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# Polysaccharide-degrading Enzymes Are Unable to Attack Plant Cell Walls without Prior Action by a "Wall-modifying Enzyme"[1]

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## ABSTRACT

**A study of the degradation of plant cell walls by the mixture of enzymes present in Pectinol R-10 is described. A "wall-modifying enzyme" has been purified from this mixture by a combination of diethylaminoethyl cellulose, Bio Gel P-100, and carboxymethyl cellulose chromatography. Treatment of cell walls with the "wall-modifying enzyme" is shown to be a necessary prerequisite to wall degradation catalyzed by a mixture of polysaccharide-degrading enzymes prepared from Pectinol R-10 or by an α-galactosidase secreted by the pathogenic fungus *Colletotrichum lindemuthianum*. The action of the "wall-modifying enzyme" on cell walls is shown to result in both a release of water-soluble, 70% ethanol-insoluble polymers and an alteration of the residual cell wall. A purified preparation of the "wall-modifying enzyme" is unable to degrade a wide variety of polysaccharide, glycoside, and peptide substrates. However, the purified preparation of wall-modifying enzyme has a limited ability to degrade polygalacturonic acid. The fact that polygalacturonic acid inhibits the ability of the "wall-modifying enzyme" to affect cell walls suggests that the "wall-modifying enzyme" may be responsible for the limited polygalacturonic acid-degrading activity present in the purified preparation. The importance of a wall-modifying enzyme in developmental processes and in pathogenesis is discussed.**

The plant cell wall is a complex structure of polymers which surrounds the cell and is separated from the cytoplasm by the cell membrane. Young cell walls are composed primarily of polysaccharide with a small amount of protein (25), while mature cell walls, in addition to these components, contain lignin (37). The cell wall has several functions. The wall counteracts physically the osmotic pressure resulting from the cell contents (30). The wall provides the rigid structure necessary to hold the plant erect (3, 4). The wall is the major component of conducting vessels (3, 4). The wall acts as an intercellular cement (3, 4). And the wall plays a complex role in plant pathogenesis by presenting a physical barrier to the infecting pathogen, by acting as a source of nutrient for the pathogen, and by controlling the production of degradative enzymes by the pathogen (5, 7).

Since the cell wall fulfills such important structural roles, and since the cell wall is quite rigid (30), morphological changes which occur during the normal course of development are accompanied by partial degradation of the cell wall. For example, growth by elongation occurs, at least in part, by weakening of the cell wall (13, 30, 32, 33), abscission results following degradation of wall polymers responsible for intercellular connection (1, 19, 36, 44), and pathogenesis is often accompanied by wall degradation (5).

Although degradation of the plant cell wall is of central importance in the biological phenomena mentioned, little is known about the reactions requisite to cell wall degradation. Studies of developmental processes, such as growth by cell elongation, have generally involved detection of gross compositional changes in in the cell wall resulting from treatment with hormones or metabolic inhibitors (14, 18, 42). Lee *et al.* (29) have demonstrated that isolated plant cell walls are capable of autolysis, but have neither isolated nor identified the enzymes responsible for the degradative process. In contrast, workers who have isolated and identified particular polysaccharide degrading enzymes, thought to be important in wall degradation, have used model substrates for identification purposes and have failed to demonstrate that these enzymes are able to catalyze degradation of cell walls (1, 16).

A large variety of polysaccharide degrading enzymes has been isolated from higher plants (2, 16), fungi (5), and bacteria (5, 21, 43). The effect of such degradative enzymes on unmodified substrate has been investigated in great detail only in the case of the cellulases secreted by the mold *Trichoderma viride* (40, 41). When grown in culture with cotton fibers, *T. viride* secretes two distinct enzymes or classes of enzymes important in the degradation of cotton. These two enzymes have been termed the $C_x$ and the $C_1$ components by Reese (38). The $C_x$ components catalyze hydolysis of model substrates such as carboxymethyl cellulose but are unable to directly effect the more highly ordered forms of cellulose such as unmodified cotton fibers. The $C_1$ component will not catalyze hydrolysis of model substrates, but its action on the more highly ordered forms of cellulose is a prerequisite for degradation of these polymers by the $C_x$ enzymes. It is significant that the $C_x$ enzymes, which specifically catalyze hydrolysis of soluble β-1,4-linked glucans, are unable to degrade unaltered cotton even though this natural product is approximately 90% β-1,4-linked glucan (15). Furthermore, the $C_1$ component, which is of primary importance in the degradation of cotton,

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cannot be detected with model substrates, nor can its action be detected in the absence of $C_x$ enzymes.

A cellulase has been isolated from the culture filtrate of the wood degrading fungus *Polyporus schweinitzii* Fr. (9, 24). This enzyme, like the $C_x$ components from *T. viride*, cannot degrade unmodified cotton fibers but can degrade regenerated cellulose.

The results of our investigation demonstrate that the occurrence of polysaccharide-degrading enzymes, which cannot degrade naturally occurring cell wall polymer substrates, is a general phenomenon. The organization of the cell wall, as may be the case with unmodified cotton fibers, prevents degradation of the wall polysaccharides by these enzymes. Degradation of structural components basic to the organization of the cell wall appears to be a prerequisite to the degradation of many cell wall polymers. There is some evidence to support this theory. Albersheim *et al.* (5) have observed that when the bean pathogen *Colletotrichum lindemuthianum* is grown in culture an α-galactosidase is excreted into the culture medium. They have found that, although this α-galactosidase is capable of catalyzing hydrolysis of *p*-nitrophenyl-α-galactoside, it is not capable of degrading the galactose containing polymers of the plant cell wall. However, pretreatment of the cell walls with an unidentified factor, produced early in the growth cycle of this fungus, enables the α-galactosidase to remove galactose from the cell wall.

The present paper describes the isolation and partial characterization of a cell wall degradation factor from a commercially available mixture of degradative enzymes. Treatment of cell walls with this factor is a necessary prerequisite for degradation of the walls by a wide variety of polysaccharide degrading enzymes.

## MATERIALS AND METHODS

### SUBSTRATES

**Phosphatase and Glycoside Substrates.** *p*-Nitrophenyl-α-galactoside, *p*-nitrophenyl-β-galactoside, *p*-nitrophenyl-β-glucoside, and *p*-nitrophenyl-β-xyloside were purchased from Koch-Light Laboratories. *p*-Nitrophenyl-α-L-arabinofuranoside was prepared by Dr. J. B. Jurale of this laboratory using methods described elsewhere (20). *p*-Nitrophenyl phosphate disodium tetrahydrate was purchased from Sigma Chemical Company.

**Polysaccharide Substrates.** Cellulose gum (CMC 7MP) was purchased from Hercules. Pectin (National Formulary) and polygalacturonic acid were the gift of Sunkist Growers. Araban, mannan (ex yeast), and galactan (ex Larch) were purchased from Koch Light Laboratories. Xylan was purchased from City Chemical Corporation and laminarin (water insoluble) from Pierce Chemical Company.

**Esterase and Peptidase Substrates.** α-*N*-Benzoyl-L-arginine ethyl ester was purchased from Sigma Chemical Company. The reagents, L-tyrosine β-napthylamide, L-lysyl β-napthylamide, L-seryl β-napthylamide, and L-hydroxyproline β-napthylamide, were purchased from Nutritional Biochemicals Corporation.

### PREPARATION OF CELL WALLS

Cell walls were prepared from the hypocotyls of *Phaseolus vulgaris* L. var. Red Kidney. Seeds were surface-sterilized by soaking for 7 min in 0.5% sodium hypochlorite (10% Clorox) solution. The seeds were planted in trays containing moist vermiculite and watered daily. Plants were grown in a Percival (model PGW 108) growth chamber with a photoperiod of 14 hr of light followed by 10 hr of dark. The temperature was maintained at 20 C during the light period and 16 C during the dark period. Plants were harvested at the end of the dark period 10 days after planting. Bean hypocotyls were removed with a razor blade, frozen in liquid nitrogen, and ground in a mortar in the presence of liquid nitrogen. The resulting powder was suspended in 10 volumes (v/w) of 0.1 M potassium phosphate (pH 7.0) and ground for 5 min in a Waring Blendor. The insoluble portion was collected on a coarse sintered glass funnel, washed five times with 10 volumes (v/w) of the suspension buffer, and resuspended in the same buffer. The material was ground again in the Waring Blendor, collected, washed with buffer, and finally washed with distilled water. The insoluble material (cell walls) was suspended in 10 volumes (v/w) of chloroform-methanol (1:1) and ground in a Waring Blendor. The cell walls were collected on a coarse sintered glass funnel, washed five times with 10 volumes (v/w) of the chloroform-methanol mixture, washed with acetone, and dried in a culture dish at room temperature. All extractions were carried out at 2 C.

### ENZYME ASSAYS

**Glycosidase and Phosphatase Assays.** α-Galactosidase, β-galactosidase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, and phosphatase activities were assayed by measuring the increase in absorbance resulting from the release of *p*-nitrophenol from the respective *p*-nitrophenyl substrate. In a typical assay, 2 ml of solution containing 1 mg of the appropriate *p*-nitrophenyl substrate was mixed with 1 ml of enzyme solution and incubated at 25 C. The reactions were terminated by the addition of 1 ml of 1.0 M ammonium chloride-ammonium hydroxide buffer (pH 9.6). The absorbance was determined at 400 nm with a Gilford model 2000 spectrophotometer.

**Polysaccharide-degrading Enzyme Assays.** Pectin hydrolase, polygalacturonic acid hydrolase, cellulase, mannanase, galactanase, arabanase, and xylanase activities were assayed by measuring the release of reducing sugars from a 0.1% (w/v) solution of the appropriate polysaccharide in 50 mM sodium acetate (pH 5.2). Reaction mixtures were incubated at 25 C. Reactions were terminated by addition of 3 ml of dinitrosalicylic acid reagent, and reducing sugars determined by the method of Miller (35). The absorbance was measured with a Klett-Summerson colorimeter fitted with filter No. 54. The amount of reducing sugar present was calculated relative to a standard concentration curve prepared with glucose. This method was used to measure between 5 and 400 μg of reducing sugar.

Degradation of laminarin was assayed by measuring the release of reducing sugars from the water-insoluble polysaccharide. Ten milligrams of laminarin were placed in a 13- × 100-mm test tube, and 3 ml of the enzyme solution to be assayed were added. At the end of the reaction period, laminarin was removed by centrifugation, and the reducing sugar released into the supernatant liquid was measured by the method of Miller (35).

