Plant Physiol. (1982) 69, 864–868
0032-0889/82/69/0864/05/$00.50/0

Studies on the Pectic Substances of Plant Cell Walls

III. DEGRADATION OF CARROT ROOT CELL WALLS BY ENDOPECTATE LYASE PURIFIED FROM *ERWINIA AROIDEAE*

Received for publication August 31, 1981 and in revised form November 19, 1981

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ABSTRACT

Pectate lyase was isolated from the cell extract of *Erwinia aroideae*. The enzyme was further purified to a high degree by a procedure involving ammonium sulfate fractionation and chromatography on CM-Sephadex C-50 and on Sephadex G-200. The enzyme attacked its substrate in an endo fashion and was more active on the sodium salt of acid-insoluble polygalacturonate or pectic acid than it was on the methoxylated pectin. The enzyme had an optimum pH at 9.3, was stimulated by calcium ions, and was completely inhibited by ethylenediaminetetraacetic acid. In addition, the reaction products showed an absorption maximum between 230 and 235 nm and reacted with thiobarbituric acid. These results indicate that the purified enzyme is an endopectate lyase. The endopectate lyase also had the ability to solubilize effectively the pectic fraction from the cell walls of carrot (*Daucus carota*) root tissue. The enzyme released 30.5% of the wall as soluble products and also liberated all of the galacturonic acid present in the walls. The total neutral sugars released by the enzyme were 10.6% of the walls, which corresponded to 71.5% of noncellulosic neutral sugars. The soluble products were separated into five fractions by DEAE-Sephadex A-50 column chromatography. Based on the analysis of sugar composition of each fraction, the pectic fraction of carrot cell wall is presented.

The pectins or pectic substances are found universally in the primary cell walls and intercellular layers of land plants. The pectic substances are composed of arabinogalactans and rhamnogalacturonans which contain neutral sugars such as arabinose, galactose, rhamnose, and xylose covalently linked to galacturonan chains. Pectate hydrolases and lyases which degrade in a random manner the α-(1 $\rightarrow$ 4) linkages between galacturonosyl moieties in the galacturonan chains are produced by many phytopathogenic microorganisms. In a number of cases, these enzymes have been shown to cause extensive cell wall breakdown and maceration, resulting in cell death of infected host tissue (1, 8, 10, 14). With those studies as a model, it is clear that the death of plant cells treated with pectolytic enzymes is due to alteration of cell walls by the enzymes (5–7, 9).

On the other hand, the structures of the pectic substances have been obtained by the analysis of polymer extracted with reagents such as EDTA, ammonium oxalate, and sodium hexametaphosphate. These procedures are time-consuming and lack sufficient sensitivity. However, endopolygalacturonases purified from Pectinol R-10 and from the culture filtrate of *Colletotrichum lindemuthianum* were able to attack isolated plant cell walls and solubilized pectic fractions as the reaction products (13, 16). Therefore, the studies on the structure of pectic substances have been made possible by availability of the purified enzymes, which are capable of degrading isolated plant cell walls. Using this method, a model has been proposed for the structure of the primary cell wall of sycamore (17).

In a previous report on the pectic substances of plant cell walls (19), we reported fractionation and characterization of polysaccharides, acidic oligosaccharides, and neutral sugars formed upon the partial acid hydrolysis of lemon pectin and pectic acid. The present paper describes the purification of an intracellular pectate lyase acting in an endo manner (endopectate lyase) from *Erwinia aroideae* and demonstrates the analysis of the components released from isolated carrot root cell walls by the action of the purified endopectate lyase.

MATERIALS AND METHODS

Preparation of Cell Walls. Carrot (*Daucus carota* L. cv. Kintoki) roots were obtained from the local gardens. The roots were sliced and homogenized in 0.1 M K-phosphate buffer (pH 7.4) containing 2.0 M NaCl and 9 mM 2-mercaptoethanol, using a mixer at 2°C. The homogenate was stirred overnight at 2°C and filtered with a sintered glass under suction; the filtrate was discarded. The residue was suspended in 90% (v/v) ethanol and heated for 20 min at 80°C to inactivate the enzymes. After the heat treatment, the cell wall preparation was washed with ethanol and acetone, until the filtrate was clear, and finally with ether.

