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Exopolygalacturonase from Suspension Cultures of *Marchantia polymorpha*[1]

ITS PRESENCE AND INVOLVEMENT IN PECTIC POLYSACCHARIDE DEGRADATION

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ABSTRACT

Polygalacturonase was isolated from cell suspension cultures of a thalloid liverwort, *Marchantia polymorpha*. The enzyme in the 'buffer-soluble' protein fraction was dialyzed at pH 5.2 and further purified 91-fold by a combination of chromatographic techniques including CM-Sephadex, Sephacryl S-200, DEAE-Sephadex, and Sephadex G-200. The purified enzyme had an optimum activity in the pH range at 3.6 to 3.8 and molecular weight of 76,000 daltons, and its activity was not stimulated by cations. The enzyme was identified as an exohydrolase from viscometric data and chromatographic analysis of the reaction products.

The polysaccharides extracted from the *Marchantia* cell walls with 2% (w/v) Na hexametaphosphate solution were separated into two fractions, neutral polysaccharides (fraction P-N) and acidic polysaccharides (fraction P-A) by a DEAE-Sephadex column. The fraction P-N was not susceptible to the purified exopolygalacturonase, whereas fraction P-A was partially degraded. This resulted in hydrolysis of 19.5% of the glycosyl linkages of fraction P-A with the release of galacturonic acids. The specific activity of exopolygalacturonase increased during the growth cycle.

Pectin or pectic substances are found universally in the primary cell wall and middle lamella of young growing plant tissues, and consist of three distinct polysaccharides: an arabinan, a galactan, and a rhamnogalacturonan (2). The pectic depolymerases catalyzing cleavage of the α-1,4-linkages between galacturonosyl residues at the interior of the pectin backbone are found in a wide variety of plant tissues. One of these enzymes, polygalacturonase, is classified into two groups on the basis of enzymic action pattern toward its substrate—a random cleaving enzyme (endopolygalacturonase) and a terminal cleaving enzyme (exopolygalacturonase)—whose classification and some properties have been described in detail by Rexová-Benková and Markovič (24). In general, most of the polygalacturonases found in higher plants are endohydrolases and many studies on the enzymes have been limited to the enzymology of fruit cell wall breakdown during ripening. One of the reasons is that endopolygalacturonase is not detectable in unripe fruits, but the activity appears when the fruits begin to soften and then increases sharply as ripening proceeds. The degradation of cell walls which results in fruit ripening is an exceedingly complex process. Although it is now generally accepted that endopolygalacturonase plays a crucial role in converting protopectin in cell walls to soluble forms during this process. On the other hand, Pressey and Avants demonstrated that endo- and exopolygalacturonases were both present in peach (19) and pear (21) fruit homogenates. They suggested that the endopolygalacturonase plays a role in solubilization of pectic fraction within cell walls, and then the exopolygalacturonase catalyzes further degradation of solubilized pectic fraction. Thus, it is conceivable that exopolygalacturonase coupled with endopolygalacturonase action may provide for the complete hydrolysis of pectin. However, polygalacturonases from carrot roots (9), cucumbers (20), oat seedlings (22), apple cortical tissues (3), and potato tubers (23) exhibited only exohydrolase activity. Inasmuch as exopolygalacturonase acting alone may be unable to degrade completely the pectic fraction in cell walls, the function of this enzyme *in situ* has been difficult to explain. The physiological role of this enzyme is still an open question. Furthermore, the studies mentioned above on polygalacturonases have been restricted to flowering plants which seem to have highly branched pectic polymers in the cell walls. It is important to determine if the polygalacturonase is universally distributed in the plant kingdom including representative taxa in mosses, liverworts, and ferns, as well as flowering plants. To gain a deeper understanding of the distribution and/or the role of polygalacturonases in plant kingdom, we decided to isolate the enzyme from a liverwort.

In this paper, we describe the purification and some properties of exopolygalacturonase from suspension cultures of a thalloid liverwort, *Marchantia polymorpha*. To elucidate the role of the enzyme, we have also attempted to analyze the pectic polysaccharides extracted from *Marchantia* cell walls and examine the enzyme action toward the pectic polysaccharides.

MATERIALS AND METHODS

Cell Culture. *Marchantia polymorpha* L. cell line, denoted as HYH-2F, in suspension culture was routinely subcultured every week using MSK-2 medium (11). Stock cultures were maintained by inoculating 15 ml of cell suspension into 500 ml of fresh medium in 700-ml flat oblong flasks with an inner thickness of 28 mm. For experiments, 7-d-old cells from stock culture were suspended in the medium at a concentration of approximately 0.2 mg cell dry weight/ml. The flask was illuminated perpendicular to the flat surface with a bank of fluorescent lamps (FL 40 w; Matushita Electric Work, Japan) at an irradiance of approxi-

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mately 90 $\mu E/m^2 \cdot s^{-1}$ (400–700 nm, 8000 lux), being determined with a Lambda Photometer (LI-185A). Aeration and agitation to keep the culture in suspension were done by passing air containing 1% CO_2 through the culture (50–100 ml/min) at 25°C.