Pectin and polygalacturonic acid lyase activities were assayed by measuring the increase in absorbance at 235 nm as previously described (3). Substrate consisted of a 0.5% (w/v) solution of the appropriate polysaccharide in 50 mM sodium acetate (pH 5.2). Reactions were incubated at 25 C.

Cellulase, pectinase, and polygalacturonase activities were also assayed by measuring the enzyme catalyzed decrease in viscosity of a 2% (w/v) solution of the appropriate polysaccharide in 50 mM sodium acetate (pH 5.2). Viscosity measurements were made with either a Wells-Brookfield micro viscometer or a Gilmont model V-2200 viscometer.

Polygalacturonic acid-degrading activities, whether assayed by increase in reducing groups, increase in absorbance, or reduction of viscosity, were measured three ways: in the presence of 160 μM calcium chloride, in the presence of 160 μM ethylenediaminetetraacetic acid, and in the absence of both.

**Esterase and Peptidase Assays.** Esterase activity was determined by measuring the increase in absorbance at 253 nm which is produced upon hydrolysis of α-*N*-benzoyl-L-arginine ethylester

(39). The substrate was prepared at a concentration of 1 mM in 50 mM sodium acetate (pH 5.2).

Peptidase activity was determined by measuring the release of β-napthylamine from the β-napthylamide derivatives of hydroxyproline, lysine, serine, and tyrosine. Substrate solutions were prepared at a concentration of 1 mM in 50 mM sodium acetate (pH 5.2). In a typical assay, 1 ml of enzyme was added to 2 ml of substrate solution and was incubated at 25 C. The reactions were terminated by the addition of an equal volume of 10% trichloroacetic acid. The resulting precipitate was removed by centrifugation, and β-napthylamine in the supernatant liquid was estimated by a modified Bratton Marshall method (22). This method detects between 5 and 50 μg of β-naphthylamine.

**Pectinesterase Assay.** Pectinesterase activity was measured at 25 C by titration of carboxyl groups liberated in 0.5% pectin (National Formulary) prepared in 50 mM sodium acetate (pH 5.2) containing 50 mM sodium chloride. In a typical reaction, 4 ml of enzyme solution were added to 8 ml of substrate solution. The pH was maintained by addition of 20 mM sodium hydroxide with a Radiometer TTT 1c titrator (28).

**Cell Wall Degradation Assays.** Two milliliters of enzyme solution were incubated with approximately 10 mg of cell wall in a stoppered 13- × 100-mm test tube. Incubation was carried out on a rotary shaker at 25 C. At the end of the reaction period, absolute ethanol was added to a concentration of 70% (v/w). The insoluble material was pelleted by centrifugation, and the supernatant liquid was removed with a pipette. The pellet was then washed with 2 ml of 70% ethanol (v/v). The supernatant liquid and the wash were combined, and the solvent was evaporated under a stream of filtered air at 55 C. The reducing sugars present in the residue were measured as described above. Amounts of reducing sugar were recorded relative to the glucose standard concentration curve as microgram glucose equivalent. The enzyme catalyzed release of reducing sugars soluble in 70% ethanol (v/v) was considered the criterion for cell wall degradation.

In some cases cell walls were treated with enzyme, the 70% ethanol-solubilized material was removed, and the remaining cell walls were subjected to hydrolysis in 2 N trifluoroacetic acid. The hydrolysate was then subjected to analysis by gas-liquid chromatography (8).

The WME[3] by itself is unable to catalyze the release of measurable reducing sugars from the cell wall. Therefore, WME was routinely assayed in the presence of a mixture of glycosidases obtained by partial fractionation of Pectinol R-10. This fraction of Pectinol R-10 is unable to degrade the cell wall, but does degrade cell walls which have been altered by treatment with WME. All quantitative assays of WME were carried out under conditions where WME was the limiting component in the assay mixture.

To detect the larger molecular weight water-soluble fragments released from the cell wall by WME, reactions were terminated by filtration on a coarse sintered glass funnel. The collected cell walls were washed with buffer. The filtrate and wash were combined and evaporated to dryness. Uronic acids were estimated directly by the modified carbazole method (12). Reducing sugars were measured after hydrolysis of the polysaccharide fragments in 2 N trifluoroacetic acid for 1 hr at 121 C. The neutral sugars liberated by hydrolysis were identified by gas-liquid chromatography (8).

## PREPARATION OF COLUMNS

**Diethylaminoethyl Cellulose.** DEAE-cellulose columns were prepared from Cellex-D (Bio Rad Laboratories) with an exchange capacity of 0.7 meq/g. Twelve grams of DEAE-cellulose were washed four times with 1-liter portions of either 50 mM (column 1) or 10 mM (column 2) sodium acetate (pH 5.2). Fine particles were removed by decanting and a 3- × 15-cm column prepared.

**Bio Gel P-100.** Bio Gel P-100 (Bio Rad Laboratories), 50 to 100 mesh, was stirred for 24 hr at 25 C in 50 mM sodium acetate (pH 5.2) which contained 0.1 M sodium chloride. A column 1.3 × 80 cm was prepared (column 3).

**Carboxymethyl Cellulose.** A CM-cellulose column was prepared from Cellex-CM (Bio Rad Laboratories) with an exchange capacity of 0.7 meq/g. Six grams of the CM-cellulose were washed five times with 4-liter portions of 2 mM potassium phosphate (pH 7.0). Fine particles were removed by decanting and a column 1.3 × 15 cm was prepared (column 4).

## ENZYME PREPARATIONS

**Pectinol R-10.** Pectinol R-10, which is a mixture of enzymes produced by *Aspergillus niger*, was the generous gift of Rohm and Haas, Philadelphia. Another source of this enzyme is Pectinol 59-L, a liquid preparation containing all of the enzymes found in Pectinol R-10.

**Peptidases.** Pronase (Grade B, Cal Biochem) was prepared in 50 mM sodium acetate (pH 5.2) at a concentration of 10 μg/ml. Trypsin (bovine pancreas, Grade B, Cal Biochem) was prepared at a concentration of 1 mg/ml in 50 mM tris (pH 8.1). Ficin (fig latex, Grade C, Cal Biochem) was prepared at a concentration of 1 mg/ml in 50 mM sodium acetate (pH 4.5) containing 25 mM cysteine.

**α-Galactosidase.** An impure α-galactosidase was prepared from the culture filtrate of the bean pathogen *Colletotrichum lindemuthianum*. The fungus was grown in shake culture with 2% galactose as the sole carbon source. The growth medium has been described previously (17). Twelve days after inoculation of the medium, mycelia were removed from the culture medium by filtration through a coarse sintered glass funnel. The culture filtrate was concentrated by lyophilization. Small molecules were removed by passing the concentrate through a 2- × 25-cm Bio Gel P-2 column prepared in 50 mM sodium acetate (pH 5.2). The protein in the void volume of this column was used as an enzyme source.

**D-III Polysaccharide-degrading Enzymes.** The mixture of polysaccharide-degrading enzymes used in the assay of WME was prepared by dissolving Pectinol R-10 in 50 mM sodium acetate (pH 5.2) at a concentration of 2 mg/ml. Five milliliters of this solution were placed on a DEAE-cellulose column (2.2 x 7 cm) prepared in the same buffer. The column was eluted with 3 void volumes of 50 mM sodium acetate (pH 5.2) followed by elution with 3 void volumes of the same buffer containing 50 mM sodium chloride. These two fractions were discarded. The column was then eluted with the same buffer containing 0.3 M sodium chloride. The eluent was collected as a single 50-ml fraction and stored at 2 C. This fraction, designated D-III, contains a large variety of glycosidases (Table II) but is unable by itself to degrade cell walls (Table III).

## HYDROLYSIS OF CELL WALL PROTEIN

Ten-day-old Red Kidney bean hypocotyl cell walls at a concentration of 10 mg/ml were subjected to hydrolysis in saturated barium hydroxide as described by Lamport (26). Following hydrolysis, the mixture was cooled to room temperature and the insoluble material was removed by centrifugation. The supernatant solution was neutralized with 1 M sulfuric acid, and the insoluble barium sulfate was removed by centrifugation. The supernatant solution was concentrated by lyophilization, and the resultant residue was dissolved in cold 0.1 M acetic acid. This

[3] Abbreviations: BAEE: α-*N*-benzoyl-L-arginine ethyl ester; CM-: carboxymethyl; DEAE-: diethylaminoethyl; WME: wall-modifying enzyme.

solution was fractionated by chromatography on a 1.5- × 110-cm Gel P-2 (Bio Rad Laboratories) column prepared in 0.1 M acetic acid. Two-milliliter fractions were collected. Hydroxyproline was estimated by the method of Bergman and Loxley (11). The absorbance of the solution was measured using a Klett-Summerson colorimeter fitted with filter No. 54. The assay was used to detect between 5 and 60 μg of hydroxyproline.

The fractionation procedure resulted in two clearly separated peaks of hydroxyproline: a higher molecular weight component, 4-*O*-(arabinosyl)$_x$-hydroxyproline, and a lower molecular weight component, free hydroxyproline.

### PROTEIN DETERMINATIONS

Protein concentrations were determined by the method of Lowry *et al.* (31). Absorbance of the solution was determined with a Klett-Summerson colorimeter fitted with filter No. 69. Protein concentrations were calculated relative to a standard prepared from crystallized bovine plasma albumin (Armour Pharmaceutical).

## RESULTS

**Purification of Wall Modifying Enzyme.** Crude enzyme is prepared by dissolving Pectinol R-10 in 50 mM sodium acetate (pH 5.2) at a concentration of 50 mg/ml. The solution is stirred for 5 min at 2 C, and the insoluble material is removed by centrifugation. The supernatant liquid is placed on a DEAE-cellulose column (column 1) and eluted with 50 mM sodium acetate (pH 5.2). The eluent is collected as a single 150-ml fraction (fraction D-I). To minimize degradation of the column material by cellulases present in the crude enzyme preparation, column 1 is eluted at a high rate (5–10 ml/min).

