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Occurrence and Properties of Polygalacturonase in *Avena* and Other Plants

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ABSTRACT

Polygalacturonase activity has been detected in a number of plants including seedlings of *Phaseolus vulgaris, Zea mays, Avena sativa,* and *Pisum sativum*. Particular emphasis was placed on characterizing the enzyme from oat seedlings. This enzyme is solubilized by 0.2 M NaCl, and its activity is highest near the apical tips of oat coleoptiles. It has a pH optimum between 5 and 5.5 and is activated by Ca^{2+}, with an optimal concentration of 0.4 mM. Cd^{2+} also activates the enzyme but less effectively than Ca^{2+}. The rate of attack is maximal for substrates with chain lengths of about 20 units and slowest for digalacturonate. The oat enzyme hydrolyzes galacturonans by removing galacturonic acid units from the nonreducing ends and progressively shortens the substrate chains.

Pectolytic enzymes in higher plants are usually associated with the conversion of protopectin to soluble pectin accompanying fruit ripening (7, 25); but exopolygalacturonases have been detected in citrus explants (20) and in the storage tissue of carrots (5, 17). After observing that exopolygalacturonase is present in developing cucumber fruit (16); we speculated that the enzyme may be involved in processes other than fruit ripening and examined a variety of vegetative tissues for activity. We found that pectolytic activity, at relatively low levels compared with that in ripe tomato fruit, is widespread in plants. We now report the detection of polygalacturonase in a number of plants and the properties of the enzyme from oat seedlings.

MATERIALS AND METHODS

Seedlings and Other Plant Materials. Seeds of *Zea mays* L. var. Seneca Chief, *Phaseolus vulgaris* L. var. Red Kidney, *Pisum sativum* L. var. Alaska, and *Avena sativa* L. var. Coker 227, Elan, and Victory were surface-sterilized in 1% NaOCl, rinsed, soaked in distilled H_2O for 1 hr, and then sowed in stainless steel trays of vermiculite. The trays were watered with distilled H_2O and kept in the dark at 25 C. After 4 to 7 days, the seedlings were harvested and immediately extracted.

The other plant materials (bean pods, corn plants, Swiss chard stalks, pokeweed stems, tomato stems, squash stalks, asparagus shoots, turnip roots, and beet roots) were obtained from local gardens. Extracts of plants were limited to young stem or stalk portions and most of the leaves were usually removed.

Extraction of Enzymes. The plant tissue (50-g portions) was cut into small pieces and blended with 150 ml of cold 0.5 M NaCl with VirTis[1] and Polytron homogenizers. All subsequent steps were conducted at 2 C. The homogenate was adjusted to pH 6 by addition of dilute NaOH or HCl and stirred for 2 hr. After centrifuging the sample at 8,000*g* for 20 min, ammonium sulfate was added to the supernatant solution to 75% of saturation. The precipitated protein was collected by centrifugation, dissolved in a small volume of 0.15 M NaCl, and dialyzed overnight against 0.15 M NaCl.

Substrates. Pectin and pectate from citrus fruits (Sigma Chemical Co.) were purified by precipitation with ethanol from aqueous solutions. The gelatinous precipitates were collected on Miracloth, squeezed dry, and redissolved in water. Each polysaccharide was precipitated and collected two more times, washed with ethanol and acetone, and dried *in vacuo* over Drierite.

Three fractions of polygalacturonates of lower mol wt were prepared from the purified pectate by hydrolysis with fungal pectinase (Sigma Chemical Co.) (14). The polygalacturonates (PGA I,[2] PGA II, and PGA III) are in order of decreasing molecular size. PGA I was prepared by treating 2 liters of 1% pectate (pH 5) with 20 mg pectinase at 35 C. The solution was brought to a boil, cooled, and the product was precipitated by adjusting to pH 2 with HCl. PGA II was prepared by treating 2 liters of 1% pectate (pH 5) with 100 mg pectinase at 35 C. The solution was heated to boiling, cooled, and the pH was adjusted to 2. Following clarification by centrifugation, the solution was cooled to 2 C and stored for 48 hr. The precipitate (PGA II) was collected and dried. The filtrate was then treated with 20 g $CaCl_2 \cdot 2H_2O$ and adjusted to pH 5. The calcium polygalacturonate was collected, dissolved, and deionized with Dowex 50-H^+. The precipitate obtained with 2 volumes of ethanol was collected and dried (PGA III). The average degrees of polymerization for pectate, PGA I, PGA II, and PGA III were 201, 79, 20, and 13, respectively.