Extraction Procedure for Enzyme. The procedure used to extract polygalacturonase is slightly modified from that of the previous report (12). Lyophilized cells (approximately 2 g) were suspended in 50 ml of 0.1 M K-phosphate (pH 7.0) containing 9 mM 2-mercaptoethanol and disrupted by sonication (at 20 kHz; model 5202 PZT, Ohtake Works, Japan) for 10 min at 0°C. All subsequent steps were conducted at 2 to 4°C and all buffers used contained 9 mM 2-mercaptoethanol. The homogenate was centrifuged at 8000*g* for 20 min, and the supernatant was collected and referred to as 'buffer-soluble' protein. To remove the phenolic matter included in the fraction, the fraction was passed through a Sephadex G-25 column (10 × 30 cm) equilibrated with 0.1 M K-phosphate (pH 7.0) and protein fractions were pooled. On the other hand, the cell wall materials were precipitated from the homogenate by centrifugation, washed thoroughly with 0.1 M K-phosphate (pH 7.0), and suspended in 50 ml of the same buffer containing 2 M NaCl and stirred overnight. The suspension was centrifuged at 8000*g* for 20 min and the supernatant was collected, referred to as 'NaCl-soluble' protein.

The buffer- and NaCl-soluble protein fractions were dialyzed for 72 h against 20 mM Na acetate buffer (pH 5.2) containing 10 mM NaCl with several changes of the buffer, respectively. Any greenish precipitate that formed during dialysis was removed by centrifugation at 8000*g* for 20 min. Each dialyzed pale greenish enzyme solution was concentrated to around 5 ml using an Amicon ultrafiltration device (PM-10 membrane; Amicon Corp.).

Purification of Polygalacturonase. All procedures were carried out at 2 to 4°C and all buffers used for dialysis and chromatography contained 9 mM 2-mercaptoethanol and 10 mM NaCl. The buffer-soluble protein fraction obtained from 183 g of lyophilized cells (15-d-old) was removed from the phenolic matter, dialyzed, and centrifuged as described above. The supernatant was concentrated to 100 ml using a Bio-Engineering ultrafiltration device (G-05 T membrane; Bio-Engineering Corp., Japan). The concentrate was dialyzed overnight against 50 mM Na acetate buffer (pH 6.0), and then passed through a CM-Sephadex C-50 ion-exchange column (3.0 × 40 cm) equilibrated with the same buffer. The polygalacturonase-active eluant (910 ml) from the CM-Sephadex C-50 column was concentrated to approximately 30 ml using an Amicon ultrafiltration device.

The concentrate was further subjected to gel filtration on a Sephacryl S-200 column (2.8 × 90 cm) equilibrated with 50 mM Na acetate buffer (pH 6.0). Polygalacturonase was filtered with the Na acetate buffer at a flow rate of 0.3 ml/min, and 10-ml fractions were collected. The fractions most active in polygalacturonase were pooled, concentrated to approximately 100 ml using an Amicon ultrafiltration device, and dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.6 at 4°C) with several changes of the buffer. The dialyzed solution was applied to a DEAE-Sephadex A-50 ion-exchange column (3.2 × 40 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.6). After washing with 450 ml of the same buffer, polygalacturonase was eluted with a 1.2 L 0.01 to 0.5 M NaCl gradient in the Tris-HCl buffer, and 15-ml fractions were collected. Active fractions were pooled and dialyzed overnight against 50 mM Na acetate buffer (pH 6.0). The dialyzed solution was concentrated to 4 ml using an Amicon ultrafiltration device. The concentrate was then subjected to gel filtration on a Sephadex G-200 column (2.0 × 90 cm) equilibrated with 50 mM Na acetate buffer (pH 6.0). 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 pooled and concentrated to 5 ml using an Amicon ultrafiltration device and dialyzed overnight against 50 mM Na acetate buffer (pH 6.0). The enzyme purified in this manner was then stored at −20°C for further use.

Preparation of Cell Walls. Lyophilized cells of 15-d-old cultures were suspended in 0.1 M K-phosphate (pH 7.0) containing 2 M NaCl and 9 mM 2-mercaptoethanol, and disrupted by sonication for 10 min at 0°C. The homogenate was centrifuged at 3000*g* for 20 min at 2°C and the pellet was collected. The pellet was suspended in 90% (v/v) ethanol and heated for 20 min at 80°C to inactivate the enzymes. After the heat treatment, the alcohol-insoluble residue of crude cell walls was collected and washed with ethanol, acetone, and finally with ether. The crude cell wall preparation was treated with Pronase E (Kaken Chemical Corp., Japan) and α-amylase (Sigma Chemical Co., pancreas type I-A) to remove protein and starch (13). The treated cell walls were stored in a desiccator over $CaCl_2$ for further use.

Extraction of Pectic Polysaccharides. The starch-free cell wall preparation (7.8 g dry weight) was dispersed in 250 ml of 2% (w/v) Na hexametaphosphate solution (pH 3.7), refluxed for 4 h at 80 ~ 90°C, and centrifuged at 8000*g* for 15 min. The supernatant was collected and the residue was subjected to further extraction. The process was repeated until all extractable pectic polysaccharides were solubilized. All extracts were combined, dialyzed for 48 h against distilled H_2O, and lyophilized.

