Characterization of a Pectic Fraction from Smooth Bromegrass Cell Walls Using an Endopolygalacturonase

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An endopolygalacturonase (PG) was isolated from a commercial enzyme preparation from *Aspergillus niger*. A fractionation scheme was developed for rapid purification of the enzyme for characterization of pectic polysaccharides from smooth bromegrass (*Bromus inermis* L.). The purified PG had a pH optimum of 4.5 and a hydrolytic temperature stability range of 25–35 °C and yielded only tri-, di-, and monogalacturonic acid after exhaustive degradation of polygalacturonic acid. A pectic polysaccharide fraction was solubilized from bromegrass cell walls with hot sodium phosphate buffer and fractionated by anion-exchange chromatography. Degradation of two uronic acid rich fractions by PG revealed a polydisperse nature of the polysaccharide components. One fraction contained a complex makeup of neutral sugars along with rhamnogalacturonans that exhibited limited degradation. The other fraction contained fewer neutral sugar components, and the rhamnogalacturonans were extensively degraded. Compositional characteristics of these polymers were similar to those of rhamnogalacturonans isolated from other plants.

INTRODUCTION

Pectins are a complex mixture of polysaccharides that contains both neutral and charged species in varying proportions. Characterization of these polysaccharides has most frequently utilized materials from dicotyledons with few examples from monocots, particulary the grasses. Synthesis of pectic polysaccharides occurs early in plant development and may play fundamental roles in cell wall expansion (Jarvis, 1984). It would appear that pectic polysaccharides are organized into regions containing long stretches of galacturonan chain interrupted by regions of frequent intramolecular substitution of rhamnose residues forming kinks in an otherwise linear molecule (Jarvis, 1984). Frequently, the rhamnose residues are substituted with neutral sugars that may be polymeric in nature (Jarvis, 1984; Rombouts and Thibault, 1986; Bacic et al., 1988; Hatfield, 1991). The unsubstituted regions of galacturonic acid can become cross-linked through ionic bonding in the wall matrix by Ca^{2+} bridges. The frequency of Ca^{2+} bridging can be regulated by the extent of methyl esterification and/or the frequency and extent of neutral sugar substitution upon the rhamnose residues in the kinked regions of the molecule.

Characterization of pectic polysaccharide fine structure is difficult in such complex arrangements. In addition, the removal of pectins from the cell wall is usually achieved by chemical means, but the harsh conditions necessary for chemical fractionation can alter the fine structure of the extracted polymers as well as the remaining wall material. Purified enzymes provide useful tools to aid in polysaccharide characterizations as well as a more selective and less harsh means of removing specific polysaccharide types. Endopolygalacturonase (EC 3.2.1.15) hydrolyzes the α-1,4 linkages between galacturonic acid units in polygalacturonic acid (PGA), releasing small oligomers. The binding site, hydrolytic pattern, and kinetics of an endopolygalacturonase (PG) from *Aspergillus niger* have been studied (Koller and Neukom, 1969; Rexova-Benkova, 1973; Thibault and Mercier, 1978). This PG produced by *A. niger* hydrolyzes unsubstituted polygalacturonic acid into limit products having a degree of polymerization (DP) equal to 3, 2, and 1, reflecting a minimum binding/hydrolytic site of four galacturonosyl residues. The objective of this work was to develop an efficient fractionation scheme for the isolation of a pectin-degrading enzyme for use in polysaccharide characterization studies. Although similar enzymes derived from *A. niger* preparations have been studied before, it was necessary to extend this work to define factors that may affect enzyme stability and hydrolytic activity. Because this enzyme would be used to characterize complex mixtures of polysaccharides, it was necessary to ensure there was a single hydrolytic activity with a well-defined substrate specificity. This PG was used to aid in the characterization of a complex buffer-soluble fraction isolated from smooth bromegrass cell walls. This enzyme has also been used to aid in the characterization of pectic polysaccharides isolated by hot buffer from alfalfa stems (Hatfield, 1991).

MATERIALS AND METHODS

Purification of PG. Three milliliters of Pectinex 5XL (a commercial enzyme preparation from *A. niger* that contains high pectinase activity; NOVO Bioindustries, Danbury, CT) containing 189 mg of protein was diluted to 25 mL with 20 mM sodium acetate buffer, pH 5.0, and separated into three fractions by $(NH_4)_2SO_4$ precipitation. Material precipitated by the addition of solid $(NH_4)_2SO_4$ (50% saturation) was pelleted by centrifugation (10000*g*), and the supernatant was removed and made 75% saturated with solid $(NH_4)_2SO_4$. The second precipitate was pelleted by centrifugation and the supernatant removed, diluted to 50 mL with 20 mM sodium acetate buffer (pH 5.0), and concentrated in an Amicon stirred cell ultrafiltration device (YM10 filter; Amicon Corp. Danvers, MA). Four 50-mL buffer exchanges of 20 mM sodium acetate buffer (pH 5.0) were carried out in the Amicon cell to remove excess salt in the supernatant fraction. The final volume was 5 mL and contained the bulk of the PG activity.

Concentrated PG was applied to a DEAE-Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) column (2.5 × 10 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0, 50 mM NaCl, 0.01% NaN_3). Unbound proteins were eluted with 30 mL

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of equilibration buffer, and bound proteins were eluted with a 400-mL linear gradient of 350–550 mM NaCl in equilibration buffer (flow rate, 20 mL/h). Protein concentration in each fraction (3 mL) was monitored by absorbance at 280 nm. Fractions containing protein were assayed for PG and (carboxymethyl)-cellulase activity (CMCase).

Fractions containing PG activity were pooled and concentrated, and the buffer was exchanged three times (50 mL each) with 10 mM sodium formate (pH 3.5, 0.01% NaN_3). The concentrated sample (10 mL) was applied to an SP-650M-TSK gel (Supelco, Bellefonte, PA) column (1 × 10 cm) equilibrated with 10 mM sodium formate (pH 3.5, 0.01% NaN_3). Proteins were eluted with a 200-mL linear gradient of 0–250 mM NaCl in equilibration buffer. Fractions were assayed for protein, PG, and CMCase before those containing PG activity were pooled. The purified PG was dialyzed against sodium acetate buffer (pH 5.0, 50 mM NaCl, 0.01% NaN_3) and stored dilute. All purification steps were carried out at 4 °C.