The oligogalacturonates were prepared from purified pectate by more extensive hydrolysis with pectinase (16). A liter of 1% pectate (pH 5) was treated with 200 mg pectinase at 30 C. The solution was heated to 100 C cooled, and centrifuged. The mixture of oligogalacturonates was precipitated by addition of 10 g $SrCl_2$ and 2 volumes of 95% ethanol. The precipitate was collected, dissolved in water, deionized with Dowex 50-H^+, and adjusted to pH 6. The solution was then applied to a column (10 × 70 cm) of DEAE-Sephadex A-50 previously adjusted to pH 6 and washed with water. The oligomers were eluted by

[1] Mention of trademark of proprietary product does not constitute a guarantee or warranty of product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may be suitable.

[2] Abbreviations: AGA: anhydrogalacturonic acid; OGA: oligogalacturonate; PGA: polygalacturonate.

increasing the NaCl concentration of the eluting solution (17). The properties of the oligogalacturonates were described earlier (17).

Reduced substrates were prepared by treating 0.3 g dissolved in 25 ml water at pH 9 and 25 C for 30 min with 0.3 g $NaBH_4$ (11). Excess reagent was destroyed by acidifying the solution to pH 4. The products were recovered as the Sr^{2+} salt followed by deionization with Dowex 50-H^+.

Pentagalacturonate was labeled with tritium by reducing with NaB^3H_4. About 8 mCi (3 mg) of the NaB^3H_4 (New England Nuclear) was diluted with 2 g of $NaBH_4$ by dissolving in 150 ml of isopropylamine. The solution was evaporated to dryness by a stream of dry N_2 and the salt was dried *in vacuo* over Drierite. Pentagalacturonate was then reduced with the diluted NaB^3H_4 according to the above procedure for reduced substrates.

Enzyme Assays. Polygalacturonase was measured by both reductometric and viscometric methods. For the former, the reaction mixture consisted of 0.2 ml 0.2 M acetate buffer (pH 5.2), 0.5 ml of enzyme solution diluted with 0.15 M NaCl, 0.1 ml 0.01 M $CaCl_2$, and 0.5 ml 1% PGA I (pH 5.2). Blanks were prepared for each sample by heating the reaction solutions in boiling water for 3 min before the addition of substrate. The samples were incubated 1 hr at 3 C, heated 3 min, and aliquots were analyzed for reducing groups by the arsenomolybdate method (12). A unit of polygalacturonase is defined as that amount which catalyzes the hydrolysis of 1 μmol of galacturonic acid glycosidic linkages under these conditions. The rate of reaction for oat polygalacturonase in the standard reductometric assay was linear with respect to enzyme concentration up to 0.45 mg protein of the Sephadex G-100 fraction (see Table III) and incubation time up to 90 min.

For the viscometric method, the quantities of the components of the reaction mixture were increased 4-fold, the concentration of the $CaCl_2$ solution was decreased to 1 mM, and the substrate was pectate. Five ml of the solution was transferred to an Ostwald viscometer immersed in a water bath at 37 C and the viscosity was read at periodic intervals.

Glycosidases were measured by the liberation of *p*-nitrophenol from *p*-nitrophenol-glycosides. The reaction mixture consisted of 0.5 ml 0.2 M acetate buffer (pH 5), 0.1 ml enzyme solution, 0.4 ml 0.15 M NaCl, and 0.4 ml 10 mM substrate in water. After 30 min at 30 C, the reactions were terminated by the addition of 2 ml 0.2 M sodium carbonate, and the absorbancy was read at 415 nm (8). A unit of glycosidase is defined as that amount which releases 1 μmol *p*-nitrophenol under these conditions.

Pectinesterase was assayed by adding enzyme solutions to 50 ml of 0.5% pectin in 0.1 M NaCl (pH 7.5) and titrating with 0.02 N NaOH at pH 7.5 for 10 min at 25 C (10). A unit of pectinesterase is defined as that amount which releases 1 μmol acid groups under these conditions. Pectin and pectate lyases were measured spectrophotometrically at 235 nm (1, 2) and by the thiobarbituric acid method (19, 23).

Proteins were measured by the biuret method (9) with BSA as the standard.

RESULTS

Solubility of Oat Polygalacturonase. The polygalacturonases in fruit tissues are strongly associated with the cell walls and are solubilized by relatively high concentrations of salts (6, 7, 15, 25). The following procedure was used to determine whether this is also true for the oat enzyme. Samples (15 g) of Elan oat seedlings harvested 5 days after planting were homogenized with 60 ml of water, 0.1 M NaCl, 0.2 M NaCl, and 0.5 M NaCl. The homogenates were adjusted to pH 6 by addition of dilute NaOH, stirred 1 hr, and centrifuged. Ammonium sulfate was added to the supernatant solutions to 75% of saturation. The precipitates were collected by centrifugation, dissolved in 15 ml of 0.15 M NaCl, and dialyzed overnight against 0.15 M NaCl. The solutions were then assayed for protein, polygalacturonase, pectinesterase, β-galactosidase, and β-glucosidase (Table I). The results are presented as the total units solubilized from each 15-g sample of seedlings.