Enzyme Assay. Polygalacturonase (poly-1,4-α-D-galacturonide galacturonohydrolase, EC 3.2.1.67) was measured by following the production of reducing groups from acid-insoluble polygalacturonate as described previously (12). The acid-insoluble polygalacturonate used as the substrate in this assay was prepared by acid hydrolysis of commercial lemon pectin (14). One unit of enzyme activity is defined as that amount which forms 1 μmol of galacturonic acid from 0.2% (w/v) substrate per h at pH 4.0 and 37°C. Protein concentration was measured by the method of Lowry *et al.* (16) using BSA as a standard. The protein concentration in the column chromatography fractions was followed by measuring the *A* at 280 nm.

Carbohydrate Analysis. Neutral glycosyl residues of cell walls and pectic polysaccharides were analyzed by GLC as their alditol acetate derivatives according to the technique of Albersheim *et al.* (1). GLC was performed with a model 163 Hitachi instrument (Hitachi Ltd., Japan) fitted with dual columns (0.3 × 120 cm) containing 0.2% polyethylene glycol succinate, 0.2% polyethylene glycol adipate, and 0.4% silicone XF-1150 coated on Gas-Chrom P, equipped with a flame ionization detector. Separation was achieved with a 40 ml/min carrier gas (N_2) flow and 1°C/min temperature program from 120 to 185°C. The integration of chromatographic peak areas was calculated with a model 834-30 Hitachi Chromato-Processor. Total carbohydrate and uronic acids were estimated by the methods of phenol-H_2SO_4 (8) and carbazole-H_2SO_4 (4), respectively, using galacturonic acid as a standard.

RESULTS

Extraction of Polygalacturonase. The polygalacturonases in plant tissues are usually bound to cell wall materials via ionic interactions; consequently, the enzymes are solubilized with salt solutions of high ionic strength such as NaCl or LiCl. This property permits the removal of the constituents solubilized with a low ionic strength buffer prior to extraction of polygalacturonase and hence facilitates the enzyme purification. To evaluate extractability of polygalacturonase in *Marchantia*, therefore, the enzyme activity in 15-d-old cultures corresponding to the end of the exponential phase of the growth cycle was assayed after extraction successively by 0.1 M K-phosphate and buffer plus 2 M NaCl. The buffer-soluble protein contained 74.7% of the total protein solubilized (8.9 ± 1.7 mg protein/g cell dry weight), and

all the polygalacturonase activity (5.8 ± 1.4 units/g cell dry weight·h^{-1}) was detected in the buffer-soluble protein fraction. The enzyme activity in the cell wall materials was measured after extraction with buffer plus NaCl, but no activity was detected. Based on the results, the polygalacturonase for other experiments in this paper was extracted using a low ionic strength buffer, 0.1 M K-phosphate (pH 7.0), free from NaCl.

Purification and Physical Characteristics of Polygalacturonase. The chromatography on cation-exchange resins such as CM-cellulose, CM-Sephadex, and CM-Sepharose provides a useful fractionation for polygalacturonase from plant tissues because the enzymes bind specifically to those exchangers at acidic pH. However, the polygalacturonase from *Marchantia* could not be bound to CM-Sephadex C-50 resin; approximately 53% of the protein, including the polygalacturonase activity, was passed through a CM-Sephadex C-50 column equilibrated with 50 mM Na acetate buffer (pH 6.0). The enzyme was then fractionated by the ion-exchange chromatography on DEAE-Sephadex A-50. A representative elution profile is illustrated in Figure 1. This step removed a pale greenish impurity in the enzyme solution. After completion of the gradient, the column was eluted with 20 mM Tris-HCl buffer (pH 7.6) containing 1 M NaCl and 9 mM 2-mercaptoethanol, but no activity was detected. Thus, the polygalacturonase-rich fractions 33 through 42 were combined, concentrated, and further fractionated by gel filtration on Sephadex G-200. The enzyme activity eluted as a symmetrical peak coincident with the protein peak. The observations obtained from the various chromatographic analysis made apparent that polygalacturonase in *Marchantia* existed in only one form. The data in Table I, summarizing a typical purification of the enzyme, show that a 91-fold increase in specific activity was achieved with a recovery of approximately 9%.

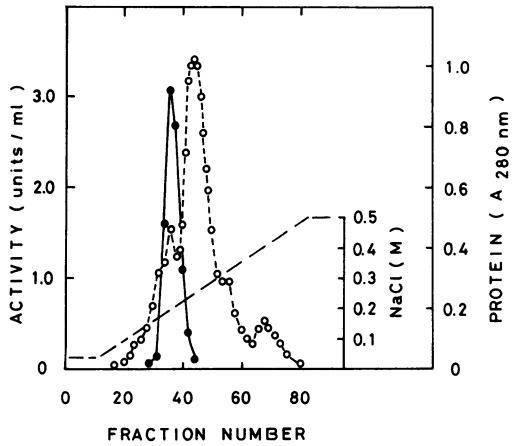

FIG. 1. Purification of polygalacturonase by a DEAE-Sephadex A-50 ion-exchange column chromatography. The enzyme solution obtained by Sephacryl S-200 gel filtration was applied to a DEAE-Sephadex A-50 column (3.2 × 40 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.6 at 4°C) containing 9 mM 2-mercaptoethanol and 10 mM NaCl. The column was washed with the Tris-HCl buffer, and then eluted with a linear 0.01 to 0.5 M NaCl gradient in 20 mM Tris-HCl buffer (pH 7.6) at a flow rate of 0.3 ml/min. The eluate was collected as 15-ml fractions, and assayed for *A* at 280 nm (O) and for polygalacturonase activity (●).