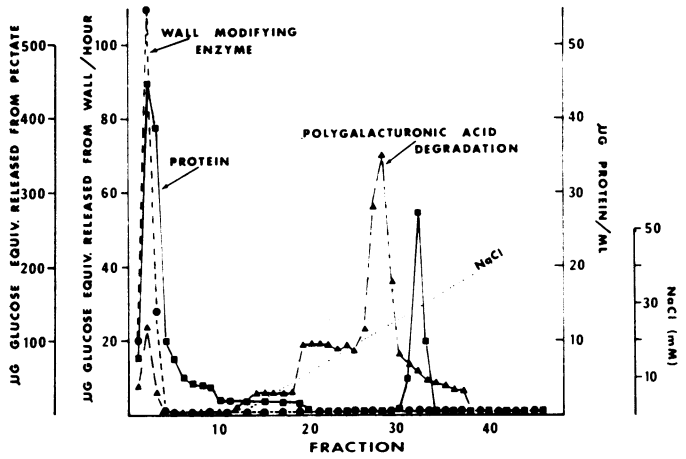


FIG. 1. Fractionation of D-I by DEAE-cellulose chromatography. A solution of Pectinol R-10 was prepared at a concentration of 2 mg/ml in 50 mM sodium acetate (pH 5.2). Five milliliters of this solution were used to prepare D-I as described in Table II. Fraction D-I was concentrated by lyophilization, dissolved in 5 ml of distilled water, and dialyzed for 6 hr against 10 mM sodium acetate (pH 5.2). Three milliliters of the nondialyzable material were placed on a DEAE-cellulose column (1.2 × 12 cm) prepared in 10 mM sodium acetate. The column was first eluted with 10 void volumes of 10 mM sodium acetate (pH 5.2), followed by elution with a linear 0 to 0.5 M sodium chloride gradient prepared in the same buffer. Seven-milliliter fractions were collected. Polygalacturonic acid degradation assays were carried out by adding 1 ml of each fraction to 2 ml of 0.1% (w/v) polygalacturonic acid in 50 mM sodium acetate (pH 5.2). The reactions were stopped after 5 min by the addition of the dinitrosalicyclic acid reagent. The amount of reducing sugars produced was determined as described in "Materials and Methods." Reaction mixtures for cell wall degradation assays were prepared by adding 1 ml of the fraction, 0.5 ml of D-III, and 0.5 ml of 50 mM sodium acetate (pH 5.2) to 10 mg of cell walls. Reactions were incubated for 2 hr on a rotary shaker at 25 C and wall degradation was measured as described in "Materials and Methods." The fractions (No. 1–9) containing WME activity were assayed for pectate lyase activity as described in "Materials and Methods." No pectate lyase activity could be detected in these fractions; the data are not included in this figure. Protein was determined by the method of Lowry *et al.* (31).

Table I. *Fractionation of Pectinol R-10 by DEAE-cellulose Chromatography (Model Substrate Assays)*

The crude enzyme solution was prepared by dissolving Pectinol R-10 at a concentration of 2 mg/ml in 50 mM sodium acetate (pH 5.2). The mixture was stirred for 5 min at 2 C, and the insoluble material was removed by centrifugation. Five milliliters of this solution were placed on a DEAE-cellulose column (1.2 × 9 cm) which had been prepared in sodium acetate buffer. The column was eluted with a stepwise gradient starting with 50 mM sodium acetate (pH 5.2), and then eluted with the same buffer containing 50 mM, 300 mM, and 500 mM sodium chloride. Each of the four elution steps was collected as a single 40-ml fraction. These fractions were designated D-I, D-II, D-III, and D-IV, respectively. Each fraction was assayed for the indicated enzyme activities by the procedures described in "Materials and Methods." Plus (+) indicates the presence of the designated activity; minus (−) indicates the absence of the designated activity; elipses (...) indicate that the designated enzyme was not assayed in that fraction. Protein was determined by the method of Lowry *et al.* (31). The total protein in each fraction is displayed as micrograms of protein per fraction.

| Enzyme Activity | Fraction from DEAE-cellulose Column | | | |
|---|---|---|---|---|
| | D-I | D-II | D-III | D-IV |
| α-Galactosidase | − | − | + | + |
| β-Galactosidase | − | − | + | + |
| β-Galactosidase | − | − | + | + |
| α-L-Arabinofuranosidase | + | − | + | + |
| Pectin hydrolase | − | + | + | + |
| Pectin lyase | − | − | + | + |
| Pectin esterase | − | ... | ... | ... |
| Polygalacturonic acid hydrolase | + | + | + | − |
| Pectic acid lyase | + | − | − | − |
| Xylanase | + | ... | ... | ... |
| Cellulase | − | + | + | + |
| α-*N*-Benzoyl-arginine ethyl esterase | + | ... | ... | ... |
| Phosphatase | − | ... | ... | + |
| Protein (μg/fraction) | 1100 | 450 | 2000 | 700 |

Fraction D-I is concentrated by lyophilization, dissolved in 10 ml of distilled water, and subjected to dialysis against 10 mM sodium acetate (pH 5.2) for 6 hr at 2 C. The nondialyzable fraction is placed on a second DEAE-cellulose column (column 2) and eluted with 10 mM sodium acetate (pH 5.2). The eluent is collected in 10-ml fractions. Since WME is eluted with the peak of protein not retained by column 2 (Fig. 1), the fractions containing this protein are pooled and concentrated by lyophilization. The preceding method provides a partially purified preparation of WME which is free of pectate lyase activity, most of the polygalacturonic acid hydrolase activity (Fig. 1), and all of the enzyme activities present in fractions D-II, D-III, and D-IV (Table I).

The concentrate from column 2 is dissolved in 5 ml of 50 mM sodium acetate (pH 5.2) which contains 0.1 M sodium chloride. This solution is placed on a Bio Gel P-100 column (column 3)

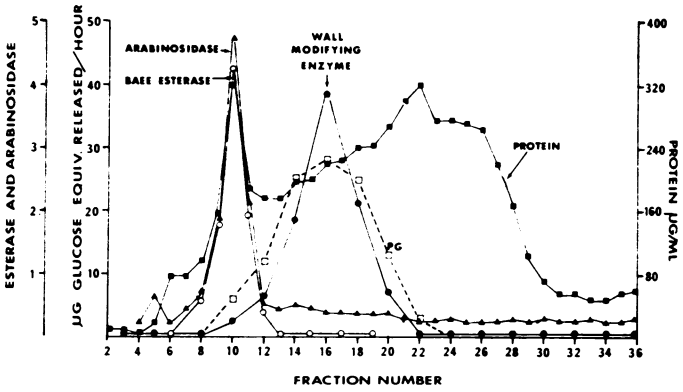


FIG. 2. Purification of WME by Bio Gel P-100 chromatography. Six milliliters of solution containing WME activity, obtained by fractionation of Pectinol R-10 through columns 1 and 2, were placed on a Bio Gel P-100 column (column 3). Column 3 was eluted with 50 mM sodium acetate (pH 5.2) which contained 100 mM sodium chloride. Six-milliliter fractions were collected. Each fraction was assayed for α-L-arabinofuranosidase and α-*N*-benzoyl arginine ethyl esterase (BAEE-esterase) as described in "Materials and Methods." One unit of α-L-arabinofuranosidase was defined as the amount of enzyme activity that caused at 25 C an absorbance change of 0.1/hr·ml of solution. One unit of BAEE-esterase was defined as the amount of enzyme activity that caused at 25 C an absorbance change of 0.001 optical density unit/min·ml of solution. Polygalacturonic acid (PG) degradation was assayed by measuring the decrease in viscosity of a 2% (w/v) solution of polygalacturonic acid. The fraction containing the most activity (fraction 16) decreased the viscosity of a 2% solution of polygalacturonic acid by approximately 40% in 30 min. Reaction mixtures for cell wall degradation assays were prepared by adding 1 ml of each fraction to 1 ml of D-III and 10 mg of cell walls. The reactions were incubated for 4 hr at 25 C on a rotary shaker, and wall degradation was measured as described in "Materials and Methods." Protein was determined by the method of Lowry *et al.* (31).

and eluted with 50 mM sodium acetate (pH 5.2) containing 0.1 M sodium chloride. The eluent is collected in-6 ml fractions, and each fraction is assayed for BAEE-esterase, α-L-arabinofuranosidase, WME, and polygalacturonase activities. The BAEE-esterase and α-L-arabinofuranosidase activities are of a larger molecular weight and are clearly separated from WME (Fig. 2). A polygalacturonic acid degrading activity is eluted from the column simultaneously with WME. Those fractions (No. 13–20) from column 3 (Fig. 2) which contain WME activity, but not BAEE-esterase and α-L-arabinofuranosidase activities, are pooled and concentrated by lyophilization.

The concentrated WME activity from column 3 is dissolved in 4 ml of distilled water and subjected to dialysis for 24 hr at 2 C against 1 mM potassium phosphate (pH 7.0). The nondialyzable fraction is placed on a CM-cellulose column (column 4), and the column is eluted with 10 void volumes of 1 mM potassium phosphate (pH 7.0), followed by 10 void volumes of the same buffer containing 50 mM sodium chloride. The column is then eluted with a linear 50 mM to 1.0 M sodium chloride gradient in potassium phosphate buffer. The eluent is collected in 6-ml fractions, and each fraction is subjected to dialysis for 24 hr against 50 mM sodium acetate (pH 5.2) before being assayed for WME activity (Fig. 3). The eluent is dialyzed because potassium phosphate buffer inhibits the ability of WME to attack cell walls. The protein (CM-I) not retained by the column contains polygalacturonic acid-degrading activity and xylanase activity (Table II). A second peak of protein (CM-II), which is eluted from column 4 at a sodium chloride concentration of approximately 0.5 M, contains WME activity (Fig. 3). The only enzyme activity identifiable using model substrates and eluting coincident with the protein in CM-II is a polygalacturonic acid degradative enzyme. The poly-