The residue after extraction of oat seedlings with 0.5 M NaCl was suspended in 60 ml of 0.15 M NaCl, stirred, and centrifuged. It was then suspended in 30 ml of 0.15 M NaCl and assayed for polygalacturonase. The assay mixture contained 1 ml of the suspension, 0.4 ml 0.2 M acetate (pH 5.2), 0.1 ml 0.01 M $CaCl_2$, and 0.5 ml 1% PGA I (pH 5.2). The sample and a heated blank were incubated 16 hr at 25 C in a shaker-water bath. The reducing groups released by the oat residue at these conditions were 0.16 μmol/ml suspension. It is clear that the amount of enzyme remaining with the insoluble fraction of oat seedlings was very low compared with that in the soluble fraction.

Polygalacturonase in Three Varieties of Oats. Seedlings of Elan, Coker 227, and Victory oats were harvested after 5 days, and 15-g portions were extracted with 0.5 M NaCl according to the standard procedure. The levels of polygalacturonase/g seedlings (fresh wt basis) in Elan, Coker 227, and Victory oats were 1.01, 1.10, and 1.03 units, respectively.

Effect of Oat Seedling Size. Trays of Elan oats planted on 3 successive days were harvested 5 days after the last planting. Equal quantities of the seedlings were extracted for polygalacturonase according to the standard procedure. The average heights were 7.8, 4.9, and 3 cm and the polygalacturonase levels were 0.75, 0.92, and 1.03 units/g tissue (fresh wt basis) for the oldest, middle, and youngest seedlings, respectively.

Distribution of Polygalacturonase in Oat Seedlings. Seedlings approximately 3 cm tall were selected from trays of Elan oats 5 days after planting and cut with a razor blade into 1-cm lengths (top, middle, and bottom). The leaves inside the coleoptiles were not removed. Extracts were prepared from 12 g of each sample and assayed for protein and polygalacturonase (Table II). The levels of pectinesterase, β-galactosidase, and β-glucosidase in the extracts are also presented. Seedlings of Coker 227 and Victory oats were analyzed in a similar manner. The difference in polygalacturonase levels between the top and bottom sections was smaller for Coker 227 seedlings and larger for Victory seedlings than for Elan seedlings.

Purification of Oat Polygalacturonase. Elan oat seedlings (63 g) were homogenized in 190 ml of 0.5 M NaCl, adjusted to pH 6, and stirred for 1 hr. The homogenate was centrifuged and ammonium sulfate was added to the supernatant solution to 40% of saturation. The precipitate that formed was removed by centrifugation and discarded. The ammonium sulfate concentration in the solution was increased to 75% of saturation. The second precipitate was collected by centrifugation and dissolved

Table I. Effect of NaCl concentration on extraction of enzymes from oat seedlings

NaCl	Protein	Polygalacturonase	Pectinesterase	β-Galactosidase	β-Glucosidase
M	mg		units		
0	84	10.0	34	120	150
0.1	100	14.1	136	170	260
0.2	102	16.1	163	160	280
0.5	98	15.8	232	180	290

Table II. Distribution of enzymes in Elan oat seedlings

Section	Protein	Polygalacturonase	Pectinesterase	β-Galactosidase	β-Glucosidase
cm	mg/g		units/g		
Top	6.6	1.6	34	14	28
Middle	5.2	1.2	21	10	21
Bottom	2.6	0.4	7	4	6

in 15 ml of 0.15 M NaCl. An aliquot of the crude extract and of the 75% ammonium sulfate fraction were dialyzed against 0.15 M NaCl and assayed for polygalacturonase (Table III).

The remainder of the 75% ammonium sulfate fraction was concentrated to 10 ml by ultrafiltration with a PM-10 membrane (Amicon Corp.) under 20 p.s.i. N_2. This concentrate was applied to a column (2.5 × 90 cm) of Sephadex G-100 adjusted to pH 6 and equilibrated with 0.15 M NaCl. Fractions eluted with 0.15 M NaCl were assayed for polygalacturonase, pectinesterase, and absorbance at 280 nm (Fig. 1). The fractions containing polygalacturonase (fractions 17 through 21) were combined, concentrated to 9 ml by ultrafiltration, and assayed for polygalacturonase (Table III).

From the elution volumes of the protein standards BSA, ovalbumin, and Cyt *c* on the above Sephadex G-100 column, the mol wt of oat polygalacturonase was calculated to be about 63,000. A single peak of pectinesterase in the solution corresponded to a mol wt slightly lower than that for the polygalacturonase (Fig. 1). Peaks of β-galactosidase, β-glucosidase, laminarinase, and invertase also overlapped with the polygalacturonase peak (data not shown). The partially purified polygalacturonase, therefore, still contained all of these enzymes.