Table I. *Summary of the Purification of Polygalacturonase from Cell Suspension Cultures of Marchantia polymorpha*

Step	Total Protein	Total Activity	Specific Activity	Yield
	mg	*units*	*units/mg protein*	*%*
Buffer-soluble protein fraction	45,047	2,352	0.052	100
Dialysis at pH 5.2	1,287	1,098	0.85	47
CM-Sephadex C-50 batch	673	697	1.04	30
Sephacryl S-200 gel filtration	302	401	1.33	17
DEAE-Sephadex A-50 column chromatography	103	243	2.36	10
Sephadex G-200 gel filtration	46.2	219	4.74	9.3

Some properties of the purified polygalacturonase were tested. The activity of the enzyme in purified and concentrated solution was stable for at least 1 month when stored at −20°C. Incubation at pH 4.0 for 30 min at temperatures up to 55°C did not inactivate the enzyme, but one-half of the activity was destroyed when heated to 65°C for 30 min, and all of it when heated to 80°C. The pH-dependent enzyme activity was examined in McIlvaine (17) or Na citrate buffers, and the pH optimum was found to be 3.6 to 3.8. After incubation of the enzyme at 30°C in McIlvaine buffer at various pH values for 20 h, the enzyme was found to be comparatively stable in the pH range of 3.0 to 6.5. The effect of various cations on the activity was examined. Salt free acid-insoluble polygalacturonate and enzyme (0.1 unit) made free from NaCl by dialysis were incubated in the presence of 0.5 or 1.0 mM cations, and the activity was measured. The following cations and EDTA at concentrations of 0.5 or 1.0 mM had no effect on the activity: Mg^{2+}, Co^{2+}, Ca^{2+}, Na^{+}, K^{+}, and EDTA (data not shown). However, 0.5 mM Hg^{2+}, Mn^{2+}, Ba^{2+}, and Cu^{2+} reduced activity by 20 ~ 53%. The mol wt of the purified enzyme was estimated by Sephadex G-150 gel filtration, by plotting the relative elution volume *versus* the log of the mol wt of the standard proteins as follows: γ-globulin, serum albumin, ovalbumin, and chymotrypsinogen. Mol wt was estimated to be 76,000 ± 3,000 D.

Mode of Action and Substrate Specificity. An approach that is commonly employed for determining the mode of polygalacturonase attack on substrate involves measuring the viscosity and reducing power of the substrate during the reaction. Figure 2 shows that a 16.3% loss in specific viscosity occurred when 0.34 μmol of reducing groups was released during a 2-h incubation at 37°C. In fact, this represents cleavage of 3.4% of the glycosidic linkages in the substrate while the viscosity decreases

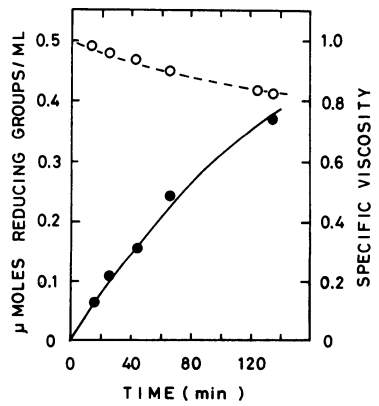

FIG. 2. Change in solution viscosity and number of reducing groups of pectic acid during degradation by the purified polygalacturonase. The reaction mixture for each assay contained 0.39 unit of enzyme and 0.4% (w/v) pectic acid in a total volume of 5.0 ml. At the times indicated, 3.0 ml of the reaction mixture was transferred to a viscometer and the remaining solution was assayed for reducing groups. (O), Viscosity; (●), reducing groups. Each point is the mean of three experiments, each run in duplicate.

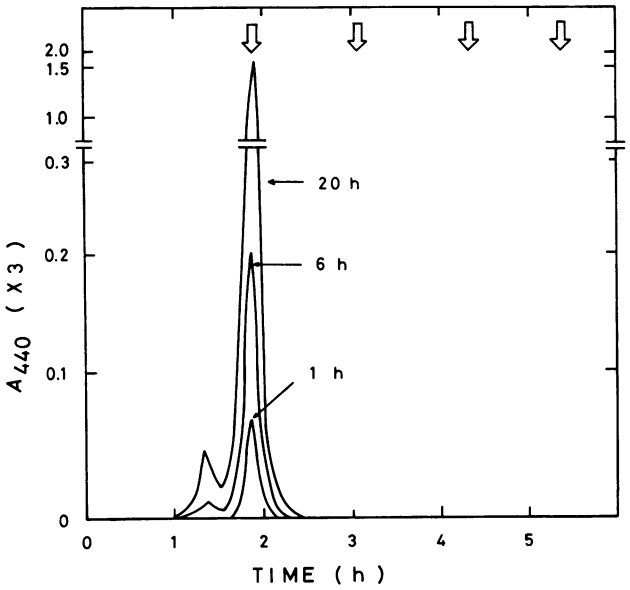

FIG. 3. Liquid chromatogram of the reaction products by polygalacturonase. Reaction mixtures were incubated for 1, 6, and 20 h at 37°C, and assayed by a liquid chromatograph. The arrow marks represent, from left to right, galacturonic acid, digalacturonic acid, trigalacturonic acid, and tetragalacturonic acid.