The wall preparation was treated with pronase and α-amylase to remove protein and starch. The wall sample (about 100 mg) was suspended in 50 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 100 mg of Pronase E (Kaken Chemical Corporation) and incubated at 30°C for 18 h with shaking in the presence of a drop of toluene (3). After the treatment, the walls were collected by filtration, then washed with distilled H_2O and once with 25 mM sodium phosphate buffer (pH 6.9). The treated walls were suspended in 50 ml of an α-amylase solution and incubated at 30°C for 20 h. The α-amylase solution consisted of 0.1 ml of α-amylase (Sigma, Pancreas Type I-A) in 100 ml 25 mM sodium phosphate buffer (pH 6.9) containing 6 mM NaCl (20). After the treatment, the walls were collected by filtration and washed with distilled H_2O. For delignification, the above walls were suspended in 0.6% (w/v) $NaClO_2$ and kept for 1 h at 70°C (28). The suspension was filtered, and the final residue was washed with distilled H_2O, followed by ethanol, acetone, and ether, and dried for 2 d in a vacuum oven at 40°C. The walls were stored in a desiccator over $CaCl_2$ or P_2O_5 for further use.

Production and Purification of Enzyme. *E. aroideae* was used in this study (23). A seed culture of the organism was maintained on slants of potato extract agar containing 2% sucrose and grown at 27°C for 3 d. A culture medium which contained 0.5% pectic acid, 0.5% peptone, 0.3% meat extract, 15 mM KH_2PO_4, and 28 mM $Na_2HPO_4 \cdot 12H_2O$ (final pH adjusted to 7.2) was used for the enzyme production. Two hundred ml of the medium in 500-ml flasks were inoculated with the organisms and grown on a shaker

at 27°C for 4 d. The cells were collected by centrifugation at 8,000*g* for 20 min and washed with 10 mM Tris-HCl buffer (pH 7.0). The harvested cells (150 g, wet weight) were suspended in 300 ml 100 mM Tris-HCl buffer (pH 7.0) containing 9 mM 2-mercaptoethanol and disrupted by sonication (at 20 kHz, model T-A-4201, Marine Instruments Corporation, Japan) for 5 min at 0°C. The homogenate was centrifuged at 8,000*g* for 20 min at 2°C, and the supernatant was collected. The cell debris was suspended in 150 ml of 10 mM Tris-HCl buffer (pH 7.0) and centrifuged at 8,000*g* for 20 min at 2°C. All subsequent steps were conducted at 2 to 4°C, and all buffers used for dialysis and chromatography contained 9 mM 2-mercaptoethanol.

The supernatants obtained were combined and dialyzed for 48 h against 20 mM sodium phosphate buffer (pH 7.0), with several changes of the buffer. To the dialyzed solution solid ammonium sulfate was added slowly with stirring until 80% saturation was reached, and the turbid solution was left overnight. The resulting precipitate was collected by filtration and dissolved in 20 mM sodium phosphate buffer (pH 7.0) and dialyzed for 48 h against the same buffer with several changes of the buffer. Any precipitate which formed during dialysis was removed by centrifugation at 8,000*g* for 20 min. The supernatant (465 ml) was applied to a CM-Sephadex C-50 column (3.2 × 20 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and then washed with the same buffer. Adsorbed protein was eluted with the same buffer containing 300 mM NaCl at a flow rate of 0.4 ml/min, and 10-ml fractions were collected. The fractions having enzyme activity were collected and dialyzed for 24 h against 20 mM sodium phosphate buffer (pH 7.0), with several changes of the buffer.