Stability of Oat Polygalacturonase. The partially purified enzyme in concentrated solutions was stable for at least several weeks when stored at −10 C. Heating the solutions at pH 6 for 5 min up to 55 C did not inactivate the enzyme, but half of the activity was destroyed when heated to 62 C and all of it when heated to 69 C. Addition of 1 mM Ca^{2+} to the enzyme solution did not affect the stability of the enzyme to heat. The stability with regard to pH was determined by diluting the enzyme solution with MES-acetate buffer and heating to 60 C for 5 min. The solutions were then diluted with more MES-acetate (pH 5.2) and assayed by adding Ca^{2+} and PGA I (pH 5.2). The enzyme was most stable in the pH range of 5 to 6.5 and least stable at pH 3.

Activation by Cations. Low polygalacturonase activity was observed when the partially purified enzyme was assayed in the absence of Ca^{2+}. This low level of activity was abolished by 30 mM citrate or EDTA in the reaction mixture. Ca^{2+} stimulated polygalacturonase activity with an optimum concentration of about 0.4 mM that produced a 7-fold increase over the control (no Ca^{2+} added) with PGA III as the substrate (Fig. 2). Activation of the enzyme by Ca^{2+} was independent of the pH, with identical optimal concentrations at pH 4, 5, 6, and 7.

Ca^{2+} at concentrations higher than 1 mM suppressed the hydrolysis of PGA III by oat polygalacturonase (Fig. 2). This effect of high levels of Ca^{2+} was dependent on the concentration and molecular size of the substrate. Plots of reaction velocity *versus* PGA I concentration at constant Ca^{2+} concentration changed from hyperbolic to sigmoidal shape with increasing Ca^{2+} (Fig. 3) because the effect was greatest at low substrate levels. Suppression of cleavage was most pronounced for the largest substrates; 2 mM Ca^{2+} completely blocked the hydrolysis of pectate. The direct relationship between substrate size and the suppression of activity suggests that the effect is due to substrate insolubilization rather than to actual inhibition of the enzyme.

The following divalent cations at concentrations of 0.14, 0.5, and 1.4 mM did not stimulate polygalacturonase activity: Sr^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Hg^{2+}. The only cation besides Ca^{2+} that activated the enzyme was Cd^{2+}. The optimal concentration of Cd^{2+} was about 0.5 mM, but the maximum effect was lower than for Ca^{2+} (Fig. 2). K^+ and Na^+ at concentrations between 7 and 56 mM, alone and in combination with 0.4 mM Ca^{2+}, did not affect the reaction rate. Higher concentrations of salts were inhibitory, with 42% inhibition by 170 mM NaCl.

Riov (20) found that polygalacturonase in extracts of citrus leaf explants could be measured only after inhibition of uronic acid oxidase present in the extracts with 1 mM hydrosulfite. Hydrosulfite added to assay mixtures of oat polygalacturonase to final concentrations of 0.6, 1.2, and 2.4 mM had no effect on the activity.

pH Optimum. The oat enzyme was optimally active between pH 5 and 5.5 both in the absence of added Ca^{2+} and in the presence of 0.4 mM Ca^{2+} (Fig. 4). The pH optimum was independent of substrate size and of Ca^{2+} concentration. Reac-

Table III. Summary of purification of oat polygalacturonase

Fraction	Volume	Protein	Activity	Yield	Purification
	ml	mg	units	%	relative
Crude	214	530	74	100	1
75% $(NH_4)_2SO_4$	16	192	52	70	1.9
Sephadex G-100	9	13	33	45	18

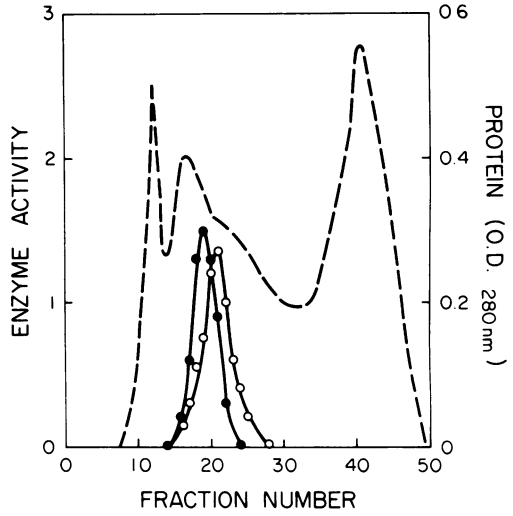

FIG. 1. Gel filtration on Sephadex G-100 of the protein precipitated from an oat extract by ammonium sulfate between 40 and 75% of saturation. The fraction volume was 10 ml. (●——●): polygalacturonase; (○——○): pectinesterase; (- - -): absorbance at 280 nm.