Table II. *Glycosyl Compositions of Marchantia Pectic Polysaccharides and DEAE-Sephadex A-50-Fractionated Pectic Polysaccharides*

The pectic polysaccharides were extracted from *Marchantia* cell walls with 2% (w/v) Na hexametaphosphate solution for 4 h at 80 ~ 90°C. Fractions P-N and P-A were fractionated from the pectic polysaccharides by DEAE-Sephadex A-50. These polysaccharides were hydrolyzed for 1 h at 121°C on 2 N trifluoroacetic acid. After hydrolysis, the noncellulosic neutral glycosyl residues were analyzed as their alditol acetates by gas-liquid chromatography, and galacturonosyl residue was analyzed by paper chromatography and estimated by the carbazole-H_2SO_4 method using galacturonic acid as a standard. The glycosyl contents are expressed as weight percentage of total sugars. Values are the mean ± SE of three separate experiments.

Glycosyl Residue	Pectic Polysaccharides	DEAE-Sephadex Column Fraction	
		P-N	P-A
Rhamnose	14.0 ± 0.9	Trace	14.5 ± 1.0
Fucose	1.16 ± 0.05	None	1.07 ± 0.07
Arabinose	16.2 ± 0.4	4.53 ± 0.19	16.5 ± 1.1
Xylose	4.50 ± 0.06	17.4 ± 1.3	1.21 ± 0.17
Mannose	0.58 ± 0.02	1.77 ± 0.22	0.099 ± 0.004
Galactose	28.2 ± 0.4	22.2 ± 1.3	25.2 ± 0.6
Glucose	11.5 ± 0.3	45.1 ± 4.6	1.61 ± 0.16
Galacturonic acid	23.9 ± 1.2	9.01 ± 0.51	39.9 ± 3.6

very slowly, suggesting a terminal hydrolysis of the substrate. In addition, paper chromatographic analysis of the reaction products with a solvent system of 1-butanol:acetic acid:H_2O (4:1:2, v/v/v) demonstrated that galacturonic acid was the sole product. This was also shown by liquid chromatography (15), which yielded an intense peak of galacturonic acid. Moreover, the amount of galacturonic acid increased with the reaction time (Fig. 3). An unknown peak was detected on the chromatogram during a 20-h reaction, but it was small in comparison with the galacturonic acid content produced by the enzyme action. On the other hand, the reaction products by exhaustive enzyme action of the polygalacturonate or pectic acid were not detected in the thiobarbituric acid assay (28) and showed no *A* at 230 nm

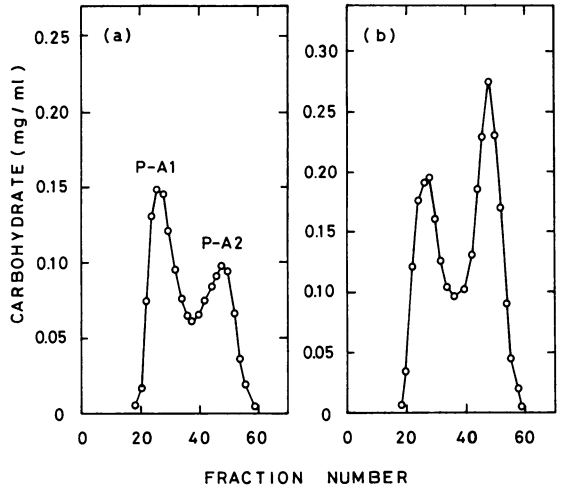

FIG. 4. Gel filtration chromatography of the acidic polysaccharides (fraction P-A) on Bio-Gel A-5m before and after the enzyme treatment. (a), Fraction P-A (9.0-mg sugar content) was dissolved in 1.0 ml 50 mM Na acetate buffer (pH 5.2) containing 20 mM EDTA and subjected to gel filtration on a Bio-Gel A-5m column (1.8 × 90 cm; V_0 = 66 ml, V_i = 189 ml). The polysaccharide was filtered with the same buffer at a flow rate of 0.2 ml/min, and 3-ml fractions were collected. The carbohydrate contained in each fraction was assayed by the phenol-H_2SO_4 method. (b), Fraction P-A (13.3-mg sugar content) was incubated with 2.47 units of the purified exopolygalacturonase in 50 mM Na acetate buffer (pH 4.0) at 30°C. A drop of toluene was added to suppress microbial growth. After the exhaustive enzyme treatment, the reaction was stopped by heating at 100°C for 5 min, and the solution was dialyzed against 50 mM Na acetate buffer (pH 5.2) containing 20 mM EDTA. The dialysate was subjected to gel filtration on Bio-Gel A-5m by the same procedure as noted above.