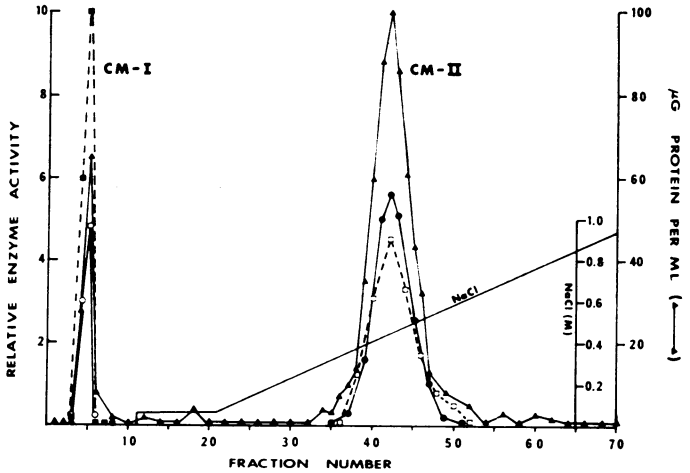


FIG. 3. Purification of WME by CM-cellulose chromatography. Four milliliters of solution containing WME activity, obtained by fractionation of Pectinol R-10 through columns 1, 2, and 3, were placed on a CM-cellulose column (column 4). Column 4 was eluted with 10 void volumes of 1 mM potassium phosphate (pH 7.0) followed by 10 void volumes of potassium phosphate (pH 7.0) containing 50 mM sodium chloride. The column was then eluted with a linear 50 mM to 1.0 M sodium chloride gradient prepared in 1 mM potassium phosphate (pH 7.0). Six-milliliter fractions were collected and the individual fractions were pooled into 5 major fractions as follows: 3 to 9 (CM-I), 11 to 20, 21 to 34, 35 to 51 (CM-II), and 52 to 70. These major fractions were dialyzed separately for 24 hr at 2 C against 50 mM sodium acetate (pH 5.2). Cell wall degradation assay mixtures were prepared by adding 1 ml of each major fraction to 1 ml of D-III and 10 mg of cell walls. Reactions were incubated for 4 hr at 25 C and wall degradation was assayed as described in "Materials and Methods." Only the fraction containing tubes 35 to 51 (CM-II) had detectable WME activity. No WME activity could be detected, even in CM-II, before dialysis. A second preparation of WME, obtained by fractionation of Pectinol R-10 through columns 1, 2, and 3, was subjected to chromatography through column 4 as described above. Fractions 35 to 51 (CM-II) were dialyzed separately for 24 hr at 2 C against 50 mM sodium acetate (pH 5.2). The nondialyzable portion of each fraction was assayed for wall-degrading activity as described above. Each of these dialyzed fractions was also assayed for polygalacturonic acid-degrading activity by adding 1 ml of enzyme solution to 2 ml of 0.1% polygalacturonic acid in 50 mM sodium acetate (pH 5.2). The reactions were incubated for 3 hr at 25 C and terminated by the addition of the dinitrosalicylic acid reagent. The presence of reducing sugars was determined as described in "Materials and Methods." One-tenth milliliter aliquots of fractions 3 to 9 (CM-I) were diluted to 1.0 ml with 50 mM sodium acetate (pH 5.2) and the diluted solutions were assayed without dialysis for polygalacturonic acid-degrading activity by following the procedure described above (the peak fraction, 5, was substrate-limited in this assay). Five-tenths milliliter aliquots of fractions 3 to 9 (CM-I) were diluted to 1.0 ml with 50 mM sodium acetate (pH 5.2) and these solutions were assayed without dialysis for xylanase activity by adding them to 2 ml of a 0.1% solution of xylan. No xylanase activity was detectable in CM-II (Table II). The reactions were incubated for 3 hr at 25 C and then terminated by the addition of dinitrosalicyclic acid reagent. The presence of reducing sugar was measured as described in "Materials and Methods." One relative unit of enzyme activity represents the following number of micrograms glucose equivalents released per hour per milliliter of the indicated enzyme: CM-I polygalacturonic acid-degrading enzyme (■), 400; CM-I xylanase (○), 200; CM-II polygalacturonic acid-degrading enzyme (□), 20; CM-II (WME) + D-III (●), 10. Protein (▲) was determined by the method of Lowry *et al.* (31).

galacturonic acid-degrading activities in CM-I and CM-II are not increased by the addition of calcium chloride. Neither CM-I nor CM-II contains pectate lyase activity. Fractions 11 to 34 and 52 to 70 (Fig. 3), which contain no detectable protein, have no effect on either cell walls or model substrates.

Table II. *Determination of Enzyme Activities Present in Peaks CM-I and CM-II from Column 4 Using Model Substrates*

The polysaccharides used in this assay were described in "Materials and Methods." Polysaccharide solutions were prepared in 50 mM sodium acetate as described in "Materials and Methods." Those fractions (No. 3–9) from column 4 (Fig. 3) constituting CM-I were combined, as were those (No. 35–51) constituting CM-II. These two solutions were dialyzed for 24 hr against 50 mM sodium acetate (pH 5.2). Reaction mixtures were prepared by combining 1 ml of the fraction to be assayed with 2 ml of the appropriate polysaccharide solution. In the assay for polygalacturonic acid-degrading activity of CM-I, 0.1 ml of solution was used. When the xylanase activity of CM-I was assayed, 0.5 ml of solution was used. In both cases, the volume of solution used for the assay was adjusted to 1 ml with sodium acetate buffer and added to 2 ml of the polysaccharide solution. Reactions were terminated by addition of the dinitrosalicylic acid reagent, and the amount of reducing sugar was determined as described in "Materials and Methods." Pectate lyase activity was measured as described in "Materials and Methods." The values in this table represent the average of duplicate reactions.

| Polysaccharide | Glucose Equivalent Reducing Sugar | |
|---|---|---|
| | Fraction CM-I | Fraction CM-II |
| | *μg/hr·ml enzyme* | |
| Araban | 3 | 0 |
| Carboxymethylcellulose | 3 | 0 |
| Galactan | 0 | 0 |
| Laminarin | 3 | 0 |
| Mannan | 0 | 0 |
| Pectin | 11 | 2 |
| Polygalacturonic acid | >4000 | 30 |
| Polygalacturonic acid (lyase assay) | 0 | 0 |
| Xylan | >600 | 0 |

**Cell Wall Degradation by Pectinol R-10.** The enzymes present in Pectinol R-10 catalyze hydrolysis of plant cell walls at pH 5.2. This can be demonstrated by either measuring the release of 70% ethanol (v/v) soluble reducing sugars from cell walls (Fig. 4) or detecting by gas-liquid chromatography the decrease in sugar yield upon acid hydrolysis of the remaining ethanol-insoluble cell wall material (Table III). The amounts of all of the monosaccharides present in the cell wall hydrolysate are reduced by pretreatment of cell walls with the Pectinol R-10 preparation.

**Characterization of Wall Modifying Enzyme.** A crude preparation of Pectinol R-10 was divided into four fractions (D-I, D-II, D-III, and D-IV) by DEAE-cellulose chromatography as described in Table I. Even though each fraction contained a number of potential cell wall-degrading enzymes, no fraction alone contained enzymes capable of liberating 70% ethanol-soluble reducing sugars from cell walls upon incubation for 1 hr at 25 C (Table IV). All combinations of fractions D-I, D-II, D-III, and D-IV were assayed for cell wall-degrading activity. Only those combinations containing D-I were able to catalyze cell wall degradation. Furthermore, a crude α-galactosidase, prepared from the culture filtrate of the bean pathogen *C. lindemuthianum*, was able to catalyze degradation of cell walls only in the presence of D-I (Table IV). It is apparent that D-I contains some enzyme or enzymes necessary for cell wall degradation. This activity in D-I has been designated "wall-modifying enzyme."

Pectinol R-10 appears to contain only a single protein with the properties of WME. Evidence in support of this was obtained by fractionating a crude preparation of Pectinol R-10 on Bio Gel P-100. Each fraction from the column was assayed for cell wall-degrading activity in the presence of D-III. A single peak of wall-degrading activity was observed. This peak of wall-degrading activity was eluted from the column in those fractions intermediate to the higher molecular weight glycosidases and the lower molecular weight cellulases present in Pectinol R-10. Both the single peak of wall-degrading activity obtained by fractionation of the crude preparation of Pectinol R-10 and the peak of WME activity obtained by fractionation of D-I (Fig. 2) were eluted from Bio Gel P-100 with the same ratio of elution volume to void volume. Both fractionations give an approximate molecular weight for WME of 40,000.

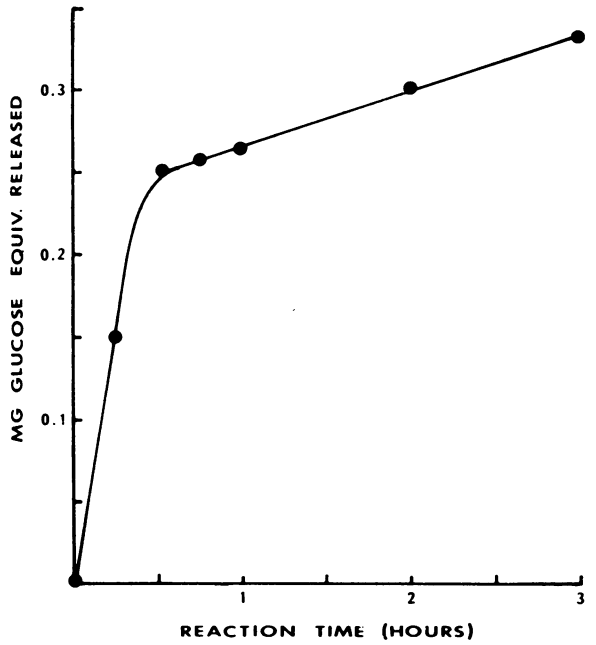


FIG. 4. Degradation of cell walls by a crude preparation of pectinol R-10. A solution of Pectinol R-10 was prepared at a concentration of 0.5 mg/ml in 50 mM sodium acetate (pH 5.2). Reaction mixtures for the cell wall degradation assay were prepared by adding 2 ml of this enzyme solution to 10-mg samples of cell wall. The reactions were incubated at 25 C and wall degradation was assayed as described in "Materials and Methods." Each point in the figure represents the average of values obtained from duplicate reactions.

The WME activity present in D-I is lost when the enzyme preparation is heated. This is shown by the data illustrated in Figure 5. In one set of reactions, cell walls were incubated with a combination of D-I and D-III. After a 4-hr reaction period, 250 μg of glucose equivalents reducing sugar were released from the cell walls. In a second set of reactions, D-I was heated for 10 min in a boiling water bath (95 C) before it was added to D-III and cell walls. No detectable 70% ethanol-soluble reducing sugar was released from the cell walls after 4 hr.

A partially purified preparation of WME is unable, by itself, to release 70% ethanol-soluble reducing sugars from cell walls. This was demonstrated with a preparation of WME purified through columns 1, 2, and 3. When cell walls were incubated with a combination of purified WME and D-III, a release of reducing sugars similar to that displayed in Figure 5 for the combination of D-I and D-III was observed. When cell walls were incubated with WME in the absence of D-III, no release of 70% ethanol-soluble reducing sugar could be observed after 4 hr (Fig. 5).