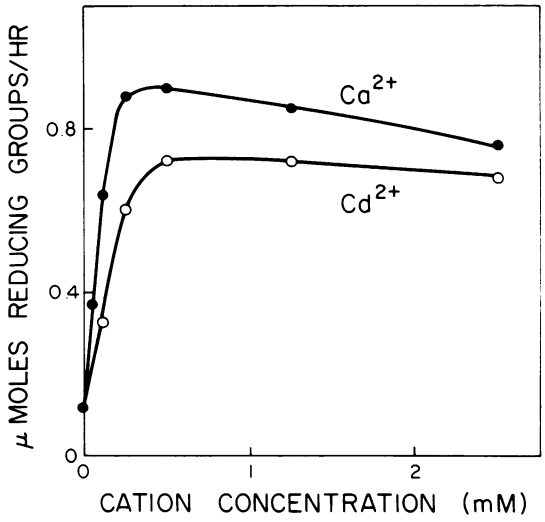

FIG. 2. Effects of Ca^{2+} and Cd^{2+} on oat polygalacturonase activity at pH 5.2. The reaction mixtures contained 0.2 ml of Sephadex G-100 fraction from oat seedlings. The substrate was PGA III; other assay conditions were standard.

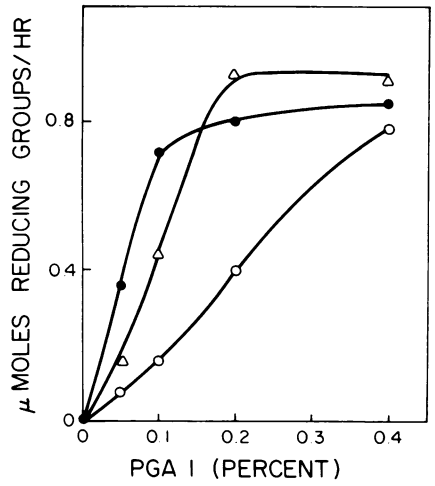

FIG. 3. Effect of Ca^{2+} concentration on the plots of polygalacturonase activity *versus* substrate concentration. The reaction mixtures contained 0.2 ml of Sephadex G-100 fraction from oat seedlings. The substrate was PGA I. (●——●): 0.3 mM Ca^{2+}; (△——△): 0.6 mM Ca^{2+}; (○——○): 1.2 mM Ca^{2+}.

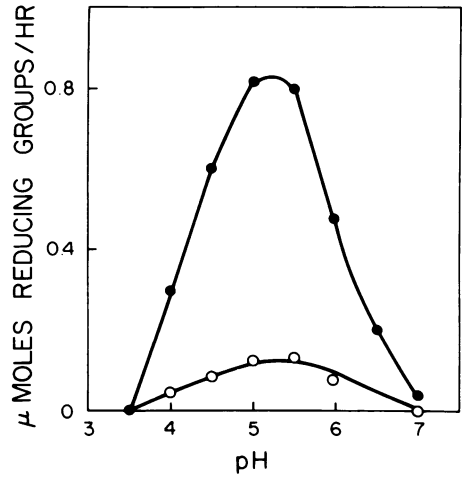

FIG. 4. Effect of pH on the activity of oat polygalacturonase. (○——○): in the absence of added Ca^{2+}; (●——●): in the presence of 0.4 mM Ca^{2+}. The reaction mixtures contained 0.2 ml of Sephadex G-100 fraction from oat seedlings. The samples contained 0.2 ml of 0.2 M MES-acetate buffer, and the substrate (PGA I) solution was adjusted to the various pH values before addition to the reaction mixtures.

tion rates were identical between pH 3.5 and 5.5 in the presence of acetate, maleate, and succinate buffers. The nature of the buffer was a factor, however, at higher pH. Compared to the activity in imidazole, MES, and tris, the reaction velocity in maleate was lower at pH 6 and sharply decreased to zero at pH 6.5.

Effects of Substrate Size. V_{max} and K*m* were determined for the oat enzyme acting on oligogalacturonates, polygalacturonates, and pectate (Table IV). For the K*m* calculations, the average mol wt of pectate, PGA I, PGA II, and PGA III were taken as 35,400, 13,900, 3,500, and 2,300, respectively. The data showed that the enzyme acted with highest velocity on moderately long substrates (PGA II), but it had the highest affinity for pectate, the longest substrate. The rate of cleavage of the dimer was by far the slowest.