(18). These results rule out the possibility that the purified enzyme preparation contains an exo- and endopectate lyase.

The sodium salt of acid-insoluble polygalacturonate (14) is a far better substrate for the enzyme than are pectic acid and 7.5% methoxylated pectin. The relative rates of degradation were acid-insoluble polygalacturonate, 100, pectic acid, 55.4 ± 7.3, and pectin, 18.2 ± 4.1 (means of four replicates ± SE), during a 1-h incubation.

The data presented indicate that the purified enzyme attacks a given substrate molecule, continuously releasing a monomer unit, *i.e.* the enzyme is clearly an exopolygalacturonase.

Fractionation and Sugar Analysis of Pectic Polysaccharides by a Combination of Chromatography with DEAE-Sephadex A-50 and Bio-Gel A-5m. The pectic substances have been studied in liverworts in a few cases (5, 7, 10, 25, 26). Therefore, the problem has been raised as to whether native polysaccharides degraded by the exopolygalacturonase are present in *Marchantia* cell walls. The starch-free cell walls and polysaccharides extracted with Na hexametaphosphate were analyzed by the alditol acetate derivatives for glycosyl residues upon complete acid hydrolysis. The noncellulosic neutral glycosyl content of cell walls was 24.9% and uronic acid was 6.6%. All the uronosyl residues in the cell walls appear to be galacturonic acid; glucuronic acid was not detected by paper chromatography, although the sensitivity of the method is not high. The extracted polysaccharides were composed mainly of rhamnose, arabinose, galactose, and galacturonic acid (Table II), this composition being typical for pectic substances and associated sugars (6).

The pectic polysaccharides (300-mg sugar content) were dissolved in 50 ml of 10 mM K-phosphate (pH 6.0), and this solution was applied to a DEAE-Sephadex A-50 ion-exchange column (4.0 × 40 cm) equilibrated with the same buffer. The column

was then washed with 2 volumes of the K-phosphate. The neutral polysaccharides which passed directly through the column were pooled, dialyzed against distilled H_2O, and concentrated by lyophilization. The acidic polysaccharides which adsorbed to the column were eluted with 0.5 M K-phosphate (pH 6.0), pooled, dialyzed against distilled H_2O, and lyophilized. Each polysaccharide fraction is referred to hereafter as P-N and P-A, respectively. This procedure yielded 55 mg of fraction P-N and 120 mg of fraction P-A. The glycosyl compositions of these fractions are given in Table II. The polysaccharides in fractions P-N and P-A were dissolved in 1.0 ml of 50 mM Na acetate buffer (pH 5.2) containing 20 mM EDTA and subjected to gel filtration on a Bio-Gel A-5m column (1.8 × 90 cm; V_0 = 66 ml, Vi = 189 ml) equilibrated with the same buffer. The fraction P-N was eluted from this column as a single carbohydrate-containing peak, whereas fraction P-A was further fractionated into two peaks P-A1 and P-A2, illustrated in Figure 4a. Conceivably, the pectic polysaccharides in *Marchantia* would be made up of a large number of highly branched heterogeneous polymers. This is similar to the pectic substances of higher plant (2). The chemical structure of pectic polysaccharides in *Marchantia* will be reported in more detail in a separate publication.

Activity of the Purified Exopolygalacturonase toward Pectic Polysaccharides. The extracted pectic polysaccharides (516-μg sugar content) were incubated at 30°C with the purified exopolygalacturonase (0.52 unit) in 50 mM Na acetate buffer (pH 4.0). After incubation for periodic intervals, the reducing groups released by the enzyme action increased with the incubation time. The reaction mixture from the enzyme treatment was clearly separated into two fractions, void and included fractions, by Bio-Gel P-2 gel filtration chromatography with 50 mM Na acetate buffer (pH 5.2) containing 20 mM EDTA (profile not shown). The void and included materials were identified with the undegraded pectic polysaccharides and galacturonic acid, respectively.

To examine the regions of the pectic polysaccharides degraded by the enzyme action, fractions P-N and P-A fractionated by a DEAE-Sephadex ion-exchange column were reacted with the enzyme as described above. After exhaustive enzyme action, fraction P-N was not susceptible to the enzyme, whereas fraction P-A was partially degraded. This resulted in hydrolysis of 19.5% of the glycosyl linkages of fraction P-A with the release of galacturonic acids. It is possible that the regions of galacturonosyl residues methyl-esterified in fraction P-A block further degradation by the enzyme. The fraction P-A was de-esterified by treatment with 0.01 N NaOH for 90 min at 0°C (14) prior to the enzyme action, but the extent of hydrolysis by the enzyme was the same as with untreated fraction P-A.