The dialyzed fraction was applied to a second CM-Sephadex C-50 column (3.2 × 20 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and washed with the same buffer. The elution was carried out with a linear gradient of 0 to 300 mM NaCl at a flow rate of 0.3 ml/min. The mixing chamber and reservoir were filled with 450 ml 20 mM sodium phosphate buffer (pH 7.0) and 450 ml of the same buffer containing 300 mM NaCl, respectively. Ten-ml fractions were collected and assayed for enzyme activity and protein. The fractions having enzyme activity were collected and dialyzed overnight against 20 ml sodium phosphate buffer (pH 7.0). The dialyzed solution was concentrated to 5.0 ml by ultrafiltration using a PM-10 membrane (Amicon Corporation). The concentrate was placed on a Sephadex G-200 column (2.0 × 90 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0). Elution was carried out with the same buffer at a flow rate of 0.2 ml/min, and 5-ml fractions were collected. The major fractions of enzyme activity were collected and concentrated to 20 ml by ultrafiltration using a PM-10 membrane and dialyzed overnight against 20 mM sodium phosphate buffer (pH 7.0). The enzyme purified in this manner was then stored at −25°C for further use.

Enzyme Assay. Pectate lyase (poly-1,4-α-D-galacturonide lyase, EC 4.2.2.2) was determined by measuring the increase in *A* at 232 nm (22). The reaction mixture (2 ml) contained 0.2% (w/v) sodium salt of acid-insoluble polygalacturonate, 0.5 mM $CaCl_2$, 50 mM Tris-HCl buffer (pH 8.6), and the enzyme solution, and it was incubated at 37°C for 10 min. A blank consisting of boiled enzyme was run with each sample. After incubation, the reactions were stopped by adding 2 ml of 100 mM sodium acetate buffer (pH 3.6), and each solution was analyzed for aldehyde groups. The increase of 2.3 in the *A* at 232 nm in a 2-ml reaction mixture under the above conditions is equivalent to the release of 1 μmol of aldehyde groups. One unit of enzyme activity is defined as that amount which formed 1 μmol of aldehyde groups (unsaturated compounds) per min at pH 8.6 and 37°C.

For the viscometric method, the quantities of the components of the standard reaction mixture were increased 2.5-fold, except that the substrate was 0.4% (w/v) pectic acid. Three ml of the reaction mixture were transferred to an Ostwald viscometer, and the viscosity was read at periodic intervals.

The preparations of the sodium salt of acid-insoluble polygalacturonate and pectic acid were described previously (18).

Enzyme Treatment of Carrot Root Cell Walls. Isolated cell walls (10 mg) were incubated at 30°C with the pectate lyase in 2 ml reaction mixtures containing 0.93 units of enzyme, 0.5 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 8.6). After incubation for periodic intervals, the wall residue was separated from the reaction products by filtration on glass fiber filter paper (Toyo Roshi, Type CG-90).

For analysis by exhaustive pectate lyase treatment of the cell walls, the reaction mixture (20 ml) contained 100 mg of cell walls, 9.3 units of enzyme, 0.5 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 8.6). After the reaction mixture was incubated at 30°C for 24 h, the wall residues and reaction products were obtained utilizing the same procedure described above. The reaction products were applied to a Bio-Gel P-2 column (2.0 × 90 cm, Vo = 65 ml) and eluted with distilled H_2O. The carbohydrate fractions were collected and lyophilized.

Estimates of the carbohydrate released in reaction mixtures were made using the phenol-sulfuric acid method (12). The non-cellulosic neutral sugars in the isolated cell walls and in the wall residues and reaction products treated with pectate lyase were analyzed as their alditol acetates by a gas-liquid chromatograph (model 163, Hitachi, Ltd.) equipped with a Chromato-Processor (model 834-30, Hitachi, Ltd.) after hydrolysis with 2 N trifluoroacetic acid at 121°C for 1 h (2). Galacturonic acid in each sample was analyzed by paper chromatography with the solvent 1-butanol:acetic acid:water (4:1:2, v/v/v) and estimated by the carbazole-sulfuric acid method (11) using galacturonic acid as a standard.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (21) using BSA as a standard. The protein concentrations in the column chromatography fractions were followed by measuring the *A* of eluates at 280 nm.