Mode of Action. An important question concerning the oat enzyme is the mechanism of its action on the galacturonan chain. Among the possibilities are random cleavage of interior linkages or specific cleavage, beginning at one end of the polymeric chain, by either hydrolysis or transelimination of pectin or its de-esterified form, pectate. Most of the pectolytic enzymes in higher plants that have been characterized are endo- or exohydrolases, although Albersheim and Killias (1) have reported that pea seedlings contain a transeliminase.

The possibility that the natural substrate for the oat enzyme is pectin, the esterified polymer, was ruled out by observations that the rate of cleavage was about four times as high for pectate as for pectin. The slow degradation of pectin may be explained by the fact that the commercial grade of pectin used in this study is not completely esterified. Another reason is that the Sephadex G-100 fraction of oat polygalacturonase contained a low level of pectinesterase and its action on pectin may have slowly rendered the pectin molecule susceptible to change.

Evidence against a mechanism of random attack on the substrate chain was obtained by comparing the rates of reaction according to the viscometric and reductometric methods. The reaction mixture contained 0.5% pectate and the Ca^{2+} concentration was decreased to 0.1 mM to eliminate gelling or precipitation that would invalidate viscosity measurement. During a 1-hr incubation at 37 C, the viscosity of the solution decreased only 7% while 2.2% of the glycosidic bonds were cleaved (Fig. 5). The small effect on the viscosity of pectate is characteristic of hydrolysis in an exo- fashion. In contrast, endopolygalactu-

Table IV. Kinetic parameters of the hydrolysis of galacturonans by oat polygalacturonase

Substrate	K_m	Maximum Velocity
	μM	Relative
Digalacturonate	290	0.2
Trigalacturonate	210	0.6
Tetragalacturonate	180	1.7
Pentagalacturonate	160	1.9
Reduced Pentagalacturonate	130	2.0
Hexagalacturonate	140	2.3
PGA III	74	3.0
Reduced PGA III	68	2.9
PGA II	46	3.8
Reduced PGA II	48	4.1
PGA I	12	3.1
Pectate	6	1.0

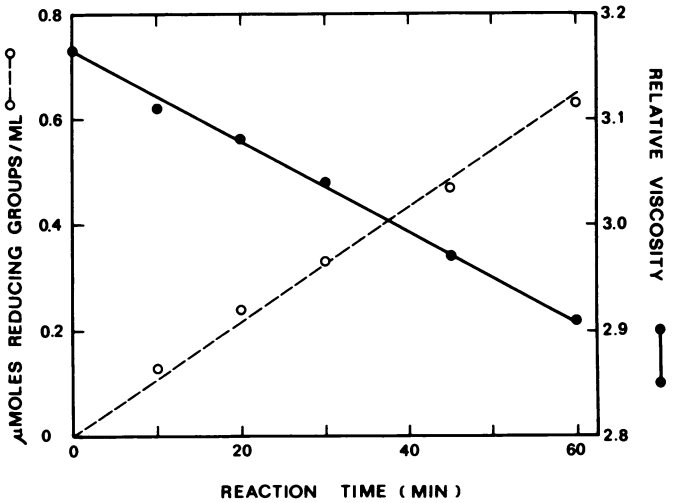

FIG. 5. Changes in the viscosity and reducing groups of pectate during degradation by oat polygalacturonase. The reaction mixture contained 2 ml of Sephadex G-100 fraction from oat seedlings, 5 ml of 1% pectate (pH 5.2), 1 ml 1 mM Ca^{2+}, and 2 ml of 0.2 M acetate (pH 5.2). After mixing, 5 ml of the solution were transferred to a viscometer, and aliquots of the remaining solution were analyzed for reducing groups at the times indicated.

ronases reduce the viscosity of pectate by 50% while hydrolyzing only 1% of the glycosidic bonds (4).

The nature of the product released by the oat enzyme was established by tests for unsaturation and by paper and ion exchange chromatography. According to the reaction with the Nelson-Somogyi copper reagent, the product contained a reducing group. Results from the UV absorption assay were negative for unsaturated oligogalacturonates (1, 19) that are produced by transeliminases. The reaction solution tested contained 0.72 μmol reducing groups/ml. Had the enzyme produced unsaturation for each reducing group formed, the increase in absorbance at 235 nm should have been 3.3 (2). Similarly, no reaction was obtained in the very sensitive thiobarbituric acid assay (23) for unsaturated oligogalacturonates and 4-deoxy-5-ketouronic acids.

