was added to 5 ml of 0.6% (w/v) PGA solution in 0.1 M-acetate buffer (pH 4.1) preincubated at 30 °C. The reaction mixture was incubated at 30 °C. The amount of aldehyde formed was determined by the hypiodite method of Jansen & MacDonnell (1945). The sample was mixed with 1 ml of 1 M- Na_2CO_3 in a glass-stoppered Erlenmeyer flask, to which was then added 5 ml of 0.1 N-iodine. The mixture was allowed to stand for 20 min, and was then acidified with 2 ml of 2 M- H_2SO_4 . The iodine remaining in the solution was titrated with 0.05 N- $\text{Na}_2\text{S}_2\text{O}_3$. One unit of polygalacturonase activity (PG unit) was defined as the amount of enzyme which produces 1 micromol of aldehyde groups/min at 30 °C. A semimicro hypiodite method was used with 0.25% (w/v) substrate (Patel & Phaff, 1959).

Viscosity decrease was measured by the method of Roboz, Barratt & Tatum (1952). The reaction conditions were exactly the same as those for the aldehyde-group assay. One viscometric unit (VR unit) was defined as the amount of enzyme which reduced the viscosity of PGA by 50% in 10 min.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with egg albumin (Katayama Kagaku Co.) as reference protein.

Analysis of reaction products. Reaction mixtures were treated with Dowex 50 (H^+ form), evaporated to dryness and dissolved in 0.5 ml water. Samples were spotted on Toyo Roshi no. 51A paper and developed by descending chromatography with a solvent system of butanol-acetic acid-water (4:2:3, by vol.). Under these conditions, GA, 2GA, 3GA* and their oxidized derivatives (the terminal aldehyde group being oxidized) were separable on paper. Identification of products on paper was made as follows. Aldehyde groups were detected by spraying with alkaline silver nitrate reagent at room temperature (Preiss & Ashwell, 1963). Carboxylic groups were located by spraying with a mixed reagent of bromocresol green and bromophenol blue (Pásková & Munk, 1960). For quantitative analysis of products, reaction mixtures treated as described above were applied to paper in streaks. Areas containing reaction products were eluted from the paper with 0.2% (w/v) ammonium oxalate on a shaker and the eluate was condensed to 0.5 ml by evaporation. Uronic acids were estimated by the carbazole method of Dische (1947) with slight modification (Matsumura, 1969). Galacturonic acid (Sigma Chemical Co.) was used as the standard. Aldehyde groups were determined by the micro hypiodite method of Hatanaka (1967).

Preparation of oligogalacturonic acids and their oxidized derivatives. Di- and trigalacturonic acids were prepared as described by Phaff & Luh (1952). Digalacturonic and trigalacturonic acids thus prepared had $-\text{COOH}/-\text{CHO}$ ratios of 1.98 and 3.05, respectively. Tetra- and pentagalacturonic acids were prepared according to the method of Hatanaka & Ozawa (1966) and had $-\text{COOH}/-\text{CHO}$ ratios of 3.85 and 4.90, respectively. Oxidized oligogalacturonic acids were prepared by the hypiodite oxidation method of Patel & Phaff (1959). The terminal aldehyde group is changed to a carboxyl group by this method. The oxidized substrates showed no reducing activity.

Procedures for purification of enzyme. The enzyme was purified by a method based on that reported by Uchino *et al.* (1966), as follows. After 72 h cultivation, mycelia were removed by centrifugation at 10000 g for 5 min. To 10 l of the culture fluid was added 1.5

* Abbreviations used: GA, monogalacturonic acid; 2GA, digalacturonic acid; 3GA, trigalacturonic acid; 4GA, tetragalacturonic acid; 5GA, pentagalacturonic acid; PGA, polygalacturonic acid.