Enzyme Assays. Substrates were prepared fresh in 20 mM sodium acetate buffer (pH 5.0) at 1 mg/mL. For PG activity, samples were diluted as necessary and 25 μL of enzyme preparation was incubated with 475 μL of PGA (Sigma Chemical Co.) for 5–20 min at 30 °C. All other assays utilized undiluted enzyme at 25 μL in 475 μL of substrate with incubations at 30 °C varying from 3 to 16 h depending upon the enzyme fraction and substrate combination. Reactions were stopped by addition of the Somogyi reagent, and activity was assessed by measuring the formation of reducing equivalents after incubation. The purified PG fraction was assayed for the presence of other hydrolase activities using arabinan (Koch-Light Laboratory, Buckinghamshire, U.K.), cellulose (powdered Whatman filter paper No. 3), carboxymethylcellulose 4M6F, carboxymethylcellulose 7MF (Hercules, Wilmington, DE), galactan (Aldrich, Milwaukee, WI), laminarin (U.S. Biochemical Corp., Cleveland, OH), arabinogalactan, barley β-glucan, locust bean gum, oat spelt xylan (Sigma Chemical Co.), and xyloglucan as substrates. Activity was assessed by measuring the formation of reducing equivalents after a 19-h incubation. Endo-activity was measured as the change in viscosity of 25 mg/mL citrus pectin (Fluka Chemical Corp., Ronkonkoma, NY) in 20 mM sodium acetate buffer. One hundred microliters of enzyme was mixed with 900 μL of substrate and the viscosity measured as the time required for the meniscus to fall from 0 to 0.14 mL in a 0.2-mL pipet.

Glycosidase activities were assayed as described by Huber and Nevins (1981) with the *p*-nitrophenyl derivatives of β-galactopyranose, β-glucopyranose, β-glucuronic acid, β-mannopyranose, β-xylopyranose, α-galactopyranose, α-arabinofuranose, and α-fucopyranose (Sigma Chemical Co.). The *p*-nitrophenyl sugars were prepared as 25 mM solutions in 10 mM citrate–phosphate buffer, pH 5.5. One milliliter of buffer was mixed with 100 μL of enzyme and 100 μL of substrate and incubated 3 h at 30 °C. The reaction was stopped by the addition of 2 mL of 200 mM sodium carbonate, and the absorbance of the free *p*-nitrophenol was measured at 400 nm.

Lyase activity was monitored spectrophotometrically at 235 nm (Ishii and Yokotsuka, 1972). Background calibration was 475 μL of 1 mg/mL apple pectin (Fluka Chemical Corp.) in acetate buffer. The assay was started by placing 475 μL of buffer into a cuvette, adding 25 μL of enzyme, and mixing thoroughly. The absorbance was recorded immediately and at 1-min intervals for 10 min and then at 5-min intervals for an additional 20 min.

Electrophoresis. Isoelectric focusing was performed using a Isobox apparatus (Hoefer Scientific Instruments, San Francisco, CA). Preformed agarose isoelectric focusing gels (Isolab, Akron, OH) were used which contained ampholytes covering the pH range 3–7. Proteins were focused for 2 h at 5 °C, and protein bands were visualized with 0.1% Coomassie Blue. The established pH gradient was determined from protein standards (isoelectric focusing kit, pH 3.6–6.6, Sigma Chemical Co.). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) of purified polygalacturonase was performed by using a 12.5% gel at pH 8.8. Protein bands were visualized by staining with Coomassie Blue.

Characterization of Hydrolytic Activity. PGA solutions at various pH values were prepared by mixing 5 mL of PGA stock solution (2 mg/mL in deionized H_2O) with 5 mL of 100 mM approximate universal buffer at pH values of 2–9 (Carmody, 1961). Final solution pH was determined and adjusted with 1 M citric acid or 1 M NaOH for each combination as necessary. The purified PG was diluted 1000-fold and 20 μL incubated with the substrate pH combinations for 20 min at 25 °C. For pH stability, 100 μL of enzyme was mixed with 100 μL of 200 mM universal buffer, pH 3–9, and incubated for up to 24 h in sealed 1.5-mL microfuge tubes at 25 °C. After the appropriate preincubation time (0, 0.5, 1.0, 2.0, 4.0, and 24 h), 20 μL of enzyme was removed and added to 480 μL of PGA (1 mg/mL in 20 mM acetate buffer, pH 4.5), and the mixture was incubated for 20 min at 25 °C. Enzyme activity was measured as reducing equivalents released. The temperature optimum was determined with 1 mg/mL PGA in 20 mM acetate buffer, pH 4.5, over the temperature range 15–60 °C. To determine temperature stability, PG was preincubated at 5, 15, 25, 35, 45, 55, and 65 °C for up to 24 h before subsamples were removed and added to PGA. Individual enzyme samples (20 μL) were incubated with 480 μL of PGA (1 mg/mL in 20 mM acetate buffer, pH 4.5) for 20 min at 25 °C. Samples of diluted enzyme (0.018 mg/mL) were placed in microcentrifuge tubes and frozen overnight. Tubes were removed from the freezer, allowed to thaw at room temperature, and refrozen overnight one to four times. The activities of the frozen/thawed enzymes were determined as above.

To test the effect of divalent cations on enzyme activity, PGA was prepared in 20 mM sodium acetate buffer, pH 4.5, containing 0.01–50 mM $CaCl_2$, $MgCl_2$, or $BaCl_2$. The enzyme was incubated with substrate for 10 min at 25 °C, and reducing sugars were measured. The ability of ammonium oxalate to reverse the effects of Ca^{2+} was tested by preparing 1 mg/mL PGA in 5 mM $CaCl_2$ and adding ammonium oxalate to a final concentration of 0.01–50 mM. The reaction was started by the addition of PG and proceeded for 10 min at 25 °C. Reducing sugars were assayed with the *p*-hydroxybenzoic acid hydrazide method (Lever, 1972), because ammonium oxalate does not interfere with this assay. Apple pectin (72% methyl ester) and citrus pectin (60% methyl ester) were incubated with PG for 10 min at 25 °C and reducing equivalents measured.