A reaction solution of PGA II and 0.73 units of oat enzyme, after a 2-hr incubation, was spotted on 3MM paper sheets which were developed with ethyl acetate-acetic acid-water (10:5:6) and butanol-acetic acid-water (3:1:1) by the descending method. Reaction products were detected by dipping the sheets in a mixed indicator solution and in diphenylamine-aniline reagent (13). The only mobile product detected was acidic and gave the same reddish brown color reaction with diphenylamine-aniline as galacturonic acid; the R_F was identical to that for galacturonic acid in both solvent systems. The origins on the chromatograms reacted as strongly acidic spots with just slight streaking down the sheets. No spots of di-, tri-, or tetragalacturonates were detected. Results were similar when a portion of the reaction solution was analyzed by the ion exchange method (17). A single peak of carbazole-positive (21) material was eluted, and corresponded to galacturonic acid standard run on the same column.

The enzyme removes monomer units from the ends of the polymeric chains. The end of the molecule that is attacked was established by the approach described earlier (15). Sodium borohydride-reduced substrates (OGA V, PGA II, and PGA III) were cleaved at the same rates as the unreduced substrates (Table IV). This suggests that cleavage might occur at the nonreducing ends, unless the modified monomer at the reducing end does not block enzymic action. The latter possibility was resolved by separating the products of partially hydrolyzed reduced PGA III, on the basis of solubility, to determine where the reducing groups were formed. A reaction solution containing 0.2 ml of the Sephadex G-100 fraction from oat seedlings and reduced PGA III as the substrate after 2 hr at 37 C was analyzed for reducing groups and AGA (anhydrogalacturonic acid), and the remainder was treated with 2 volumes of ethanol. The precipitate was collected and dissolved in water. Analyses of the alcoholic supernatant and the solution of the precipitate showed that 95% of the reducing groups and only 13% of the AGA were in the alcoholic solution. Cleavage at the reducing end would have produced a reducing group on the residual molecule and the reducing value for a short chained substrate, such as PGA III, would have been much higher. Furthermore, at least part of the reducing groups associated with the precipitate may have been due to co-precipitated galacturonic acid and the actual value may have approached zero.

Evidence supporting the mechanism of removal of galacturonic acid units from the nonreducing ends was obtained with studies on tritium-labeled OGA V. A solution of labeled OGA V, after a 2-hr reaction with the Sephadex G-100 fraction of oat polygalacturonase, was applied to a column of DEAE-Sephadex A-50 and eluted with a linear gradient of 0.05 to 0.20 M NaCl (16). The fractions were analyzed for AGA and the peaks identified by comparison with patterns for standards. Results with two levels of enzyme (Table V) show that the next lower oligogalacturonate, OGA IV, predominates after a moderate amount of reaction as with the lower level of enzyme. The dimer appears only after considerable reaction has occurred. These observations confirm that single galacturonic acid units are removed from the nonreducing ends of the substrate chains and all chains are progressively shortened. The radioactivity remains with the substrate residue after removal of the first monomer unit and increases as hydrolysis proceeds.

Polygalacturonase in Oat Plants. One hundred forty g of stem tissue from 6-week-old field-grown Elan oat plants (approximately 30 cm tall) were extracted with 0.5 M NaCl at pH 6. The level of polygalacturonase activity in the crude extract was equivalent to 0.57 unit/g fresh wt. The crude extract was fractionated with ammonium sulfate and the 40 to 75% ammonium sulfate fraction was filtered through a column (2.5 × 90 cm) of Sephadex G-100 in 0.15 M NaCl. A single peak of activity was obtained, corresponding to a mol wt of approximately 65,000. The enzyme had a pH optimum between 5 and 5.5 and was activated by both Ca^{2+} and Cd^{2+}.

Polygalacturonase in Oat Seeds. Oat seeds ground with a Wiley mill were extracted with 0.5 M NaCl. The activity of polygalacturonase extracted was 5.1 units/g. The seed enzyme was more effective in reducing the viscosity of pectate than the seedling enzyme, with a 34% decrease in the viscosity while producing 0.66 μmol reducing groups/ml. Possibly oat seeds contain an endopolygalacturonase, but most of the activity appeared to be due to an enzyme similar to that found in seedlings. The seed enzyme was activated by Ca^{2+} and Cd^{2+}, had a pH optimum between 5 and 5.5, and was most active on PGA II and III.

Polygalacturonase Activity in Other Plants. Concentrates of protein from various plant materials were prepared by extraction with 0.5 M NaCl followed by precipitation with ammonium sulfate at 75% of saturation. The precipitates were dissolved in 0.15 M NaCl, dialyzed against 0.15 M NaCl, and assayed for polygalacturonase in acetate buffer over the pH range of 3.5 to 6 in the absence and presence of 0.6 mM Ca^{2+} (Table VI).