Table 1. *Summary of purification of polygalacturonase*

Step	Volume (ml)	Protein (mg)	Total enzyme activity (PG units)	Specific enzyme activity ($\frac{\text{PG units}}{\text{mg protein}}$)	Yield (%)
Culture filtrate	10 000	46 700	5 600	0.12	100
Acetone precipitate	500	21 840	4 360	0.20	78
Duolite CS-101 eluate	1 000	5 600	2 800	0.50	50
Starch zone electrophoresis	10	606	728	1.20	13

volume of acetone (-20°C). The precipitate formed was collected by centrifugation at 10000 *g* for 10 min, dissolved in 300 ml of distilled water, and dialysed against distilled water for 72 h at room temperature with frequent changes of outer medium. The dialysed solution (about 500 ml) was then applied on to a column of Duolite CS-101 (60×270 mm) which had been equilibrated with 0.05 M-sodium acetate buffer (pH 4). The column was washed with 2800 ml of 0.2 M-acetate buffer (pH 5.0) and then eluted with 0.4 M-acetate buffer (pH 6.0) at a flow rate of 240 ml/h. The fractions containing enzyme activity were pooled. To the solution was added 1.5 volume of cold acetone (-20°C). The precipitate formed was recovered by centrifugation at 10000 *g* for 10 min, dissolved in distilled water and dialysed against distilled water. Ten ml of the dialysed preparation obtained from 10 l of the culture fluid was mixed with 12 g of dried starch grain (Maruishi Pharmaceutical Co., Osaka, Japan) and applied on to a starch block for zone electrophoresis. Electrophoresis was carried out on a starch block ($15 \times 100 \times 400$ mm) in 0.1 M-phosphate buffer (pH 7.0) with 60 mA, 340 V for 24 h. The block was cut into slices (10 mm thick) which were frozen once at -20°C and thawed. The protein was extracted with distilled water and those fractions which had enzyme activity were pooled, precipitated with 1.5 volume of acetone and dialysed against 0.1 M-acetate buffer (pH 4.1).

RESULTS

Purity of enzyme

From the results on starch zone electrophoresis, the fungus appeared to produce only one kind of extracellular polygalacturonase. Table 1, giving the data on the purification procedure, shows that with a loss of 87 % of the original activity a 10-fold increase in specific activity had been achieved. The purity of polygalacturonase was examined in a SPINCO model E analytical ultracentrifuge. The schlieren pattern of the preparation showed a single peak with a sedimentation coefficient (s_{20}^w) of 5.8 (Fig. 1). The purity of the preparation was also examined on a CM-cellulose column chromatogram with a linear pH gradient from 5.0 to 7.0. As shown in Fig. 2, there was a good coincidence between the protein patterns and the activity patterns measured by using two different methods. The result strongly indicates that the enzyme preparation was homogeneous.

Effect of pH and temperature on enzyme activity

The effect of pH on the polygalacturonase activity was studied by means of the hypoiodite assay under the standard conditions except that 0.1 M-citric acid- Na_2HPO_4 buffers with various pH values were used. The optimum pH was found to be approximately 4.2. The enzyme showed no activity at pH 2 and 6. The effect of pH on the stability of the enzyme

Fig. 1. Ultracentrifugation of purified polygalacturonase. Protein solution (5.0 mg/ml in 0.1 M-acetate buffer, pH 4.5) was centrifuged at 59780 rev./min with a Spinco model E analytical ultracentrifuge. Temperature was 16 °C and photograph was taken with a bar angle of 60° after 64 min.

was also studied. The enzyme preparation was treated with either 0.1 M-citric acid- Na_2HPO_4 (pH 2.0 to 8.0) or 0.1 M-tris-HCl buffer (pH 7.5 to 9.0) for 24 h at 30 °C. After this treatment the preparation was dialysed against acetate buffer (pH 4.1) and enzyme activity was measured using the standard assay condition. The enzyme was stable over a pH range from 2.5 to 7.5. Fifty per cent inactivation was observed at pH 9.0.