Analysis of Enzyme Digestion Products. PGA was degraded with PG (16 h), the digest was assayed for reducing sugar and total uronic acid, and a portion was applied to a 2.5 × 115 cm Toyopearl HW-40 TSK gel column (Supelco). The oligomers were eluted with 200 mM acetate, pH 3.75, and column fractions were assayed for uronic acid and reducing sugar. A separate sample was treated a second time with additional enzyme (16-h incubation). All long-term incubations were conducted in the presence of 0.1% benzalkonium chloride, because it, unlike azide, does not interfere with the uronic acid assay. The digest was assayed and fractionated on the HW-40 column as above.

Characterization of Bromegrass Polysaccharide Solubilized by Hot Buffer. *Cell Wall Isolation.* Smooth bromegrass (*Bromus inermis* L.) plants were grown in a greenhouse under high-pressure sodium lamps using a 14-h day/10-h night light regime. Plants were harvested 5.0 cm above the soil line at leaf stage of development when stems had not begun to elongate, freeze-dried, and separated into leaves and sheaths. Leaf material harvested from different pots were pooled, cut into pieces 5–10 mm long, and used for cell wall isolation. Cell walls were isolated using the procedure as described by Hatfield (1991). Briefly, the leaf material was homogenized in cold (5 °C) phosphate buffer (10 mM NaH_2PO_4 plus 50 mM NaCl, pH 7.0, 50 mL/g of dry tissue) with a Waring blender. Cell wall material (CW) was collected on two Teflon mesh filters (52 μm, Spectrum Medical Industries, Los Angeles, CA) and washed with cold 50 mM NaCl (2000 mL, 5 °C), to remove cytoplasmic contaminants, followed by acetone (−20 °C) until all of the residual pigment was removed. Chloroform/methanol (2:1, 500 mL) was added and allowed to drain slowly from the sample. After the final wash, air was pulled through the residue until dry. Half of the CW material was transferred to a jar mill (Model 753RM Norton, Akron, OH) with 80% ethanol, brought to a final volume of 400 mL, and milled until the particle size was approximately 500 μm (5–6.5 h) as determined by microscopic examination of multiple subsamples. Milled CW was collected on a glass fiber filter with vacuum filtration, washed with 80% ethanol followed by acetone,

Table I. Total Protein and Enzyme Activities at Different Steps of Endopolygalacturonase (PG) Purification from Pectinex 5XL

fraction	total protein, mg	enzyme-specific activities: enzyme activities, mg of RS[a] min^{-1} (mg of protein)$^{-1}$ xylanase	CMCase	PG	total PG activity	% of original
crude Pectinex	189	0.18	0.01	15.32	2835	100
50% $(NH_4)_2SO_4$ pellet	20	0.46	0.02	15.46	310	11
75% $(NH_4)_2SO_4$ pellet	74	0.15	0.06	7.36	548	19
75% $(NH_4)_2SO_4$ soluble	48	0.02	0.01	32.30	1550	55
DEAE-endopolygalacturonase	20	ND[b]	0.01	66.54	1330	47
SP-endopolygalacturonase	9	ND	ND	98.86	840	30

[a] RS, reducing sugar equivalents. [b] ND, not detected.

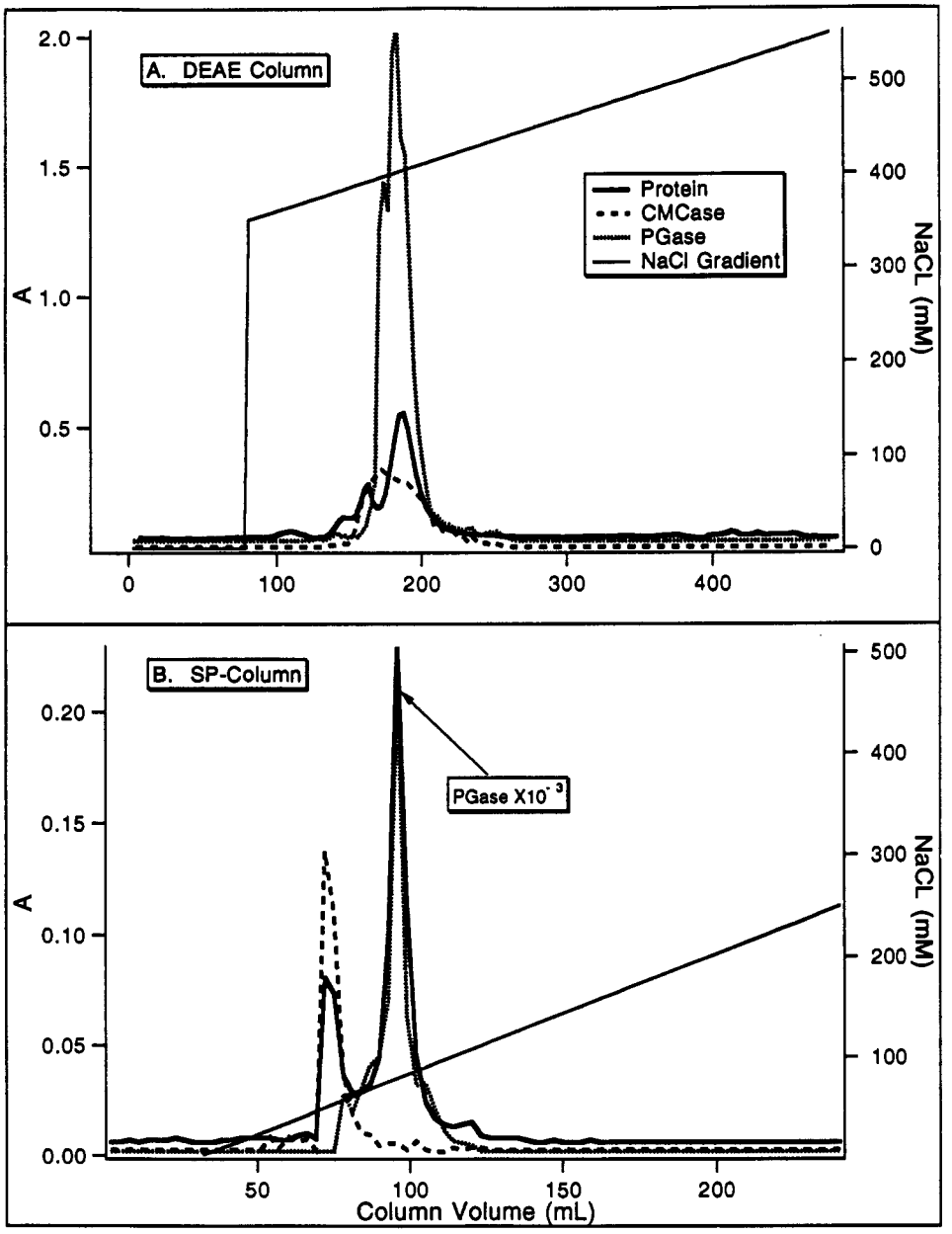

Figure 1. (A) DEAE-Sepharose CL-6B column profile of the supernatant from the 75% ammonium sulfate precipitate. (B) SP-TSK column profile of the pooled PG fraction from the DEAE-Sepharose column.

air-dried, and transferred to 250-mL centrifuge bottles. The remaining CW material was treated in the same manner.