The release of reducing sugars catalyzed by the combination of WME and D-III results from the degradation of cell walls and not from material in D-III or in the fractions of column 3 possessing WME activity. This was ascertained by incubating for 4 hr ali-

Table III. *The Degradation of Cell Walls by a Crude Preparation of Pectinol R-10*

Pectinol R-10 was prepared in 50 mM sodium acetate (pH 5.2) at a concentration of 1 mg/ml. Reaction mixtures were prepared by adding 2 ml of the enzyme preparation or 2 ml of 50 mM sodium acetate, (pH 5.2) to 10 mg of cell walls. Reaction mixtures were incubated at 25 C on a rotary shaker. After a 3-hr incubation period, reactions were terminated by the addition of ethanol to a concentration of 70% (v/v). The cell walls were pelleted by centrifugation, and the supernatant liquid was removed with a pipette. The walls were then washed with 2 ml of 70% ethanol (v/v). The supernatant liquid and the wash were discarded. The remaining ethanol was removed from the cell walls by passing a stream of filtered air over the walls at 55 C. The cell walls were then subjected to hydrolysis in a sealed tube in 2 ml of 2 N trifluoroacetic acid at 121 C for 1 hr. The hydrolyzed sample was made 70% with respect to ethanol, and the residual cell wall material was removed by centrifugation. The supernatant liquid was collected with a pipette, and the cell walls were washed with an additional 2 ml of 70% ethanol. The supernatant liquid and wash were combined, and the solvent was evaporated by passing a stream of filtered air over the solution at 55 C. The monosaccharides present in the resulting residue were acetylated and analyzed by gas-liquid chromatography as described previously (6). The amounts of each monosaccharide released from the cell wall material by acid hydrolysis are displayed as mg % (mg of monosaccharide/100 mg of cell wall × 100). The values represent the average of duplicate reactions.

| Monosaccharide | Untreated Walls | Pectinol R-10-treated Walls |
|---|---|---|
| | *mg %* | *mg %* |
| Arabinose | 1.8 | 0.9 |
| Fucose | 0.3 | 0.2 |
| Galactose | 4.5 | 1.6 |
| Glucose | 2.7 | 2.3 |
| Mannose | 0.9 | 0.8 |
| Rhamnose | 0.9 | 0.4 |
| Xylose | 5.5 | 4.6 |

quots of fractions 14 to 20 (column 3) with D-III and cell walls or by incubating aliquots of these fractions with D-III in the absence of cell walls. In the presence of D-III and cell walls, the peak of WME activity displayed in Figure 2 was observed. When cell walls were omitted from the reaction mixture, no release of reducing sugar was detected in any fraction.

The requirement for WME in the cell wall degradation process is partially removed when cell walls are subjected to acidic or basic conditions prior to treatment with D-III. This was demonstrated by incubating cell walls with the various buffers described in Figure 6. After 30 min at 25 C, ethanol was added to a concentration of 70% (v/v). The cell walls were collected by centrifugation, and the supernatant liquid was discarded. The walls were then washed twice with 2-ml portions of 70% ethanol (v/v). Finally, the walls were incubated for 4 hr at 25 C with D-III in 50 mM sodium acetate (pH 5.2). A release of reducing sugar was observed when cell walls were pretreated with buffers having a pH below 4.5 or above 10.0 (Fig. 6).

WME has a pH optimum at or below pH 4.7 (Fig. 6). This was demonstrated by treating cell walls with WME at pH values from 4.7 to 7.8. After 4 hr, ethanol was added to a concentration of 70% (v/v). The cell walls were collected by centrifugation and washed with 2 ml of 70% ethanol (v/v). The supernatant liquid and the wash were discarded. The cell walls were then incubated for 4 hr with D-III in 50 mM sodium acetate at pH 5.2. WME was only active below pH 6.0. With the buffers tested, the optimal activity was observed at pH 4.7. Assays of WME activity were not carried out below pH 4.5, since below that pH the need for WME is partially eliminated.

Table IV. *Fractionation of Pectinol R-10 by DEAE-cellulose Chromatography (Cell Wall Degradation Assays)*

Fractions D-I, D-II, D-III, and D-IV were prepared as described in Table I. In the wall degradation assays, 1 ml of each fraction was added to 1 ml of 50 mM sodium acetate (pH 5.2) and 10 mg of cell walls. In the recombination experiments, 1 ml of one fraction was added to 1 ml of a second fraction and 10 mg of cell walls. The reactions were incubated for 1 hr on a rotary shaker at 25 C and were terminated by the addition of ethanol to a concentration of 70% (v/v). Cell walls were pelleted by centrifugation and the supernatant liquid was removed with a pipette. The walls were then washed with an additional 2 ml of 70% ethanol. The supernatant liquid and the wash were combined and the solvent was evaporated by passing a stream of filtered air over the solution at 55 C. The reducing sugars present in the residue were measured as described in "Materials and Methods." The method of preparation of the α-galactosidase (CL) was described in "Materials and Methods." The values in this table are the average of duplicate reactions. All combinations of the four fractions were assayed for cell wall-degrading activity, but only those containing D-I were able to release 70% ethanol-soluble reducing sugars from the wall.

| Fraction | Glucose equivalent of Reducing Groups Released into 70% Ethanol-soluble Fraction |
|---|---|
| | *μg* |
| D-I | 0 |
| D-II | 0 |
| D-III | 0 |
| D-IV | 0 |
| D-I + D-II | 20 |
| D-I + D-III | 80 |
| D-I + D-IV | 50 |
| CL | 0 |
| D-I + CL | 90 |

The requirement for WME in cell wall degradation is also partially removed by treatment of walls with pronase. Ten-milligram samples of cell walls were pretreated for 4 hr at 25 C with 10 μg of pronase in 2 ml of 50 mM sodium acetate (pH 5.2). At the end of the reaction period, ethanol was added to a concentration of 70% (v/v). The cell walls were collected by centrifugation and washed with 2 ml of 70% ethanol (v/v). The walls were then incubated for 4 hr at 25 C with 1 ml of D-III and 1 ml of 50 mM sodium acetate (5.2). The enzymes present in D-III were able to release nearly 50 μg of glucose equivalent reducing sugar from cell walls which had been pretreated with pronase. No reducing sugar was released from cell walls which had been similarly pretreated with trypsin.

Even though WME can be replaced, at least in part, by pronase, treatment of cell walls with WME has no apparent effect on extensin (25), the hydroxyproline containing protein of the cell wall. This was demonstrated by incubating separate 500-mg samples of cell wall with WME (CM-II) or with buffer. The reactions were terminated by filtration on a coarse sintered glass funnel. The soluble material was evaporated to dryness, and the residue was dissolved and subjected to hydrolysis in 6 N hydrochloric acid. Insoluble material produced during hydrolysis was removed by centrifugation, and the supernatant liquid was assayed for hydroxyproline (11). Treatment of cell walls with WME did not result in the release of hydroxyproline-containing peptides from the cell wall. The cell wall samples which had been collected by filtration were removed from the sintered glass funnels, subjected to hydrolysis in a saturated solution of barium hydroxide, and

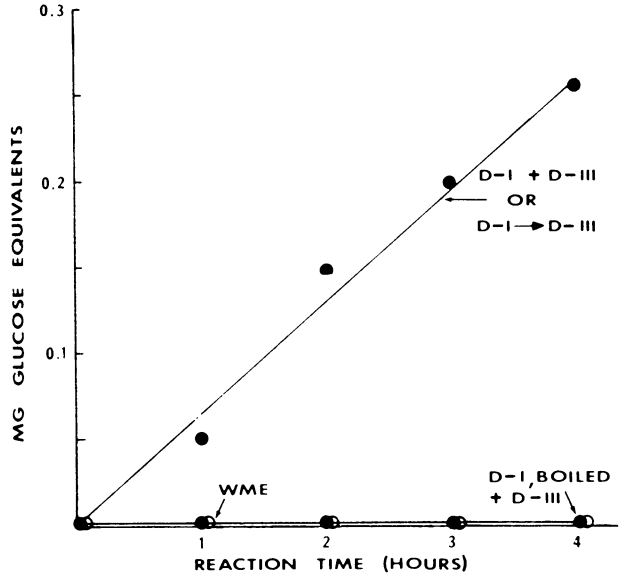


FIG. 5. Heat lability of WME; D-III is required for the production of 70% ethanol-soluble sugars. Fraction D-III was prepared as described in "Materials and Methods." Fraction D-I was prepared by subjecting 5 ml of a crude preparation of Pectinol R-10 (10 mg/ml in 50 mM sodium acetate [pH 5.2]) to fractionation by DEAE-cellulose chromatography. In the first set of experiments (D-I + D-III), reaction mixtures were prepared by adding 0.25 ml of D-I, 0.5 ml of D-III, and 1.25 ml of 50 mM sodium acetate (pH 5.2) to 10 mg of cell walls. The reactions were incubated at 25 C and wall degradation was measured as described in "Materials and Methods." The D-I → D-III designation indicates that the same total release of reducing suagr was observed regardless of whether cell walls were incubated with D-I first, followed by incubation with D-III, or D-I and D-III were added to the cell walls at the same time. In the second set of reactions, D-I was heated in a boiling water bath for 10 min before being added to D-III and cell walls. Cell wall degradation assays were carried out as described above for the combination of D-I + D-III. The WME used in the third set of reactions was prepared by fractionation of Pectinol R-10 through columns 1, 2, and 3. Reaction mixtures were prepared by adding 0.2 ml of WME solution, 0.5 ml of D-III, and 1.3 ml of 50 mM sodium acetate (pH 5.2) to 10 mg of cell walls. Another set of reactions, incubated at 25 C, was prepared as described above but without the 0.5 ml of D-III and with an additional 0.5 ml of the sodium acetate buffer (designated in the figure by WME). Wall degradation was assayed as described in "Materials and Methods." A set of reactions containing both WME and D-III gave results similar to those obtained by the combination of D-I and D-III; the data are not presented.