The changes of the molecular size and glycosyl compositions of fraction P-A were tested on this enzyme degradation. After the galacturonic acids produced by the enzyme action were removed by dialysis against 50 mM Na acetate buffer (pH 5.2) containing 20 mM EDTA, the enzyme-treated fraction P-A was subjected to gel filtration on Bio-Gel A-5m by the same procedure as noted earlier. The polysaccharide was separated into two peaks, and the elution profile was almost identical with that of the native fraction P-A. Nevertheless, the degradation affected the ratio of fractions P-A1 to P-A2 in the polysaccharides (Fig. 4b). The profiles indicate that fraction P-A upon treatment with the enzyme did not exhibit, compared to native fraction P-A, a remarkably reduced molecular size of the polymers. The fractions for each peak were pooled, dialyzed against distilled H_2O, and analyzed for the glycosyl compositions upon complete acid hydrolysis (Table III). The data show that fraction P-A1 is richer in neutral glycosyl residues, while fraction P-A2 is richer in galacturonosyl residues. The galacturonosyl residues contained in both fractions, in particular fraction P-A2, were markedly reduced by the enzyme action, but there was an increase in the ratios of arabinose and galactose residues detected in fraction P-A2 by treatment with the enzyme.

Table III. *Glycosyl Compositions of Fractions P-A1 and P-A2 Fractionated from the Acidic Polysaccharides by Bio-Gel A-5m Gel Filtration Chromatography before and after the Enzyme Treatment*

Fractions P-A1 and P-A2 were dialyzed against distilled H_2O, and concentrated by lyophilization. Glycosyl compositions of each fraction were as in Table II.

Glycosyl Residue	Native Fractions		Enzyme-Treated Fractions	
	P-A1	P-A2	P-A1	P-A2
Rhamnose	20.6 ± 4.2	9.51 ± 0.98	22.8 ± 4.2	8.09 ± 1.14
Fucose	Trace	Trace	0.58 ± 0.25	1.21 ± 0.05
Arabinose	22.2 ± 6.7	7.69 ± 0.95	26.3 ± 6.8	25.2 ± 0.7
Xylose	1.55 ± 0.20	Trace	1.52 ± 0.65	0.91 ± 0.02
Mannose	Trace	Trace	0.10 ± 0.03	0.53 ± 0.12
Galactose	28.4 ± 4.6	12.4 ± 0.2	30.7 ± 7.0	35.7 ± 1.3
Glucose	1.04 ± 0.27	0.08 ± 0.02	1.12 ± 0.07	1.35 ± 0.01
Galacturonic acid	26.1 ± 1.3	70.3 ± 3.3	16.9 ± 0.5	27.0 ± 0.5

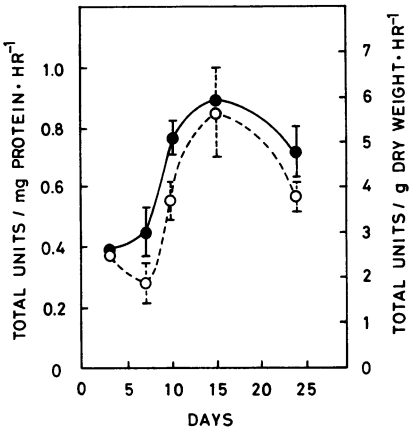

FIG. 5. Changes in the activity of exopolygalacturonase during culture growth. The polygalacturonase in the buffer-soluble protein fraction was assayed at 50 mM Na acetate buffer (pH 4.0) and 37°C. (●), Total units/mg protein · h^{-1}; (○), total units/g dry weight · h^{-1}. Each point is the mean of two separate experiments, each run in duplicate. Vertical bars represent ± SE.

Change in Exopolygalacturonase Activity during the Cell Culture. The exopolygalacturonase activity in *Marchantia* was measured at all stages and changed significantly during the growth cycle (Fig. 5). The value of enzyme activity per cell dry weight decreased slightly whereas the specific activity remained low during the initial 7 d of culture after transfer to fresh nutrient medium. After the lag phase, however, the enzyme activity increased rapidly until d 15, which corresponded to exponential phase during cell growth previously reported by Katoh *et al.* (11), and then declined again. This represented a 3-fold enhancement of its specific activity.

DISCUSSION

Inasmuch as the studies on the pectolytic enzymes thus far focused on flowering plants based on fruit ripening, the polygalacturonase in lower plant kingdom, such as mosses and liverworts, has received very little attention. Our experiments show that the polygalacturonase could be highly purified from cell suspension cultures of a thalloid liverwort, *M. polymorpha*, and that the enzyme was a terminal cleaving (exo-) hydrolase from

viscometric data and liquid chromatographic analysis of reaction products (Figs. 2 and 3). Furthermore, the enzyme was capable of *in vitro* degradation of pectic polysaccharides extracted from *Marchantia* cell wall preparation.

The polygalacturonases in homogenates of plant tissues have a strong affinity for cell wall materials, whereas the enzyme in cell cultures of *Marchantia* was extracted completely with a low ionic strength buffer. This is similar to the earlier observation on the exopolygalacturonase in carrot cell cultures (12). Presently, we speculate that the notably different extractability of the enzymes between intact plants and cell cultures is due to a change in cell wall structure rather than in enzymic properties.