Isolation of Buffer-Soluble Polysaccharides. Milled CW samples (15 g/bottle) were suspended in phosphate buffer (10 mM KH_2PO_4 plus 0.02% NaN_3, pH 7.0, 10 mL/g of CW) and heated in a boiling water bath for 1 h to solubilize easily extracted polysaccharides. Samples were cooled to 60–65 °C, 10 IU of α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1, Sigma A-3403) was added to each, and then the samples were placed in a water bath at 55 °C for 1.5 h. After the initial incubation, pH was adjusted to 4.75, 20 IU of amyloglucosidase (1,4-α-D-glucan glucohydrolase EC 3.2.1.3, Sigma A-3514) was added, and the samples were returned to the water bath for an additional 1.5 h. These enzyme treatments were imposed to remove possible starch contamination. Using the conditions described, neither enzyme treatment exhibited CW hydrolase activity. CW material

was pelleted at 2500g for 10 min following amyloglucosidase treatment. The supernatant was decanted into a filtration flask fitted with a glass fiber filter (GF4, Fisher Scientific, Pittsburgh, PA). Thirty milliliters of deionized H_2O was added to each bottle and mixed thoroughly and CW pelleted as before. After four wash cycles, the CW was transferred to a freeze-drying flask and lyophilized. The buffer extract and washes were combined, dialyzed against H_2O (8–10 L) for 48 h, shell-frozen, and lyophilized.

Fractionation of Buffer-Soluble Polysaccharides. All fractionation steps were carried out at room temperature. The buffer-soluble extract was fractionated on a DEAE Spectra/Gel M (Spectrum Medical Industries) column (2.5 × 15 cm). Carbohydrate samples (80 mg) were dissolved in 10 mL of 20 mM sodium acetate buffer (pH 5.75) and applied to the column, which was equilibrated with the same buffer. Elution was started with the equilibration buffer (80 mL) and continued with a linear gradient of NaCl (0–1 M, total volume of 200 mL) in the same buffer. Elution profiles were determined by measuring the total neutral sugar and total uronic acid residues in even-numbered fractions. Fractions corresponding to peaks of total carbohydrate were pooled, dialyzed against water, and lyophilized.

Uronic acid rich fractions pooled from the DEAE column were subjected to gel filtration chromatography. Samples (5–6 mg) were dissolved in 5 mL of 200 mM sodium acetate buffer (pH 3.75) and applied to a Toyopearl HW-55 TSK gel (Supelco) column (1.7 × 110 cm) equilibrated with the same buffer. Polysaccharides were eluted from the column with 225 mL of equilibration buffer at a constant flow of 25 mL/h. Three-milliliter fractions were collected and assayed for total neutral sugars and total uronic acid residues. Additional DEAE fractions were first degraded with purified PG and then fractionated on the same column run under identical conditions. Fractions containing peaks of total carbohydrate were pooled, dialyzed, and lyophilized. Carbohydrate present as oligomers in the later eluting fractions was recovered as ethanol precipitates.

General Methods. Total carbohydrate was assayed according to the phenol–sulfuric acid method (Dubois et al., 1956), and total uronic acids were assayed according to the method of Blumenkrantz and Asboe-Hansen (1973). Reducing sugars were measured using the Nelson method (1944) as modified by Somogyi (1952) with galacturonic acid as standard. Proteins were precipitated with 15% trichloroacetic acid (TCA) and assayed with BCA protein assay reagents (Pierce, Rockford, IL) with bovine serum albumin as standard. Isolated polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1.5 h at 120 °C and monosaccharides analyzed by gas–liquid chromatography (GLC) as their alditol acetates using the procedure of Blakeney et al. (1983).

RESULTS AND DISCUSSION

Purification of Endopolygalacturonase. Crude Pectinex solution was separated into three fractions by $(NH_4)_2SO_4$ precipitation. Most of the PG activity remained soluble in 75% saturated $(NH_4)_2SO_4$ (Table I). All three fractions contained (carboxymethyl)cellulase activity (CMCase), but most of the activity was precipitated by 75% $(NH_4)_2SO_4$. The Pectinex preparation did not exhibit activity against crystalline cellulose. Xylanase activity present was mostly partitioned into the 50% $(NH_4)_2SO_4$ pellet. Ammonium sulfate fractionation resulted in a 2-fold purification of PG (Table I). DEAE-Sepharose chromatography achieved a partial resolution of the contaminating CMCase from PG (Figure 1A). This CMCase activity was virtually eliminated from the PG activity by a single pass through the SP column (Figure 1B). Small amounts of CMCase that were in the pooled PG fractions could be removed by a second pass through the SP column. The entire procedure required less than 3 days and yielded sufficient enzyme for several characterization studies.

PG Properties. Properties of PG isolated in this work are summarized in Table II. The purified PG migrated as a single band on both denaturing and isoelectric focusing gels. On the basis of the migration of protein standards, the molecular weight of PG was approximately 40 000 with a p*I* of 4.0. PG exhibited maximum activity at pH 4.5 (Figure 2A) and was relatively stable in buffers with pH ranging from 3.0 to 7.0. Only in samples at pH values above 7 did PG begin to lose activity after a 24-h preincubation (Figure 2A, inset). The temperature optimum for PG was 45 °C (Figure 2B) and remained relatively constant up to 60 °C. However, PG was unstable above 35 °C (Figure 2B, inset). Long-term hydrolytic incubations were most stable when performed at 25–30 °C. These enzyme characteristics are similar to those obtained by previous workers using *A. niger* preparations [Koller and Neukom, 1969; Thibault and Mercier, 1978; summarized in Pilnik and Rombouts (1979)]. Differences in some properties may be due to different forms of the PG.