RESULTS

Purification of Pectate Lyase. The enzyme was separated into two fractions (CM-I and CM-II) on CM-Sephadex C-50 column chromatography (Fig. 1). These activities may represent isozymes and have not been studied further. The major fraction, CM-II, was further purified by Sephadex G-200 gel filtration. The enzyme activity present in peak CM-II eluted as a symmetrical peak coincident with the protein peak. The data in Table I, summarizing a typical purification of the enzyme, shows that a 200-fold increase in specific activity was achieved with a recovery of about 18%. The activity of the enzyme in purified and concentrated solution was stable for at least 1 month when stored at −25°C.

Characterization of the Purified Pectate Lyase. The pH for optimal enzyme activity was found to be 9.3. After incubation of the enzyme at 4°C at various pH values (pH 3.6–5.5, acetate buffer; pH 6.0–8.0, phosphate buffer; pH 7.5–8.8, Tris-HCl buffer; pH 8.5–10.2, glycine-NaOH buffer) for 24 h, the enzyme was found to be stable in the pH range of 7.0 to 7.4 and least stable below pH 4.0. The heat stability of the enzyme was determined. Incubation at pH 8.6 for 10 min at temperatures up to 35°C did not inactivate the enzyme, but one-half of the activity was destroyed when heated to 45°C for 10 min, and all of it was destroyed when heated to 70°C.

The enzyme required cations for activity. Ca^{2+} stimulated the enzyme activity with an optimum concentration of 0.5 mM, causing a 21-fold increase over the control (no Ca^{2+} added). On the other hand, 0.5 mM Mn^{2+}, Co^{2+}, Cu^{2+}, and Na^{+} had no effect on the activity, but Hg^{2+}, Mg^{2+}, and Ba^{2+} reduced the activity by approximately 25 to 44%. The addition of 2.5 mM EDTA inhibited the activity completely.

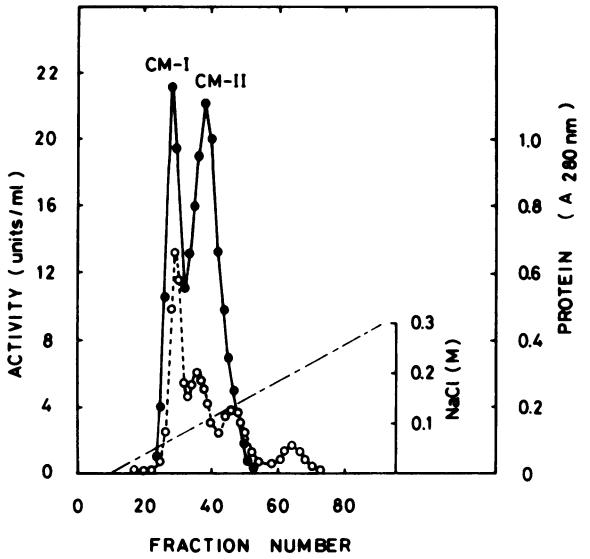

FIG. 1. Fractionation of pectate lyase by CM-Sephadex C-50 ion exchange chromatography. The enzyme solution (465 ml) obtained by ammonium sulfate fractionation was applied to a CM-Sephadex C-50 column (3.2 × 20 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 9 mM 2-mercaptoethanol. The column was eluted with a linear 0 to 0.3 M NaCl gradient prepared in the same buffer at a flow rate of 0.3 ml/min, and the eluate was collected as 10-ml fractions. Assays were for *A* at 280 nm (○) and for lyase activity (●).