DISCUSSION

The polygalacturonase in oat seedlings does not have a strong affinity for the insoluble cell wall debris of seedling homogenates because most of the activity was extracted with water (Table I). β-Galactosidase was similar to polygalacturonase in solubility,

Table V. The products of reduced pentagalacturonate hydrolysis by oat polygalacturonase

Product	0.73 unit enzyme		1.8 units enzyme	
	AGA	radioactivity	AGA	radioactivity
	mg	cpm/μg AGA	mg	cpm/μg AGA
Galacturonate	1.35	0	2.56	0
Digalacturonate	0	---	0.28	62
Trigalacturonate	0.80	38	1.88	39
Tetragalacturonate	3.44	33	3.06	29

Table VI. Polygalacturonase activity in a variety of plant tissues

Tissue	Polygalacturonase	pH optimum	Activation by 0.6 mM Ca^{2+}
	units/g		%
Red Kidney bean hypocotyls	0.52	5-5.5	65
Blue Lake bean pods	0.34	5	160
Seneca Chief corn seedlings	0.54	5-5.5	43
Seneca Chief corn plants	0.62	5-5.5	86
Alaska pea seedlings	0.24	4.5-5	100
Swiss Chard stalks	0.21	5-5.5	44
Pokeweed stems	0.17	5-5.5	---
Better Boy tomato stems	0.18	5.5	84
Butterbar squash stalks	0.10	4.5	---
Asparagus shoots	0.72	5.5	66
Turnip roots	0.42	4.5-5	64
Red beet roots	0.30	5	---

but β-glucosidase and pectinesterase, in particular, were less soluble in water. The polygalacturonase activity in oat seedlings did not vary greatly with the variety of oats. However, the activity in extracts of whole seedlings decreased with seedling age and height because the activity was highest near the tips of the coleoptiles (Table II). The amount of protein solubilized by our extraction procedure decreased in the 1-cm segments of coleoptiles downward from the tips, but polygalacturonase and the three other enzymes measured (β-galactosidase, β-glucosidase, and pectinesterase) decreased considerably more (Table II). Polygalacturonase was also found in stem tissue of young oat plants. This apparent association of polygalacturonase with rapidly growing tissues of oats suggests that this enzyme may be involved in plant growth.

The properties of the polygalacturonase in oat seedlings are consistent with those of an exohydrolase. The data in Table V do not indicate that transferase action is significant at the conditions of the assay. Apparently when a glycosidic bond in the substrate is cleaved by the enzyme, both the monomer and residual substrate molecule dissociate from the enzyme. The substrate fragment can then form a new complex with the enzyme, and another bond can be cleaved. This enzyme therefore acts by a multichain mechanism (22) in which single units of galacturonic acid are removed from the nonreducing ends of the substrate chains and all of the molecules are progressively shortened. It appears to be specific for linkages involving deesterified monomers, but the effects of ester groups elsewhere in the molecule are unknown. Ca^{2+} is required for the enzyme reaction. The activation by Cd^{2+} is a rare example of such an effect by this cation on an enzyme from higher plants (24).

Earlier reports described exopolygalacturonases from carrots (5, 17), peaches (15), cucumbers (16), and pears (18). The oat enzyme is similar to these enzymes in many respects. All attack the substrate chains at the nonreducing ends, removing monomer units. They act optimally on moderately long substrates (in the degree of polymerization range of 13 to 20) and cleave digalacturonate very slowly. With the exception of carrot exopolygalacturonase, the enzymes are activated by Ca^{2+}. The pH optima are all in the range of 4.5 to 5.5.

Riov (20) detected polygalacturonase in the separation layer of citrus leaf explants. He found that extracts of this tissue contain a uronic acid oxidase and developed a method for measuring polygalacturonase by inhibiting the oxidase with 1 mM sodium hydrosulfite. The enzyme was localized in the separation layer. It was present at low levels in freshly excised explants, but it increased after an induction period and the increase was accelerated by ethylene. Riov concluded that the enzyme was an exopolygalacturonase because the only product detectable on thin layer chromatograms was galacturonic acid. His data are in units of activity per 125 explants, and therefore it is not possible to compare the levels of activity in the explants to that in oat seedlings. However, judging from the 4- to 6-hr incubation period of his assay, the activity was relatively low. The effects of cations on the activity of the citrus explant enzyme were not determined.

Datko and Maclachlan (3) reported the occurrence of pectolytic activity in *Pisum sativum* seedlings. The activity was measured viscometrically with sodium polypectate as the substrate in 16.8 mM sodium citrate (pH 6). They found a low level (0.39% loss in viscosity after 2 hr at 35 C) of pectolytic activity in segments of epicotyl immediately below the apex. The activity in the segments of decapitated seedlings increased during the first 2 days and then decreased. In contrast, the activity in decapitated seedlings treated with IAA increased steadily and after 4 days was 20 times higher than in the control segments. Datko and Maclachlan (3) did not characterize the enzyme, but the small effect on the viscosity of pectate suggests that it hydrolyzed the substrate in an exo- fashion.

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