Table II. Properties of Endopolygalacturonase from Pectinex

property	value	property	value
mol wt	40 000	temp optimum, °C	45
isoelectric point	4.0	temp stability range, °C	5–35
pH optimum	4.5	hydrolytic limit, DP	4
pH stability range	4–6		

For long-term storage PG seemed to be most stable at 4–6 °C in 20 mM acetate buffer (pH 5.0, 50 mM NaCl, 0.01% NaN_3). At a low protein concentration (0.18 mg of protein/mL) the enzyme retained 74% of its original activity after 13 months of storage at 4 °C. Freeze/thaw cycles resulted in the loss of enzyme activity (18% after four cycles), and prolonged storage at −20 °C resulted in a 75% loss of activity in 12 months.

Enzyme Activity and Specificity. The viscosity of a pectin solution decreased exponentially when incubated with purified PG, indicative of an endopolygalacturonase (Mill and Tuttobello, 1961). When PGA was exhaustively degraded with PG, the digest eluted as three peaks on an HW-40 column with the third peak coeluting with galacturonic acid. The ratios of uronic acid to reducing sugar in the peaks were 3, 2, and 1, corresponding to tri-, di-, and monosaccharide end products. Incubation with additional enzyme did not change the relative amounts of the products, indicating that a tetrasaccharide was the smallest oligosaccharide that the enzyme can use as a substrate. This concurs with the findings of Koller and Neukom (1969) and Thibault and Mercier (1978). There are reports of other forms of PG that can hydrolyze trigalacturonosyl oligosaccharides (Mill and Tuttobello, 1961; Rexova-Benkova and Markovic, 1976).

Enzyme activity was reduced in the presence of calcium concentrations greater than 0.1 mM and was completely inhibited by 5 mM $CaCl_2$. Gel formation was visible at concentrations greater than 0.1 mM Ca^{2+}. The PG activity was unaffected by Mg^{2+} concentrations up to 10 mM but was inhibited by 50 mM $MgCl_2$, while $BaCl_2$ was inhibitory at concentrations of 1 mM and higher. Thibault and Mercier (1978) found that neither divalent cations nor EDTA had an effect on enzyme activity, but they tested only one concentration of each (0.1 mM). The effect of divalent cations was probably the result of physical interaction with the substrate and not a direct effect on the enzyme. The inhibition of enzyme activity in 5 mM Ca^{2+} was partially recovered in the presence of ammonium oxalate concentrations of 1 mM and was completely recovered at 10 mM.

When PG was incubated for 19 h with a arabinan, arabinogalactan, β-glucan, cellulose, CMC, galactan, lam-

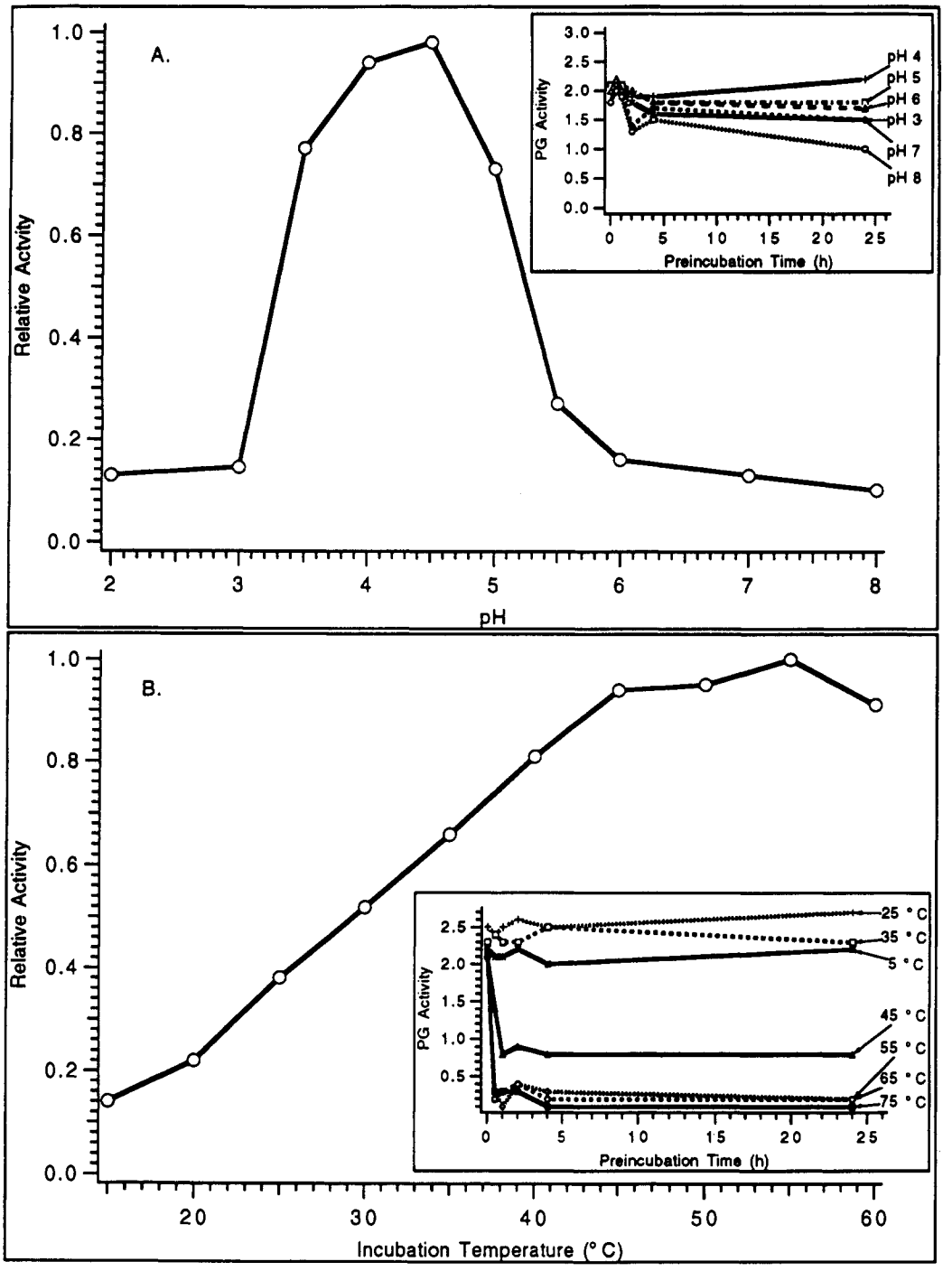

Figure 2. (A) pH optimum of purified PG; (inset) stability of PG preincubated in various pH buffer solutions for up to 24 h. (B) Temperature optimum of purified PG; (inset) temperature stability of PG preincubated at various temperatures (5–75 °C) for up to 24 h.