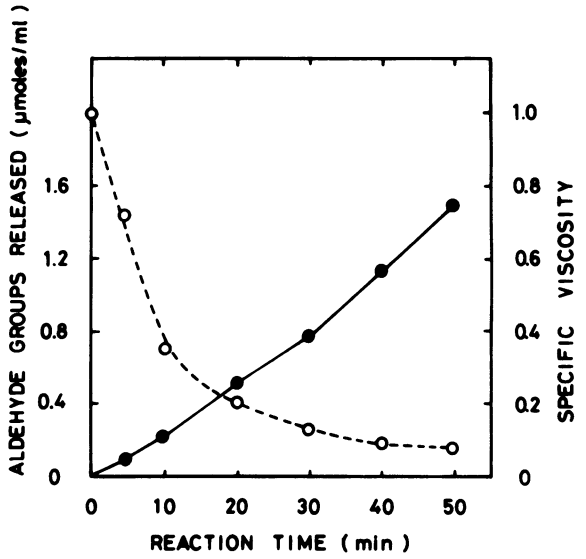

FIG. 2. Change in solution viscosity and number of aldehyde groups released from pectic acid during degradation by pectate lyase. At the times indicated, 3 ml of the reaction mixture was transferred to a viscometer, and the remaining solution was analyzed for aldehyde groups released. Enzyme solution contained 0.09 units. ○, Viscosity; ●, aldehyde groups. Each point is the mean of three experiments, each run in duplicate.

Table I. *Summary of the Purification of Pectate Lyase from Erwinia aroideae*

Step	Total Protein	Total Activity	Specific Activity	Yield
	mg	*units*	*units/mg protein*	%
Ammonium sulfate fractionation	13,252	3,536	0.27	100
CM-Sephadex C-50 batch	104	1,508	14.5	43
CM-Sephadex C-50 column chromatography CM-II	26.6	1,219	45.8	34
Sephadex G-200 gel filtration	11.4	621	54.5	18

Mode of Action and Substrate Specificity. An approach that is commonly employed for determining the mode of enzyme attack on the substrate involves measuring the viscosity and reducing power of substrate during the reaction. Figure 2 showed that the viscosity of the reaction mixture decreased 65% while 0.23 μmol of aldehyde groups were released during 10 min at 37°C. This represents cleavage of 1.42% of the glycosidic linkages in the substrate. Random cleaving enzymes (endo type) have a much greater effect on the viscosity of pectic acid than do terminal cleaving enzymes (exo type) at comparable rates of reducing group formation. Thus, the purified enzyme hydrolyzes the substrate in an endo, rather than an exo, fashion. The endo products of the enzyme action of both pectic acid and the sodium salt of acid-insoluble polygalacturonate had an absorption maximum between 230 and 235 nm, which is the same as that of 4,5-unsaturated galacturonides (24). In addition, when the products were treated with thiobarbituric acid by the method of Weissbach and Hurwitz (27), the pink color which developed had the absorption maximum around 550 nm. On paper chromatographic analysis of the reaction products with a solvent system of pyridine:ethyl acetate:acetic acid:H_2O (5:5:1:3, v/v/v/v), unsaturated mono-, di-, and trigalacturonic acid were detected.

Table II. *Degradation of Isolated Carrot Cell Walls by the Purified Pectate Lyase*

Carrot root cell walls (100 mg) were incubated for 24 h at 30°C with 9.3 units of pectate lyase in 20 ml of 50 mM Tris-HCl buffer (pH 8.6) containing 0.5 mM $CaCl_2$. After incubation, the wall residues were separated from the reaction products by filtration. Sugar concentrations in the residues and products are expressed as mg per 100 mg of the untreated control. The values are the mean ± SE of three separate experiments.