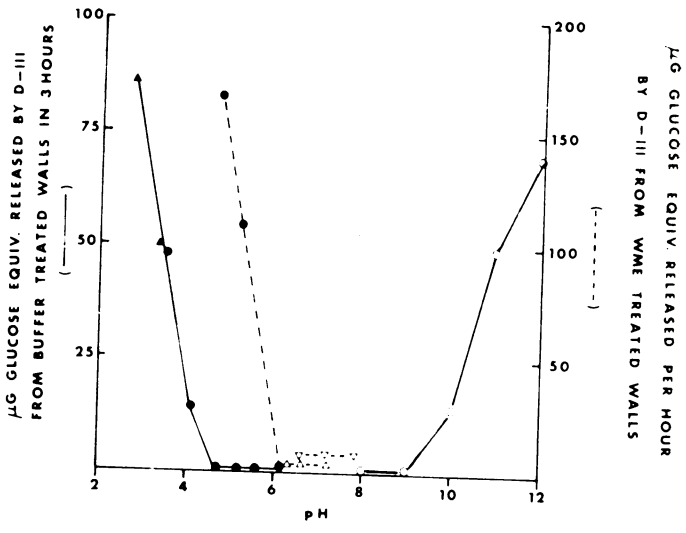


FIG. 6. Partial removal of the WME requirement in the wall degradation process by subjection of cell walls to acidic or basic conditions; pH optimum of WME activity. In the first set of reactions, 10-mg portions of cell walls and 2 ml of the appropriate buffer (▲: 50 mM sodium tartrate; ●: 50 mM sodium acetate; △: 50 mM potassium phosphate; ▽: 50 mM tris; ○: potassium hydroxide) were incubated in a 13- × 100-mm test tube at 25 C. After 30 min, ethanol was added to a concentration of 70% (v/v). Cell walls were pelleted by centrifugation in a clinical centrifuge, and the supernatant liquid was removed with a pipette. The cell walls were washed with 70% ethanol and again pelleted by centrifugation. The wash solution was removed with a pipette. The cell walls were then incubated with 1 ml of D-III and 1 ml of 50 mM sodium acetate (pH 5.2) for 3 hr at 25 C. Wall degradation was assayed as described in "Materials and Methods." In the reactions used to determine the pH optimum of WME, 1-ml aliquots of WME, which had been prepared by fractionation of Pectinol R-10 through columns 1 and 2, were added to 10-mg samples of cell walls suspended in 1 ml of the buffers described above to give a series of reactions with a final pH ranging from 4.7 to 7.8. The reactions were incubated for 3 hr at 25 C and were terminated by the addition of ethanol to a concentration of 70% (v/v). The cell walls were pelleted by centrifugation and the supernatant liquid was removed with a pipette. The pelleted walls were washed with 2 ml of 70% ethanol and the wash solution was discarded. The washed walls were incubated at 25 C for 3 hr with a combination of 1 ml of D-III and 1 ml of 50 mM sodium acetate (pH 5.2). Reactions were terminated by the addition of ethanol to a concentration of 70% (v/v) and the presence of reducing sugar in the supernatant solution was Measured as described in "Materials and Methods."

fractionated by Bio Gel P-2 chromatography as described in "Materials and Methods." The material solubilized by base-catalyzed hydrolysis of WME-treated cell walls contained about the same amount of total hydroxyproline and about the same ratios of (arabinosyl)$_x$-hydroxyproline to free hydroxyproline as the materials released upon base hydrolysis of buffer-treated cell walls.

The inability of WME to act as a peptidase was further demonstrated by the fact that purified WME is unable to degrade model substrates which are degraded by the peptidases pronase and ficin. WME was unable to release β-naphthylamine from the β-naphthylamide derivatives of L-lysine, L-hydroxyproline, L-serine, and L-tyrosine. Under the same reaction conditions, 10 μg of pronase or 1 mg of ficin catalyzed the release of β-naphthylamine from these derivatives at a rate of approximately 10 μg/hr. As demonstrated in Figure 2, the fractions containing WME activity did not contain enzymes capable of catalyzing degradation of α-*N*-benzoyl-L-arginine ethyl ester.

Purified WME does catalyze the release of water-soluble, but 70% ethanol-insoluble, polysaccharides from the cell wall. This was demonstrated by incubating 10 ml of WME (CM-II) in 50 mM sodium acetate (pH 5.2) with 100 mg of cell wall for 4 hr at 25 C. The reactions were terminated by filtration on a coarse sintered glass funnel. The soluble material was assayed for uronide-containing polymers with the modified carbazole method (12). The solubilized polymers contained approximately 7 mg of uronic acid which represents between 80 and 90% of the total polymeric carbohydrate released by the action of WME. When cell walls were incubated for 4 hr with buffer in the absence of WME, less than 0.5 mg of uronic acid was extracted from the cell wall. The water-soluble polymers released by the action of WME on cell walls were assayed for neutral sugars by gas-liquid chromatography (8). The monosaccharides detected in these polymers include: galactose, glucose, and smaller amounts of arabinose and rhamnose. The total yield of the monosaccharides was about 1 mg. The purified WME used in these experiments contained no carbazole positive materials or detectable neutral sugars.

The action of WME on cell walls results not only in a release of water-soluble polysaccharides, but also in an alteration of the insoluble cell wall residue. Both the water-soluble polysaccha-

Table V. *The Action of WME on Cell Walls*

In the primary treatment, 10-mg samples of cell walls were treated either with a combination of 1 ml of purified WME and 1 ml of 50 mM sodium acetate (pH 5.2) or with 2 ml of the sodium acetate buffer in the absence of WME. Reactions were incubated for 4 hr on a rotary shaker at 25 C. At the end of the reaction period, the cell walls were pelleted by centrifugation in a clinical centrifuge, and the supernatant liquid was collected with a pipette. The cell walls were then washed twice with 2-ml portions of 50 mM sodium acetate (pH 5.2). The washes and the supernatant liquid were combined, and any insoluble wall material was removed by filtration through a coarse sintered glass funnel. The solvent was then evaporated at 55 C by passing a stream of filtered air over these samples. The residue was dissolved in 1 ml of the sodium acetate buffer. Each original reaction was therefore represented by two samples. The first contained water-soluble polymers released from cell walls by the WME treatment, and the second contained the residual cell walls. In the secondary treatment, half of the WME-treated and half of the buffer-treated samples were further treated with 1 ml of D-III and 1 ml of the sodium acetate buffer. The other half of the WME-treated and of the buffer-treated samples were incubated with 2 ml of buffer in the absence of D-III. The reactions were incubated for 4 hr at 25 C on a rotary shaker and were terminated by the addition of ethanol to a concentration of 70% (v/v). The presence of reducing sugars in the D-III-treated water-soluble polymers was assayed as described in "Materials and Methods" following evaporation of the samples to dryness. The D-III-treated wall residues were pelleted by centrifugation, and the supernatant liquid was removed with a pipette. The wall residues were then washed with an additional 2 ml of 70% ethanol, and the supernatant liquid and wash were combined. The solvent was evaporated at 55 C by passing a stream of filtered air over the solution. Reducing sugars present in the dried samples were measured as described in "Materials and Methods." The values displayed in this table represent the average of triplicate reactions.

| Primary Treatment | Secondary Treatment | Glucose Equivalent Reducing Sugar Released | |
|---|---|---|---|
| | | Water-soluble polymers | Cell walls |
| | | μg | μg |
| WME | D-III | 70 | 234 |
| WME | Buffer | 0 | 0 |
| Buffer | D-III | 0 | 8 |
| Buffer | Buffer | 0 | 0 |

rides released by the action of WME and the remaining cell walls are susceptible to degradation by the glycosidases present in D-III. This was demonstrated by incubating cell walls with WME for about 4 hr at 25 C. After the reaction period, cell walls were collected by centrifugation. The supernatant liquid was transferred with a pipette and the cell walls were washed twice with 2-ml portions of 50 mM sodium acetate (pH 5.2). The supernatant liquid and the washes were combined, and insoluble cell wall material floating in the solution was removed by filtration through a coarse sintered glass funnel. The solvent was evaporated at 55 C from the WME-solubilized materials by passing a stream of filtered air over the solution. The resulting residue was dissolved in 1 ml of 50 mM sodium acetate (pH 5.2). The water-soluble polysaccharides and the residual cell walls were incubated separately with D-III for 4 hr. The results displayed in Table V indicate that although WME does release polysaccharides which are degraded by the glycosidases in D-III, most of the observed degradation in the normal assay results from direct attack of the enzymes in D-III on the WME-altered cell wall material.

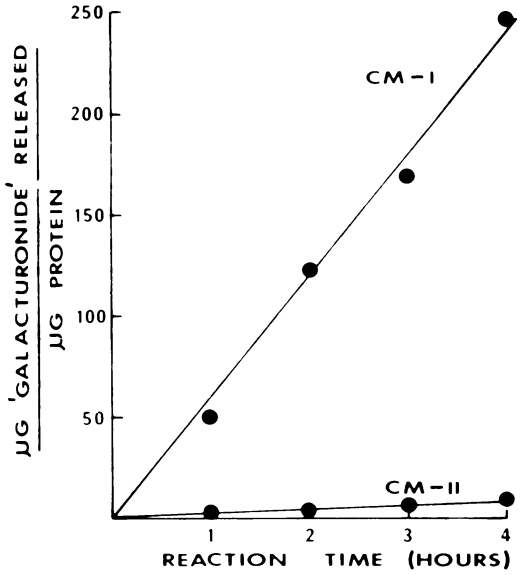


FIG. 7. Polygalactouronic acid degrading activity in CM-I and in CM-II from column 4. Fractions 3 to 9 and 35 to 51 from column 4 (Fig. 3) were pooled to yield two separate fractions, CM-I and CM-II. These fractions were dialyzed against 50 mM sodium acetate (pH 5.2) for 24 hr at 2 C. Reaction mixtures were prepared by adding either 0.01 ml of CM-I (diluted to 1 ml with sodium acetate buffer) or 1 ml of CM-II to 2 ml of 0.1% polygalacturonic acid (w/v) prepared in the sodium acetate buffer. Reactions were terminated by the addition of the dinitrosalicyclic acid reagent, and reducing sugars were measured as described in "Materials and Methods." The protein present in each fraction was determined by the method of Lowry *et al.* (31). The results are displayed as micrograms of galacturonic acid released per microgram of protein. Each point represents the average of values obtained from duplicate reactions.

The WME activity is eluted from CM-cellulose (column 4) coincident with a peak of polygalacturonic acid-degrading activity (Fig. 3). However, polygalacturonic acid is a relatively poor substrate for this enzyme. In a 4-hr reaction period, the polygalacturonic acid-degrading enzyme present in CM-I had released from polygalacturonic acid 100 times more reducing groups per microgram of protein than the enzyme present in CM-II (Fig. 7). The ability of the CM-I and CM-II polygalacturonic acid-degrading enzymes to reduce the viscosity of a 2% (w/v) solution of polygalacturonic acid in 50 mM sodium acetate was also measured. In 30 min, the polygalacturonic acid-degrading enzyme in CM-I had reduced the viscosity of the polygalacturonic acid solution by 40%. In the same time period, a comparable portion of the protein present in CM-II had caused only a 3% reduction in the viscosity of the polygalacturonic acid solution.