Fig. 3 shows the effect of heat on the stability of the enzyme. Without PGA the enzyme was stable up to 40 °C, but lost more than 80 % of its activity at 50 °C. However, the enzyme retained more than 90 % of its activity at 40 °C in the presence of PGA, a substrate of the enzyme. The result shows that the enzyme was protected by its substrate from heat inactivation.

Effect of metal ions and chelating reagents on enzyme activity

The following metal ions (1 mM) did not show any significant inhibitory effect on the enzyme activity under the conditions of the standard assay: Li^+ , Na^+ , K^+ , Ag^+ , Mg^{2+} ,

Fig. 2. Chromatogram of purified polygalacturonase on CM-cellulose column. The protein solution was adsorbed on to a CM-cellulose column (Brown Co., Berlin, New Hampshire, U.S.A., 0.6 m-equiv./g, 22 × 200 mm) which had been equilibrated with 0.01 M-sodium phosphate buffer (pH 5.0). The protein was eluted with a 0.1 M-sodium phosphate buffer gradient (pH 5.0 to pH 7.5, 600 ml). Approximate flow rate was 10 ml/h and 5 ml fractions were collected. ●—●, Absorbance at 280 nm; ○—○, PG units/ml; △—△, VR units/ml.

Fig. 3. Heat stability of polygalacturonase. The enzyme (3.8 PG units) dissolved in 0.1 M-acetate buffer (pH 4.1) was heated for 5 min at the indicated temperatures with or without 0.5 % (w/v) PGA. Enzyme assay was made under the standard condition with the hypoiodite method. ○—○, In the absence of PGA; ▲—▲, in the presence of PGA.

Fig. 4. Time course of hydrolysis of polygalacturonic acid by enzyme. The decrease of viscosity and the increase of aldehyde group were assayed under the standard conditions. ▲—▲, Aldehyde group formed assayed by the hypiodite method; ○—○, viscosity decrease assayed by the viscometric method.

Ca^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Pb^{2+} , Al^{3+} , Fe^{3+} and Sn^{4+} . The effect of chelating reagents on the enzyme activity was also studied. The following reagents (1 mM) showed no significant effect: iodoacetate, *N*-ethylmaleimide, $\alpha\alpha'$ -dipyridyl and 8-hydroxyquinoline.

Substrate specificity of enzyme action

The enzyme showed no activity towards pectin. A comparison was made of the viscosity decrease and the increase in the hydrolysis of PGA. As shown in Fig. 4, 50% loss in viscosity was observed when 5% of the glycosidic bonds had been hydrolysed. The large decrease in viscosity accompanied by a small increase in the amount of aldehyde group indicates that the enzyme hydrolyses internal bonds of PGA. The reaction products were quantitatively analysed on paper chromatograms. As shown in Fig. 5, GA, 2GA and 3GA were the main end products. The amount of aldehyde group formed which is estimated from the formation of these oligomers showed good agreement with the amount of glycosidic bond hydrolysed (Fig. 5). Pentagalacturonic acid, which is formed in the early period of the incubation, quickly disappeared accompanied by an increase of smaller oligomers. The disappearance of 4GA was accompanied by the formation of 3GA and GA but not 2GA, suggesting the cleavage of 4GA to 3GA and GA.

Fig. 5. Time course of hydrolysis of polygalacturonic acid. Incubation was under the standard condition. The amount of each product was determined with carbazole reagent after chromatographic separation on paper as described under Methods. The degree of hydrolysis of PGA was estimated by the hypiodite method. $\times$ --- $\times$, Degree of hydrolysis (per cent of total glycosidic bond in PGA). The amount of products was expressed as per cent of PGA added. The values were corrected for the amount of water for the hydrolysis of PGA. $\bullet$ — $\bullet$, GA; $\circ$ — $\circ$, 2GA; $\triangle$ — $\triangle$, 3GA; $\blacktriangle$ — $\blacktriangle$, 4GA; $\square$ — $\square$, 5GA; $\blacksquare$ — $\blacksquare$, higher oligogalacturonic acid.