inarin, locust bean gum, xylan, and xyloglucan, no increase in reducing sugars could be detected. The detection limit of this assay was 26 nmol mL^{-1} (after the 19-h incubation). No glycosidase or pectin lyase activities were detected in the purified PG preparation. Methyl esterification of the acid groups on PGA resulted in decreased hydrolysis by PG. A 60% methyl esterification reduced PG activity by 40%. The inability of the enzyme to hydrolyze highly methylated pectins is in agreement with other studies (Koller and Neukom, 1969; Thibault and Mercier, 1978). Rexova-Benkova (1973) demonstrated that the binding site has four subsites which form a complex with the galacturonic acid units of PGA, and Koller and Neukom (1969) suggested that the binding site involves two carboxyl groups. If the carboxyl groups are involved in cross-linking by Ca^{2+} or are methylated, the substrate would be prevented from complexing with the enzyme. This would explain the decreased activity against methyl esterified polygalacturonase and calcium pectates. This may also explain why PG released only small amounts of pectin from alfalfa stem cell walls and pretreatment with ammonium oxalate or incubation in buffer containing ammonium oxalate increased the ability of PG to solubilize pectic polysaccharides (data not shown).

Characteristics of Buffer-Soluble Fraction from Smooth Bromegrass. Treatment of bromegrass cell walls with hot 10 mM phosphate buffer resulted in the solubilization of wall matrix polysaccharides (31 g/kg of cell wall). This initial extract was composed of 516 g/kg total uronosyl residues, indicative of pectic polysaccharides. The

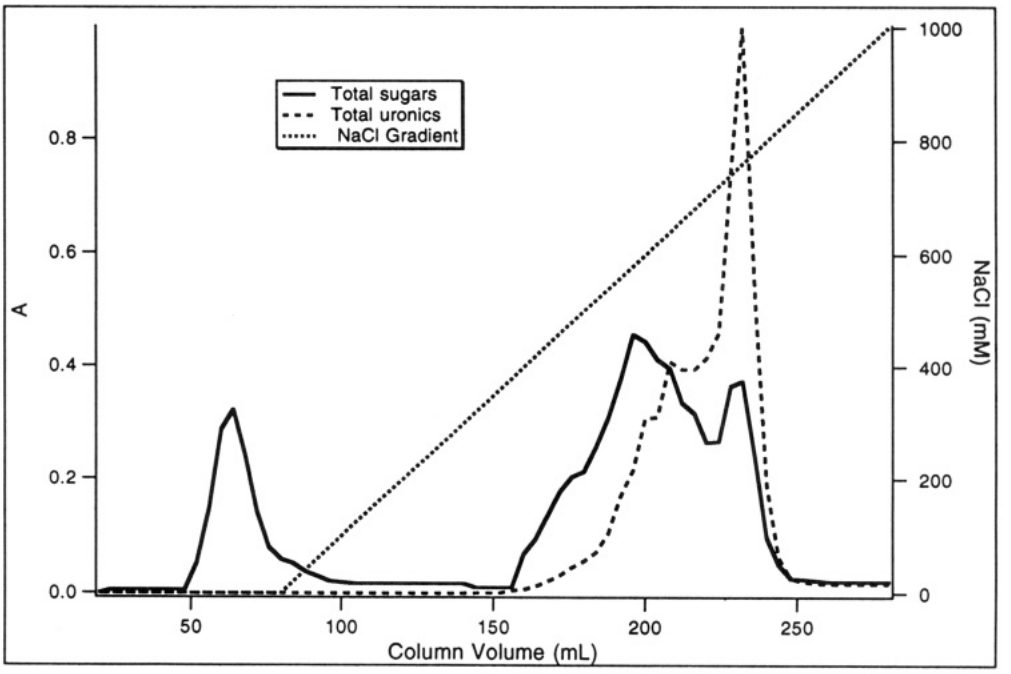

Figure 3. DEAE-Spectra Gel column profile of hot buffer extract from bromegrass cell walls. Eluted peaks were pooled into fractions as follows: F1 (48–80 mL), F2 (156–204 mL), F3 (205–220 mL), and F4 (221–248 mL). Total sugars, total neutral sugars in the fractions; total uronics, total uronic acids in the fractions.

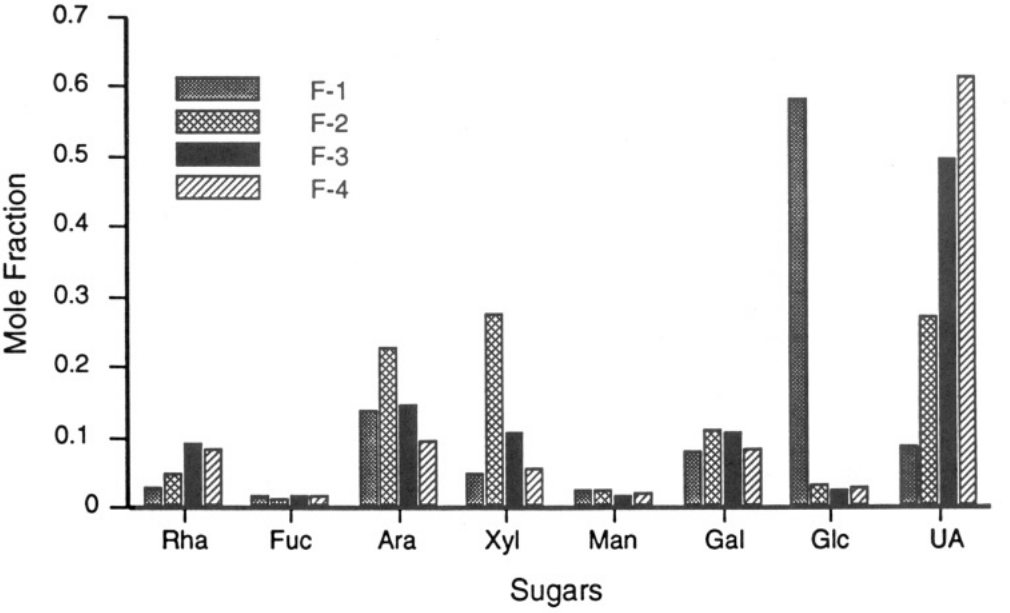

Figure 4. Carbohydrate composition of DEAE column fractions F1, F2, F3, and F4.