Although the ability of the WME-active fractions from column 4 (CM-II) to degrade polygalacturonic acid is poor, an experiment was conducted to determine whether or not polygalacturonic acid might, in fact, be a poor substrate for WME. This was accomplished by determining whether concentrations of polygalacturonic acid, which saturate the polygalacturonic acid-degrading enzyme of CM-II, inhibit the WME activity of CM-II. The polygalacturonic acid-degrading activity in CM-II is saturated by concentrations of polygalacturonic acid exceeding 0.06% (w/v) (Fig. 8). The ability of polygalacturonic acid to inhibit the action of WME was ascertained by incubating cell walls with 1 ml of WME (CM-II) in the presence of polygalacturonic acid at final concentrations of 0, 0.05, 0.1 0.12, and 0.25% (w/v) in 50 mM sodium acetate (pH 5.2). At the end of the 3-hr reaction period, the walls were pelleted by centrifugation. The supernatant liquid was removed with a pipette and discarded and the walls

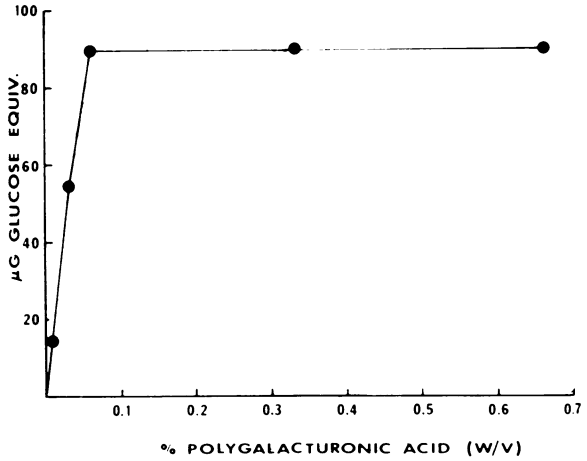


FIG. 8. Polygalacturonic acid-degrading activity in CM-II (column 4): substrate concentration curve. Fraction CM-II was prepared as described in Figure 7. A 1-ml portion of the enzyme was added to 2 ml of polygalacturonic acid prepared in 50 mM sodium acetate (pH 5.2) to give the final percentage polygalacturonic acid concentration (w/v) indicated in Figure 8. The reaction mixtures were incubated for 3 hr at 25 C and were terminated by the addition of the dinitrosalicyclic acid reagent. The amount of reducing sugars present was measured as described in "Materials and Methods."

were washed three times with 4-ml portions of 50 mM sodium acetate (pH 5.2). The walls were then incubated for 3 hr with D-III. Concentrations of polygalacturonic acid equal to or greater than 0.1% completely inhibit the ability of WME to act on the cell wall (Table VI). At 0.05% polygalacturonic acid (a concentration slightly less than that required to saturate the polygalacturonic acid-degrading enzyme of CM-II) the inhibition of WME activity is about 60%.

Table VI. *Inhibition of WME Action on Cell Walls by Polygalacturonic Acid*

Those fractions (No. 35–51) from column 4 (Fig. 3) constituting CM-II were combined and dialyzed for 24 hr against 50 mM sodium acetate (pH 5.2). The nondialyzable portion of CM-II contained the WME activity used in this experiment. Reaction mixtures were prepared by adding 1 ml of the solution containing WME activity to 10 mg of cell walls which were suspended in 1 ml of 50 mM sodium acetate (pH 5.2). The sodium acetate buffer contained polygalacturonic acid in varying amounts such that the following final concentrations (w/v) of polygalacturonic acid were achieved: 0, 0.05, 0.10, 0.12, 0.25, and 0.50%. The reactions were incubated at 25 C for 3 hr. At the end of the reaction period, the cell walls were pelleted by centrifugation, and the supernatant liquid was removed with a pipette. The walls were washed 3 times with 4-ml volumes of 50 mM sodium acetate (pH 5.2). The supernatant liquid and the washes were discarded. One milliliter of D-III and 1 ml of 50 mM sodium acetate (pH 5.2) were added to the washed cell walls. These reaction mixtures were incubated for 3 hr at 25 C. Cell wall degradation was then assayed as described in "Materials and Methods." Values obtained from three separate experiments, using one CM-II preparation for experiment 1 and a second CM-II preparation for experiments 2 and 3, are given.

| Final Concn (w/v) of Polygalacturonic Acid in the Reaction Mixture | Glucose Equivalent Reducing Sugar Released | | |
|---|---|---|---|
| | Experiment 1 | Experiment 3 | Experiment 3 |
| % | μg | μg | μg |
| 0.0 | 173 | 50 | 50 |
| 0.05 | ... | ... | 20 |
| 0.10 | ... | ... | 0 |
| 0.12 | 0 | 0 | ... |
| 0.25 | 0 | 0 | ... |
| 0.50 | 0 | 0 | ... |

## DISCUSSION

Studies of the enzyme-catalyzed degradation of cell walls have been difficult in the past owing to the lack of a convenient assay for wall degradation. Previous studies have involved analysis of cell wall residues to determine whether a portion of the wall has been removed as a result of enzyme treatment (10, 23). These procedures are time-consuming and lack sufficient sensitivity, since small losses must be detected in the presence of the large background of residual cell wall material.

A moderately convenient and much more sensitive procedure for detecting wall degradation has been developed. In this procedure, the enzyme solution to be assayed is incubated with cell walls, and the reactions are terminated by the addition of ethanol. The release from the walls of 70% ethanol-soluble reducing sugar is the criterion for wall degradation. The examination of only 70% ethanol-soluble reducing sugars makes a rapid assay possible but also limits the types of wall degradation which can be examined. For example, any alteration of the wall which fails to release wall components, such as the cleavage of linkages between polymers, could go unobserved. Even the release of water-soluble polymers would be undetected if these polymers are insoluble in 70% ethanol. These limitations were overcome by the addition of a mixture of polysaccharide-degrading enzymes to the wall degradation assays. The enzymes used consisted of those present in fraction D-III of Pectinol R-10 (Table I). The enzymes in D-III are unable to attack unaltered, undegraded cell walls (Table III). However, the enzymes in D-III are able to degrade water-soluble polymers released from the cell wall, and, perhaps more importantly, they are particularly effective in degrading cell walls which have had critical linkages broken. The products of the further degradation of the water-soluble polymers and of the altered cell walls are 70% ethanol-soluble monosaccharides and oligosaccharides. These products are readily detected by the reducing group method (35). Thus, this assay, although still somewhat laborious, is a sensitive measure of changes in cell wall integrity.

The sensitivity of this assay for measuring slight modifications in the structure of native cell walls is demonstrated by the fact that cell walls may be detectably modified by relatively mild treatments. For example, drying of cell walls under slightly acidic conditions (pH 5.2) or exposure of the walls to solutions where the pH is below 4.5 or above 10.0 (Fig. 6) renders normally resistant walls susceptible to attack by the enzymes in D-III. Thus, during the isolation of cell walls to be used as substrate in this assay procedure, it is important that conditions of isolation be chosen to insure that the native wall structure is retained.

A significant but limited degradation of cell walls by some D-III preparations was observed in the absence of added WME. It appears that this limited cell wall-degrading ability of D-III results from a combination of several factors, such as incomplete fractionation of Pectinol R-10 on DEAE-cellulose and variation in cell wall preparations. In no case did the level of wall-degrading ability of D-III exceed 20% of that observed when walls were incubated in the presence of both D-III and WME.

A table summarizing the purification, yield, and specific activity of WME at each step of purification has not been included, for meaningful results of this sort are not obtainable. The total amount of protein, as measured by the method of Lowry (31), increases significantly during each step in the purification up to and including column 3. This increase is thought to be the result of the action of proteases such as BAEE-esterase. The polygalac-

turonic acid enzymes present in D-I and separated from WME on column 2 degrade WME-treated walls. When these polygalacturonic acid-degrading enzymes are removed, their contribution to total wall-degrading activity is lost and the measure of WME activity is erroneously lowered. In fact, any quantitative measure of WME would be misleading in that such activity would reflect any degradative enzyme contaminating WME and the interaction of the products of such degradative enzymes with the enzymes of D-III. Furthermore, the activity of even highly purified WME is hidden by the fact that for each bond broken by the direct action of WME, many more are cleaved by the action of the D-III enzymes.

With the assay procedure described above, it has been demonstrated that a large variety of polysaccharide-degrading enzymes present in Pectinol R-10, including a number of cellulases, pectinases, and hemicellulases, are not able to attack native cell walls (Tables I and IV). A combination of many such enzymes, represented by those found in fraction D-III (Table I), are unable to release 70% ethanol-soluble reducing sugars from cell walls in a 4-hr reaction period (Fig. 5). Besides those enzymes isolated from Pectinol R-10 and described in "Results," it has been found that a large variety of polysaccharide-degrading enzymes isolated from rapidly elongating mung beans (*Phaseolus aureus*) are unable to degrade native cell walls (unpublished data of this laboratory). It is evident from these results that the ability of enzymes to degrade model substrates cannot be directly correlated with the ability of the enzymes to degrade similar polymers in the cell wall (34). This is an important consideration since a number of workers have implicated polysaccharide-degrading enzymes in physiological processes on the basis of data accumulated with model substrate assays, without demonstrating that the enzymes could actually degrade cell walls (*e.g.*, 1, 16).

Even though a crude preparation of Pectinol R-10 is able to degrade cell walls (Table III and Fig. 4), none of the four subfractions obtained from this preparation (Table I) is able, by itself, to carry out wall degradation in a 1-hr reaction period (Table IV). (Fraction D-I is able to release detectable 70% ethanol-soluble reducing sugars from the cell wall after a 3-hr reaction period. This property is lost when most of the polygalacturonic acid-degrading enzymes are separated from WME upon chromatography on DEAE-cellulose [Fig. 3].) Fraction D-I contains WME, a component required for degradation of cell walls by other polysaccharide-degrading enzymes. This component has been purified by a combination of DEAE-cellulose, Bio Gel P-100, and CM-cellulose chromatography. The WME activity was identified at each state of purification by its ability to permit cell wall degradation by the enzymes present in D-III.

Treatment of cell walls with purified WME results in the release of water-soluble polymers which can be further degraded by the enzymes in D-III (Table V). Thus, WME plays an analogous role to that proposed for the $C_1$ component in cellulose degradation (38). WME has the additional effect of rendering the remaining insoluble portion of the cell walls susceptible to attack by the polysaccharide-degrading enzymes in D-III (Table V), an alteration which would not have been detectable by any previous method used for the assay of wall degradation.

The results presented are consistent with WME being a polygalacturonic acid-degrading enzyme. The evidence which supports such a conclusion includes the following observations. Protein peak CM-II from column 4 (Fig. 3), which contains WME activity, is eluted from the column as a single symmetrical peak of protein in a linear salt gradient. Both the polygalacturonic acid-degrading activity and the WME activity present in peak CM-II elute as symmetrical peaks coincident with the protein peak. Indeed, WME and the polygalacturonic acid-degrading enzyme of CM-II have the same ion exchange properties on both DEAE- and CM-cellulose, and both activities have the same elution volumes upon gel filtration. Additional evidence comes from the fact that the water-soluble polymers released from the cell wall by the action of WME are rich in uronic acids (see "Results"). And polygalacturonic acid acts to inhibit the ability of WME to attack cell walls. In fact, polygalacturonic acid inhibits WME at the same concentration of the polyuronide that was found to saturate the ability of CM-II to catalyze degradation of polygalacturonic acid (Fig. 8 and Table VI). However unlikely, it still remains possible that the WME and polygalacturonic acid-degrading activities of CM-II are not catalyzed by the same enzyme.