The chromatography on anion- and cation-exchanger has been often used for the fractionation of polygalacturonase at acidic side (24). In our experiments, the fractionation during anion-exchange chromatography at the acidic side resulted in a substantial loss of enzyme activity, so that the specific activity did not rise. The ion-exchange chromatography on DEAE-Sephadex A-50 at pH 7.6 was successful to ensure stability of enzyme (Fig. 1). The behavior of exopolygalacturonase upon anion- and cation-exchange chromatography indicates that the enzyme is a rather acidic protein. The purified polygalacturonase, separated by polyacrylamide disc gel electrophoresis, was stained with the periodic acid-Schiff reagent according to the method of Zacharius *et al.* (29), suggesting a glycoprotein nature of the enzyme (data not shown). The pH optimum of exopolygalacturonases which are found in higher plant tissues (24) and cell cultures (12) lies between 4.5 and 5.5, regardless of substrate size, and the enzymes display minimal activity below pH 4.0. The exopolygalacturonase from *Marchantia* exhibited activity with an optimum at pH 3.6 to 3.8, and its activity is stable up to pH 3.0. Thus, the enzymic property differs greatly from that of exopolygalacturonases reported earlier. The mol wt was estimated to be 76,000 D by chromatography on a Sephadex G-150 column. The value is higher than that of exopolygalacturonases (36,000 to 68,000) from carrot cell cultures (12), peach (19), cucumber (20), and potato (23). The reasons for this discrepancy are probably due to the glycoprotein nature of the enzyme, and/or to the different origin of the enzyme.

There has yet to appear a detail assessment of the physiological function of exopolygalacturonase in plant tissues. Thus, because the polygalacturonase from *Marchantia* cell cultures exhibited only exohydrolase activity, it is important to learn the role of the enzyme. The exopolygalacturonase in this paper, as well as that in carrot suspension cultures (unpublished data), could not degrade the respective starch-free cell walls. Data from Bartley (3) demonstrate that the exopolygalacturonase in apple cortical tissues released uronic acid residues from its cell wall preparation. According to the current knowledge of plant cell wall structure (2, 6), pectic substances are made up of a large number of highly branched and partially methyl-esterified polymers, in which the terminal monomers of galacturonosyl residues seem to be covalently and/or ionically attached to other wall components. The exopolygalacturonase catalyzes the splitting of α-(1 $\rightarrow$ 4) glycosidic bonds of nonmethoxylated galacturonosyl polymer, starting at the nonreducing end, so that the enzyme is not able to attack directly the cell walls in a general way. On the other hand, Wallner and Nevins (27) reported on the dramatic change of β-glucosidase and β-galactosidase activities in rose suspension cultures during an 18-d culture cycle. They proposed that these enzymes serve to mediate the degradation of cell walls. Because of these considerations, presumably the apple exopolygalacturonase or cell wall preparation was contaminated with some wall-degrading enzymes such as β-glycosidases or hemicellulases.

The acidic polysaccharides (fraction P-A), fractionated by a DEAE-Sephadex column of pectic polysaccharides extracted from *Marchantia* cell walls, were susceptible to the purified exopolygalacturonase. The degradation corresponded to cleavage of 13.4% of the glycosidic linkage of total extracted pectic fraction. The change of the acidic polysaccharides was then examined by Bio-Gel A-5m gel filtration chromatography before and after the enzyme action. After the enzyme treatment, the enzyme-susceptible polymer in native fraction P-A1 was partially degraded following a release of galacturonic acids and converted into a polymer relatively enriched in neutral glycosyl residues such as arabinose and galactose. On the other hand, the native fraction P-A2 was scarcely degraded, and the molecular size of this polymer did not change by the enzyme action. The degraded polymer in fraction P-A1 caused the slight reduction in the molecular size and was eluted with the undegraded fraction P-A2 volume on Bio-Gel A-5m gel filtration (Fig. 4 and Table III). The degradation did not proceed to completion after exhaustive enzyme action, and so the degree of polymerization distribution estimated by Bio-Gel A-5m gel filtration did not clearly change. If the polysaccharides were de-esterified by treatment with base prior to the enzyme action, the extent of cleavage was the same as with untreated polysaccharides. Thus, the degradation was not affected by the methyl esters of galacturonosyl residues within the polysaccharides. It is conceivable that the neutral sugars and/or side chains, dispersed along the length of pectic backbone, would block further degradation by the enzyme action.

Katoh *et al.* (11) previously demonstrated with cell suspension cultures of *Marchantia* that the cell mass (mg dry weight/ml culture) increased exponentially from the start of the culture until d 13, and the values became almost steady till d 28. The average cell volumes (μm^3/cell) also increased from the exponential phase to the first stationary phase through cell growth (data not shown). In view of the relation between cell mass and growth, particularly noteworthy is the fact that a rise in exopolygalacturonase activity occurred in parallel with the cell growth until d 15, and then the activity declined rapidly (Fig. 5). It is possible that exopolygalacturonase, together with other wall-degrading enzymes, serves in a partial degradation of pectic polysaccharides for remodeling and/or loosening of cell wall structure throughout cell growth.

Tissue cultures are useful tools for various biochemical studies, but the cell cultures may differ from the intact plants in amount and kind of pectic substances. In this paper, large quantities of a homogeneous intact plant of the liverwort were obtained with difficulty; thus, cell suspension cultures were used. Studies are further planned to investigate the difference in the pectic substances between the two.

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