Table 2. *Substrate specificity of polygalacturonase*

Reaction mixture contained 0.25 % (w/v) substrate and 4.2 PG units of the enzyme in 2 ml of 0.1 M-acetate buffer (pH 4.1). Enzyme assay was made at 30 °C with the semimicro hypiodite method.

Substrate	Aldehyde group formed ($\mu\text{mol}/5 \text{ min}$)
2GA	0.00
3GA	0.00
4GA	0.80
5GA	1.00
PGA	3.50

The specificity of the enzyme action was further studied by using oligogalacturonic acids as substrates. As shown in Table 2, the enzyme showed no activity towards 2GA and 3GA. Oligomers longer than, and including, the tetramer seemed to be substrates of the enzyme. Products from 4GA and 5GA were then studied by paper chromatography. As shown in Fig. 6, 4GA produced 3GA and GA. In the case of 5GA hydrolysis, GA, 2GA, 3GA and 4GA were formed with a molar ratio of approximately 1:4:4:1 (Table 3). The result shows that 5GA can be split into either 4GA and GA or 3GA and 2GA and the splitting of 5GA into 3GA and 2GA was 4 times as fast as that into 4GA and GA.

Because the enzyme could not hydrolyse 3GA but seemed to need at least three galacturonic acid units next to the glycosidic bond to split it, the inhibitory effect of 3GA on the

Fig. 6. Paper chromatogram of products from tetra-, penta- and oxidized pentagalacturonic acids.

Table 3. *Products from penta- and oxidized pentagalacturonic acids*

Reaction mixture contained 0.25 % (w/v) substrate and 25.2 PG units of enzyme in 2 ml of 0.1 M-acetate buffer (pH 4.1). Enzyme assay was made for 5 min at 30 °C. Products were determined with carbazole reagent.

Substrate	Product (μmol)*							4GA and oxidized 4GA	5GA and oxidized 5GA
	GA	Oxidized GA	2GA	Oxidized 2GA	3GA	Oxidized 3GA	Oxidized 4GA		
5GA	0.27	—	1.13	—	1.13	—	0.25	(3.22)	
Oxidized 5GA	0	0	0	1.10	1.30	0	< 0.05	(3.9)	

* The amount of product was estimated on the basis of the molecular formula and the amount of GA unit found in the product.

enzyme action was studied. However, no inhibitory effect of 3GA on the hydrolysis of 4GA and PGA was observed.

The K_m values for 4GA, 5GA and PGA are presented in Table 4. Because PGA is such a large molecule that many enzyme molecules can react on the same molecule of substrate, it is not adequate to use K_m value. Therefore the calibration was made by using the concentration of galacturonic acid unit in the reaction mixture. The result indicates that the chain length of the substrate has a significant effect on the enzyme action.

From the results described above, we may conclude that the enzyme can split any glycosidic bond inside the third galacturonic acid unit from one of the terminals of PGA. To determine on which terminal (reducing or non-reducing terminal) the enzyme needs three galacturonic acid units, the terminal aldehyde group of oligomers was oxidized to the carboxylic acid group. The hydrolysis of oxidized 4GA did not occur at all, while oxidized 5GA did. Quantitative analysis of the products was made by paper chromatography in

Table 4. K_m and V_{max} values for oligo- and polygalacturonic acids

Reaction mixture contained the substrate indicated in the table and 6.3 PG units of enzyme in 2 ml of 0.1 M-acetate buffer (pH 1.4). Substrate concentration was 0.25 %, 0.20 %, 0.15 % and 0.10 %, respectively. Enzyme assay was made at 30° C with the semimicro hypoiodite method.