major neutral sugars in this fraction were arabinose, xylose, and glucose (104, 126, and 123 g/kg, respectively). Rhamnose and galactose were also present but in lesser amounts (36 and 70 g/kg, respectively). The large proportions of glucose and xylose suggested a complex mixture of rhamnogalacturonans, glucans, and xylans. Polysaccharides in the extract were resolved into neutral and charged fractions using DEAE anion-exchange chromatography.

A significant portion of the extract did not bind to the column and eluted in the void fraction of the DEAE column (Figure 3). This subfraction (F1) was composed primarily of glucose (Figure 4) but also contained arabinose, xylose, and galactose. Treatment of F1 with amylase/amyloglucosidase did not release reducing sugars, indicating that it was not solubilized starch. Partial degradation, as estimated by increased reducing equivalents, by a partially purified (1,3);(1,4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73) suggests that this fraction is primarily mixed-linked β-glucan. This fraction was not characterized in greater detail.

Polysaccharides that bound to the column eluted in partially resolved peaks (Figure 3). There was a continuous increase in uronic acid content in the eluted fractions as the NaCl concentration increased. Fraction F4 contained the highest concentrations (61.5 mol %) and eluted as a reasonably well-defined peak. Polysaccharides contained in fractions F2 and F3 were more complex. F2 contained only small amounts of uronosyl residues (27 mol %), while F3 contained nearly equal proportions of neutral sugars and uronosyl residues (49.6 mol %). Neutral sugar composition of the bound fractions varied considerably (Figure 4). Composition of F2 was primarily arabinose and xylose with smaller amounts of galactose and rham-

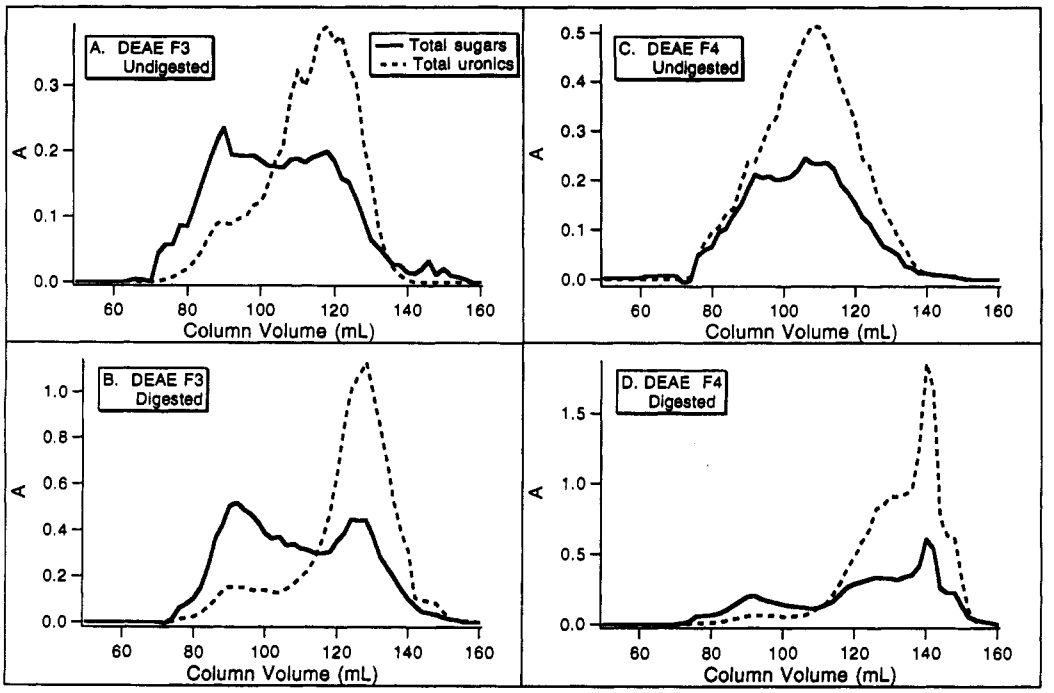

Figure 5. HW-55 column profile of DEAE column fractions F3 and F4 polysaccharides before and after treatment with purified PG. (A) DEAE F3 before PG treatment; (B) DEAE F3 after degradation by PG; (C) DEAE F4 before PG treatment; (D) DEAE F4 after degradation by PG. Eluting fractions from PG-treated DEAE F3 were pooled for analysis as follows: F3-a (72–80 mL), F3-b (81–100 mL), F3-c (101–114 mL), F3-d (115–126 mL), and F3-e (127–145 mL). Eluting fractions from PG-treated DEAE F4 were pooled for analysis as follows: F4-a (72–82 mL), F4-b (83–110 mL), F4-c (111–132 mL), F4-d (133–144 mL), and F4-e (145–156 mL). Total sugars, total neutral sugars in the fractions; total uronics, total uronic acids in the fractions.

nose. This fraction was most likely glucuronoarabinoxylans with small amounts of rhamnogalacturonans mixed in. Fractions F3 and F4 contained nearly equal proportions of rhamnose, galactose, and arabinose (Figure 4). Similar neutral sugar compositions of pectic polysaccharide fractions have been found in wall isolations from other species (DeVries et al., 1982; Thibault, 1983; Rombouts and Thibault, 1986; Massiot et al., 1988; Hatfield, 1991).

To help assess the structural features of the polysaccharides in DEAE, F3 and F4 samples were degraded with purified PG. Figure 5A,C illustrates the elution profiles, on a HW-55 column, of F3 and F4 subsamples before degradation by PG. As predicted, fraction F3 was a much more heterogeneous mixture of polysaccharides with a high molecular weight fraction that is predominantly neutral and a lower molecular weight acidic fraction. Fraction F4 is also polydisperse but would appear to be similar in composition on the basis of the ratio of total uronic acids to total neutral sugar.