Components	Untreated Control	Wall Residue	Product
Rhamnose	2.19 ± 0.35	0.10 ± 0.02	2.04 ± 0.03
Fucose	0.09 ± 0.01	trace	0.08 ± 0.02
Arabinose	4.82 ± 0.07	0.50 ± 0.10	4.26 ± 0.12
Xylose	1.21 ± 0.12	0.82 ± 0.32	0.16 ± 0.02
Mannose	0.41 ± 0.11	0.58 ± 0.17	nil
Galactose	5.08 ± 0.18	0.79 ± 0.31	4.01 ± 0.45
Glucose (noncellulosic)	1.02 ± 0.24	2.08 ± 0.43	0.054 ± 0.004
Galacturonic acid	15.8 ± 3.7	nil	19.8 ± 0.5

The sodium salt of acid-insoluble polygalacturonate is a far better substrate for the enzyme than are pectic acid and 7.5% methoxylated pectin. The relative rates of degradation were: sodium salt of acid-insoluble polygalacturonate, 100; pectic acid, 50.3; and 7.5% methoxylated pectin, 28.5; during a 30-min reaction.

Degradation of Isolated Carrot Cell Walls by the Purified Pectate Lyase. Cell walls were incubated with the enzyme for specific periods of the time, and the reaction mixture was examined for unsaturated uronides and total carbohydrate released. When 0.93 units of the enzyme were used for 10 mg of isolated carrot root cell walls, the solubilization of the enzyme-susceptible wall components was complete in 2.5 h. Analysis of the reaction products by exhaustive enzyme treatment of the cell walls demonstrates that all of the galacturonic acid and the neutral sugars corresponding to 10.6% of the cell walls were released (Table II). The neutral sugars were rhamnose, arabinose, and galactose, in addition to smaller amounts of fucose, xylose, and glucose. Man-

nose was not released by the enzyme. There was almost no change in the neutral sugar analysis of enzyme-treated walls, but there was an apparent increase in the mannose (1.4-fold) and glucose (2-fold) detected in the wall residue of enzyme-treated walls. When the reaction products were applied to Bio-Gel P-2 column, the majority of the carbohydrate was eluted in the void volume.

Chromatography of the Soluble Products on DEAE-Sephadex A-50. The soluble products (92.8 mg) were dissolved in 3 ml 25 mM K-phosphate buffer (pH 6.0), and this solution was applied to a DEAE-Sephadex A-50 column (2.0 × 20 cm) equilibrated with the same buffer. The column was eluted with 25, 100, 250, and 500 mM K-phosphate buffer (pH 6.0) in this order. The soluble products were separated into five fractions (P-1, P-2, P-3, P-4, and P-5) by the ion exchange chromatography (Fig. 3). The five fractions were concentrated by lyophilization, and salt was removed from each fraction by Bio-Gel P-2 gel filtration. This procedure yielded 4.5 mg of fraction P-1, 16.8 mg of fraction P-2, 22.8 mg of fraction P-3, 26.1 mg of fraction P-4, and 8.3 mg of fraction P-5. Thus, the eluted fractions accounted for 84.6% of the starting materials. Fractions P-1, P-4, and P-5 showed different galacturonic acid contents and gave rhamnose, arabinose, and galactose in addition to smaller amounts of fucose, xylose, and glucose upon complete acid hydrolysis (Table III). On the other hand, fractions P-2 and P-3 were composed only of galacturonic acid.