Substrate	K_m		V_{max}
	g/l	M	
4GA	8.4	0.0084	0.46
5GA	4.8	0.0048	0.50
PGA	2.1	—	0.91

streaks. A part of the paper was sprayed with mixed reagent of bromocresol green and bromophenol blue. Besides the 5GA, two spots corresponding to oxidized 2GA and 3GA were detected. Areas corresponding to each of the oxidized and non-oxidized oligomers and GA were eluted with 0.2 % (w/v) ammonium oxalate, and the amount of galacturonic acid residue and aldehyde group were determined separately. As shown in Table 3, major products were 3GA and oxidized 2GA with the molar ratio of 1:1. The molar ratio of non-oxidized GA residue in these products was close to 1:3. Aldehyde group in 3GA and oxidized 2GA fractions was also determined. Aldehyde (0.96 μ mol) was found in 1 μ mol of 3GA, while no aldehyde group was found in oxidized 2GA fraction. These results clearly show the cleavage of oxidized 5GA into oxidized 2GA and 3GA. The rate of cleavage of oxidized 5GA was comparable to that of non-oxidized 5GA (Table 3).

DISCUSSION

Substrate specificity of the polygalacturonase purified from a strain of *Acrocylindrium* is restricted to those oligogalacturonic acids which have four or more galacturonic acid units. Tetragalacturonic acid was hydrolysed to 3GA and GA, while 5GA was hydrolysed to either 3GA and 2GA or 4GA and GA. The rate of hydrolysis of 4GA, the smallest substrate, was one-fourth of that of PGA. These results show that the enzyme can hydrolyse any glycosidic bond which has more than three galacturonic acid residues next to it.

In order to determine on which side of the glycosidic bond to be hydrolysed the oligomer needs three galacturonic acid units, oxidized 5GA which has a carboxylic group instead of an aldehyde group at a terminal, was used. The hydrolysed products of oxidized 5GA were exclusively 3GA and oxidized 2GA, showing the requirement of three galacturonic acid units at the non-reducing end of the substrate for the enzyme action. The rate of hydrolysis of oxidized 5GA was about 70 % of that of 5GA. This suggests the same manner of cleavage in both cases.

The hydrolysis of oxidized 4GA and that of oxidized 5GA into 4GA and oxidized GA were not observed. This must have been due to the change by oxidation of a galacturonic acid unit adjacent to the bond to be hydrolysed. If the enzyme had the ability to release GA from the non-reducing end of an oligomer, GA formation would have been observed even with oxidized oligomers.

As shown in Table 3, 5GA was preferentially hydrolysed into 3GA and 2GA and the rate of splitting into 4GA and GA was about one-fourth of that into 3GA and 2GA. The fact that the rate of splitting of 4GA into 3GA and GA was almost comparable to the rate of hydrolysis of 5GA, suggests that the enzyme should be able to hydrolyse 5GA into 4GA and GA at a comparable rate. A possible explanation for the anomaly is that the longer

galacturonic acid chain on the non-reducing side would lower the rate of the hydrolysis. Although the possibility cannot fully be excluded, it is unlikely, because PGA showed the smallest K_m value and largest V_{max} among substrates tested even when the estimation was made per galacturonic acid unit. If a larger chain on the non-reducing side inhibits enzyme action, the rate of hydrolysis of PGA would have been slower than that of oligomers. Another possible explanation for the anomaly is as follows: if the enzyme has a higher affinity for the second bond from the reducing terminal than for the first bond, the hydrolysis of the first bond would be competitively inhibited by the second bond which is located about 5 nm away. Although we do not know the K_m value for the first bond of 5GA, the K_m value for the first bond of 4GA was about twice as large as that for the hydrolysis of 5GA in which the 2GA-3GA split is predominant. However, such a difference in K_m values does not seem enough to account for the preferential hydrolysis of 5GA into 3GA and 2GA.

The enzyme showed the smallest K_m value for PGA. Trigalacturonic acid showed no inhibitory effect for the hydrolysis of 4GA and PGA, which means that 3GA shows no significant affinity towards the enzyme. These results suggest that although the enzyme seems to need at least 3 galacturonic acid units near its active centre for its action, a longer galacturonic acid chain on the side of the reducing terminal contributes to higher affinity of substrates for the enzyme.

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