Treatment of F3 and F4 with PG resulted in a shift in the elution profile to lower molecular weight products (Figure 5B,D). However, this shift was not as clearly defined in F3 as in F4. The neutral sugar composition of subfractions F3-a–e was complex (Table III). Fractions F3-a and F3-b did not appear to be degraded by PG. These fractions contained significant amounts of rhamnose as well as arabinose, xylose, and galactose. The total uronic acid content of F3-a and F3-b was low, 3.1 and 14.6 mol %, respectively. The relatively high amounts of rhamnose in these fractions (Table III, 14 and 19%) may be indicative of small amounts of highly substituted rhamnogalacturonans that are coeluting on this column with glucuronoarabinoxylans. There are reports that glucuronoxylans from alfalfa contain rhamnose substitutions (Aspinall and McGrath, 1966), but similar structural features have not been found in glucuronoarabinoxylans in grasses. Recent work has indicated that galacturonosyl acid residues of grass pectins may be esterified to the arabinosyl

Table III. Carbohydrate Composition of PG-Degraded Bromegrass Fractions DEAE F3 and F4 Separated on an HW-55 Column (Pooled Fractions Are Defined in the Legend of Figure 4)

	carbohydrate component, mol fraction							
fraction	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[a]
F3-a	0.144	0.0	0.273	0.215	0.077	0.125	0.136	0.031
F3-b	0.192	0.0	0.257	0.124	0.027	0.196	0.057	0.146
F3-c	0.150	0.021	0.212	0.129	0.023	0.178	0.048	0.238
F3-d	0.061	0.028	0.093	0.079	0.040	0.088	0.060	0.551
F3-e	0.107	0.030	0.062	0.056	0.038	0.029	0.071	0.607
F4-a	0.077	0.041	0.331	0.168	0.081	0.167	0.136	0.046
F4-b	0.173	0.018	0.186	0.061	0.057	0.254	0.061	0.191
F4-c	0.120	0.030	0.078	0.038	0.032	0.070	0.056	0.578
F4-d	0.155	ND[b]	0.037	0.022	0.025	0.025	0.058	0.677
F4-e	0.183	ND	0.049	0.038	0.027	ND	0.065	0.637

[a] UA, uronic acids. [b] ND, not detected.

residues of glucuronoarabinoxylans (Kim and Carpita, 1992). These fractions may represent a portion of these cross-linked polysaccharides that where solubilized by the hot buffer treatment of the cell wall material. Further work will be needed to completely characterize these polysaccharides and determine if there are cross-links between the two types of polysaccharides and the specific sugar residues involved. It would seem likely that F3-b and F3-c contain polysaccharides similar to the rhamnogalacturonan type I isolated from sycamore cell suspension cultures and described by McNeil et al. (1984). In both F3-b and F3-c the mole ratio of rhamnose to uronic acids is close to unity, consistent with the altering pattern of galacturonic acid with rhamnose in the rhamnogalacturonan type I molecules (McNeil et al., 1984). This would explain the limited degradation of these fractions.

The uronic acid rich fractions were degraded by the PG treatment (Figure 5A,B). After degradation, a major

component of F3-d and F3-e was uronic acid residues, comprising 55 and 60%, respectively, of each fraction. Neutral sugar composition (Table III) was primarily rhamnose, arabinose, galactose, and xylose, reflecting rhamnogalacturonans mixed with small amounts of other polysaccharides such as xylans. The shift in molecular weight was not as pronounced as in DEAE F4 (Figure 5). This could be due to neutral sugar substitution on the rhamnogalacturonan backbone and/or methyl esterification of a portion of the uronic acid groups.

Degradation of DEAE F4 was more complete, with the majority of material eluting close to the included volume (200 mL) of the HW-55 column. This fraction would represent small oligosaccharides indicative of a high degree of hydrolysis of F4 by PG. Neutral sugar composition of prominent fractions F4-d and F4-e (Table III) was primarily rhamnose, with smaller amounts of arabinose and galactose also present. Not all of the material in F4 was hydrolyzed as extensively by PG. Fraction F4-a contained large amounts of arabinose followed by xylose, galactose, and glucose. Rhamnose was also present along with relatively small amounts of uronic acid residues (5%). This most likely represents a mixture of polysaccharides, mainly arabinoxylans and arabinogalactans. Fraction F4-b contains increased amounts of rhamnose and galactose along with higher amounts of uronic acid residues (19%). This may represent highly substituted regions on a rhamnogalacturonan backbone. Similar regions have been identified on other pectic polysaccharides (DeVries et al., 1982; Thibault, 1983; Rombouts and Thibault, 1986; Hatfield, 1991) and referred to as the "hairy regions" of the molecule (DeVries et al., 1982). A characteristic of these regions in alfalfa pectic polysaccharides is a ratio of rhamnose to galacturonic acid that approaches unity (Hatfield, 1991). The mole fractions of rhamnose and uronic acids in this bromegrass isolate (F4-b) were similar. Fractions F4-c–e were largely uronic acid residues (58, 68, and 64%, respectively, Table III), with the major neutral sugar being rhamnose. Fraction F4-c, which eluted as a higher molecular weight fraction, contained slightly higher amounts of arabinose and galactose compared to F4-d and F4-e. The restricted degradation of this material may be due to neutral sugar substitution on the rhamnogalacturonan backbone or the esterification of blocks of galacturonic acid residues sufficient to limit degradation (Jarvis, 1984).

The fractionation scheme developed in this work allows for the purification of milligram quantities of PG with ease and simple laboratory equipment. Hydrolytic characteristics of this enzyme were the same as in earlier papers (minimum binding site of four unsubstituted galacturonosyl residues), allowing the extensive degradation of open galacturonosyl regions of rhamnogalacturonans. Degradation of isolated polysaccharide mixtures from smooth bromegrass with purified PG quickly revealed the structural nature of individual fractions. Fractions separated by DEAE chromatography varied in complexity of polysaccharide composition. Fraction DEAE F4 was less complex and more extensively degraded by PG, resulting in predominantly oligosaccharide formation. Fraction DEAE F3, on the other hand, contained a mixture of polysaccharides including a group of rhamnogalacturonans with limited degradation.

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