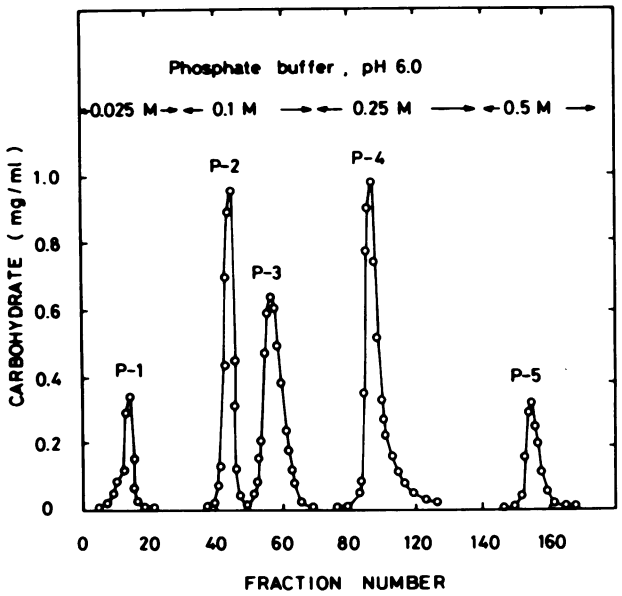

FIG. 3. DEAE-Sephadex A-50 column chromatography of the soluble products released by pectate lyase from carrot cell walls. Three ml of the soluble products (92.8 mg) released from carrot cell walls by pectate lyase was applied to a DEAE-Sephadex A-50 column (2.0 × 20 cm) equilibrated with 0.025 M K-phosphate buffer (pH 6.0) and eluted with 0.025, 0.1, 0.25, and 0.5 M of the same buffer, respectively. The fractions were 5 ml. The carbohydrate contained in each fraction was assayed by the phenol-sulfuric acid method.

Table III. *Sugar Compositions of Fractions P-1, P-4, and P-5 Separated from the Soluble Products by DEAE-Sephadex A-50 Column Chromatography*

Fractions P-1, P-4, and P-5 were concentrated by lyophilization and salt was removed from each fraction by Bio-Gel P-2 gel filtration. Sugar values are expressed as weight percentage of total sugars. The values are the mean ± SE of the three separate experiments.

Components	P-1	P-4	P-5
Rhamnose	0.88 ± 0.18	17.1 ± 3.9	8.09 ± 0.51
Fucose	0.65 ± 0.24	trace	0.56 ± 0.12
Arabinose	23.4 ± 7.8	32.2 ± 7.8	9.11 ± 0.67
Xylose	0.39 ± 0.15	trace	0.39 ± 0.12
Mannose	nil	nil	nil
Galactose	51.5 ± 14.3	27.6 ± 7.5	59.0 ± 15.7
Glucose (noncellulosic)	19.5 ± 4.6	nil	trace
Galacturonic acid	3.58 ± 0.16	22.9 ± 0.5	22.7 ± 0.5

DISCUSSION

Pectate lyase acting in an endo manner (endopectate lyase) has been detected in the culture fluid of the phytopathogenic bacteria (25). There are also reports on an intracellular endopectate lyase, *Aeromonas liquefaciens* (15), *E. carotovora* (22), and *Pseudomonas* sp. (24). However, the intracellular enzymes have been little studied, except for the above works.

We found high pectolytic lyase activity in cell extracts of *E. aroideae* under growth conditions with pectic acid as the inducer. Two peaks of enzyme activity (CM-I and CM-II) were obtained by fractionation on CM-Sephadex C-50 (Fig. 1). *E. aroideae* may produce at least two isozymes of pectolytic lyase. In this paper, the major fraction, CM-II, was isolated in a highly purified state by a procedure including fractionation with ammonium sulfate and chromatography on CM-Sephadex C-50 and on Sephadex G-200 (Table I). The purified enzyme attacked its substrate in an endo fashion, because the viscosity of pectic acid solution was rapidly reduced, while the cleavage of glycosidic bonds was small (Fig. 2). In addition, paper chromatographic analysis of the reaction products demonstrated that the initial and terminal products were predominantly unsaturated mono-, di-, and trigalacturonic acid (data not shown). On the basis of substrate specificity, pectolytic lyases are classified into two groups—pectate lyase and pectin lyase—whose classification and action pattern have been described in detail by Rexová-Benková and Markovič (25). The pectate lyase splits nonmethoxylated substrate, such as polygalacturonate or pectic acid, more rapidly than it does methoxylated pectin. The data presented indicate that the purified enzyme is clearly an endopectate lyase. Generally speaking, characteristic properties of endopectate lyase are high pH optimum and a requirement for Ca^{2+} to maintain catalytic activity (25). Furthermore, the addition of EDTA at low concentration strongly inhibited the reaction (22). These enzymic properties are consistent with those of the endopectate lyase purified from *E. aroideae*.

It is well known that plant pathogens can produce several enzymes which degrade plant cell wall constituents (9). The enzyme characteristic necessary for causing cell injury is the ability to degrade α-(1 → 4) linked galacturonic acid in a random manner (endo type) and cause tissue maceration. When the endopectate lyase described in this paper was used for cell walls isolated from carrot root tissue, the time course of release of aldehyde groups from the walls was in agreement with those of total carbohydrate released (data not shown). It is concluded that the enzyme has the ability to solubilize effectively pectic fraction within carrot cell walls. The soluble products were eluted in a void volume following slight 'tail' on Bio-Gel P-2 gel filtration. This indicates that the major products solubilized from cell walls have comparative high mol wt. Any small molecular products are scarcely produced by the action of the enzyme from cell walls, which is at variance with the degradation of polygalacturonate or pectic acid. Probably, methoxyl content or branching of any kind in galacturonosyl polymers in the products would block further degradation of the products by the enzyme action. However, Talmadge *et al.* (26) reported that the soluble products from endopolygalacturonase treatment of sycamore cell walls clearly separated into two fractions (void and included fraction) by the same procedure. The difference seems to be due to different action patterns and specificity of the two enzymes acting on the plant cell walls.

In many cases, galacturonic acid has been detected in pectic and

hemicellulosic fractions of plant cell walls (4). The purified endopectate lyase released 30.5% of the cell walls as soluble products and all of the galacturonic acid present in the walls (Table II). Data from Basham and Bateman (7) indicate that all of the galacturonic acid in tobacco pith cell walls are solubilized by endopectate lyase purified from *E. chrysanthemi* culture fluid. According to the cell wall model (17), we proposed that the galacturonic acid in the walls of carrot root, as well as that in tobacco pith, is only present in the pectic fraction.

The total neutral sugars released by the enzyme were 10.6% of carrot cell walls, which corresponded to 71.5% of noncellulosic neutral sugars. The main constituent neutral sugars associated with the hemicellulosic fraction (xylose, mannose, and glucose) were scarcely solubilized by the enzyme. Nevertheless, there was an apparent increase in the mannose and glucose detected in the wall residue of the enzyme-treated wall. This fact is in agreement with the report by Basham and Bateman (7) on degradation of tobacco cell walls. They have suggested that endopectolytic enzymes cause a massive alteration of the plant cell wall structure. However, we could not ascertain whether the speculation holds true in all endopectolytic enzymes.

Upon the ion exchange chromatography (Fig. 3), the soluble products were separated into five fractions, and the neutral sugars of fractions P-1, P-4, and P-5 were covalently attached to galacturonosyl residues (Table III). The fraction P-1 comprises a high arabinose-to-galactose ratio and very little galacturonic acid, which probably comes from a part of either the arabinan or the galactan. The fractions P-2 and P-3, which represent 50.4% of the products, contained only galacturonic acid, so that these fractions seem to be galacturonosyl polymers. The sugar composition of fraction P-4 also showed a typical rhamnogalacturonan, whereas fraction P-5 had a very high galactose content in comparison with other neutral sugars. It is conceivable that galactose-rich, acidic polysaccharide regions might be present in the pectic fraction of carrot root cell walls.

From the results obtained in this work, it is clear that the pectic substance of carrot root cell walls is made up of a large number of highly branched polymers. The endopectate lyase purified from the cell extract of *E. aroideae* is also useful for the elucidation of the existance and/or the chemical structure of pectic substances between plant species.

Acknowledgment—We are very grateful to Professor Alan D. Elbein, of the University of Texas, for his careful reading and improvement of our manuscript.

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