It is apparent that unaltered cell walls contain polymers which are potential substrates for the D-III enzymes, but, because of the structural organization of the cell wall, the polymers are not susceptible to degradation by the enzymes of D-III. The WME-catalyzed alteration of the cell wall, which permits subsequent degradation of the wall polymers by the D-III enzymes, does not require the presence of the D-III enzymes. Approximately the same degree of D-III-catalyzed degradation is observed when cell walls are pretreated with WME as observed when both D-III and WME are present simultaneously.

It appears that WME cleaves bonds important to the structural integrity of the wall; once these linkages are broken, remaining wall polysaccharides are susceptible to degradation by the enzymes in D-III. The bonds broken by the action of WME could be responsible for crosslinking of wall polymers. Since a polygalacturonic acid-degrading enzyme appears to be responsible for the wall-loosening effect, galacturonic acid might be a constituent of the crosslinking polymers. However, the enzyme in CM-II has only a poor ability to degrade polygalacturonic acid (Fig. 7), and it has no measurable ability to degrade pectin (Table II). This would suggest that if the crosslinking saccharides contain galacturonic acid, the galacturonide residues will be in an environment distinctly different from the galacturonide residues of the model substrate, citrus polygalacturonic acid.

It seems unlikely that the WME described here would have the same substrate specificity as a similar enzyme produced by higher plants. The enzyme described here is produced by a microorganism for the purpose of polysaccharide degradation, while a plant enzyme would catalyze an alteration of the cell walls which should be repairable (30). It has been demonstrated that the structural peptidoglycan of bacterial cell walls can be hydrolyzed at a number of different bonds by a variety of enzymes, all leading to the weakening of the wall (21, 43). In analogy, degradation of one of several different linkages in the critical structural polymers of the plant cell wall could result in wall weakening. This would suggest the possibility of a series of different WME enzymes. Such a possibility is supported by the fact that pronase, which is known to degrade the wall protein extensin (27), can partially replace the requirement for WME in the wall degradation process.

Since the successful infection of a plant by a pathogen is often accompanied by degradation of the host cell wall (10, 23), it is quite likely that enzymes with WME activity play a role in pathogenesis. In this respect, it is important to note that the possible existence of such WME enzymes was first suggested as a result of experiments carried out with enzymes secreted by the bean pathogen *Colletotrichum lindemuthianum* (5). Pathogen-secreted enzymes extracted from the lesion area of infected plants (10) and prepared from the media of cultured pathogens (5) have been demonstrated to degrade cell walls. It is likely that these enzyme preparations contain an enzyme with WME activity. Pathogenic enzymes containing WME would not necessarily be polygalacturonic acid-degrading enzymes. It will be necessary to determine, in each case, the enzyme activity necessary for wall degradation. Knowledge of the control of this class of degradative enzyme

(WME) is likely to lead to a clearer understanding of the role that the host cell wall plays in host-pathogen interactions.

Our evidence suggests that many polysaccharide-degrading enzymes cannot directly attack cell walls. Since cell wall degradation plays an important role in plant physiology, it is likely that enzymes with properties similar to those displayed by WME are present in plants. Abscission, which results from the degradation of the cell wall in the abscission zone (36), would be an example of a physiological process in which WME activity could be important. Cell wall degradation is also known to occur during growth by elongation (32, 33). It has been suggested that indoleacetic acid-induced loosening of the wall, which is responsible for growth by elongation (13), results from the breakage of cross-linkages between wall polymers (30). An enzyme affecting linkages similar to those degraded by WME could well result in elongation growth; the amount or activity of such an enzyme in the wall could be controlled by hormones such as auxin.

No WME activity has yet been reported in higher plants. However, it is most interesting that the enzymes extracted from the cell walls of sycamore cells (*Acer pseudoplatanus*) are able to catalyze at least partial degradation of sycamore cell walls (unpublished data of this laboratory). Such an enzyme preparation may contain WME activity. A search must be made for higher plant wall-loosening enzymes, and their role in cell wall metabolism must be defined.

## LITERATURE CITED


1. ABELES, F. B. 1969. Abscission: role of cellulase. Plant Physiol. 44: 447–452.
2. AGRAWAL, K. M. L. AND O. P. BAHL. 1968. Glycosidases of *Phaseolus vulgaris* II. Isolation and general properties. J. Biol. Chem. 243: 103–111.
3. ALBERSHEIM, P. 1965. Biogenesis of the cell wall. *In*: J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, Inc., New York. pp. 298–321.
4. ALBERSHEIM, P. 1965. The substructure and function of the cell wall. *In*: J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, Inc., New York. pp. 151–186.
5. ALBERSHEIM, P., T. M. JONES, AND P. D. ENGLISH. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7: 171–194.
6. ALBERSHEIM, P. AND U. KILLIAS. 1962. Studies relating to the purification and properties of pectin transeliminase. Arch. Biochem. Biophys. 97: 107–115.
7. ALBERSHEIM, P., D. J. NEVINS, AND P. D. ENGLISH. 1968. The biochemistry of plant cell wall polysaccharides: A basis for disease resistance. *In*: The Phytopathological Society of Japan, Biochemical Regulation in Diseased Plants or Injury, Tokyo. pp. 297–311.
8. ALBERSHEIM, P., D. J. NEVINS, P. D. ENGLISH, AND A. KARR. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340–345.
9. BAILEY, P. J., W. LIESE, R. ROESCH, G. KEILICH, AND E. G. AFTING. 1969. Cellulase ($\beta$-1,4-glucan 4-glucanohydrolase) from the wood degrading fungus *Polyporus schweinitzii* Fr. I. Purification. Biochim. Biophys. Acta 185: 381–391.
10. BATEMAN, D. F., H. D. VAN ETTEN, P. D. ENGLISH, D. J. NEVINS, AND P. ALBERSHEIM. 1969. Susceptibility to enzymatic degradation of cell walls from bean plants resistant and susceptible to *Rhizoctonia solani* Kuhn. Plant Physiol. 44: 641–648.
11. BERGMAN, I. AND R. LOXLEY. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal. Chem. 35: 1961–1965.
12. BITTER, T. AND H. M. MUIR. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4: 330–334.
13. BONNER, J. 1961. On the mechanics of auxin-induced growth. *In:* Plant Growth Regulation. 4th International Conference on Plant Growth Regulation. Iowa State University Press, Ames. pp. 307–328.
14. CLELAND, R. 1968. Hydroxyproline formation and its relation to auxin-induced cell elongation in *Avena* coleoptiles. Plant Physiol. 43: 1625–1630.
15. COWLING, E. B. 1963. Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis. *In*: E. Reese, ed., Enzymic Hydrolysis of Cellulose and Related Materials. The Macmillan Company, New York. pp. 1–32.
16. DATKO, A. H. AND G. A. MACLACHLAN. 1968. Indoleacetic acid and the synthesis of glucanases and pectic enzymes. Plant Physiol. 43: 735–742.
17. ENGLISH, P. D. AND P. ALBERSHEIM. 1969. Host-pathogen interactions: I. A correlation between $\alpha$-galactosidase production and virulence. Plant Physiol. 44. 217–224.
18. EVANS, M. L. AND P. M. RAY. 1969. Timing of the auxin response in coleoptiles and its implications regarding auxin action. J. Gen. Physiol. 53: 1–20.
19. FACEY, V. 1950. Abscission of leaves in *Fraxinus americana* L. New Phytol. 49: 103–116.
20. FIELDING, A. H. AND L. HOUGH. 1965. The synthesis of *p*-nitrophenyl-$\alpha$-L-arabinofuranoside. Carbohyd. Res. 1: 327–329.
21. GHUYSEN, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. 32: 425–464.
22. GREEN, M., K. C. TSOU, R. BRESSLER, AND A. M. SELIGINAN. 1955. The colorimetric determination of leucine aminopeptidase activity with L-leucyl-$\beta$-naphthylamide hydrochloride. Arch. Biochem. Biophys. 57: 458–474.
23. HANCOCK, J. G. 1968. Degradation of pectic substances during pathogenesis by *Fusarium solani* f. sp. cucurbitae. Phytopathology 53: 62–69.
24. KEILICH, G., P. J. BAILEY, E. G. AFTING, AND W. LEISE. 1969. Cellulase ($\beta$-1,4-glucan 4-glucanohydrolase) from the wood degrading fungus *Polyporus schweinitzii* Fr. II. Characterization. Biochim. Biophys. Acta 185: 392–401.
25. LAMPORT, D. T. A. 1965. The protein component of primary cell walls. Advan. Bot. Res. 2: 151–218.
26. LAMPORT, D. T. A. 1967. Hydroxyproline-*O*-glycosidic linkage of the plant cell wall glycoprotein extensin. Nature 216: 1322–1324.
27. LAMPORT, D. T. A. 1969. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzyme degradation of primary cell walls. Biochemistry 8: 1155–1163.
28. LEË, M. AND J. D. MACMILLAN. 1968. Mode of action of pectic enzymes. I. Purification and certain properties of tomato pectinesterase. Biochemistry 7: 4005–4010.
29. LEE, S., A. KIVILAAN, AND R. S. BANDURSKI. 1967. *In vitro* autolysis of plant cell walls. Plant Physiol. 42: 968–972.
30. LOCKHART, J. A. 1965. Cell extension. *In*: J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, Inc., New York. pp. 826–849.
31. LOWRY, O. H., N. J. ROSEBROUGH, L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
32. MACLACHLAN, G. A. AND C. T. DRUDA. 1965. Changes in concentration of polymeric components in excised pea-epicotyl tissue during growth. Biochim. Biophys. Acta 97: 288–299.
33. MACLACHLAN, G. A. AND M. YOUNG. 1962. Breakdown and synthesis of cell wall during growth. Nature 195: 1319–1321.
34. MCNAB, J. M., D. J. NEVINS, AND P. ALBERSHEIM. 1967. Differential resistance of cell walls of *Acer pseudoplatanus*, *Triticum vulgare*, *Hordeum vulgare*, and *Zea mays* to polysaccharide degrading enzymes. Phytopathology 57: 625–631.
35. MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426–428.
36. MORRÉ, D. J. 1968. Cell wall dissolution and enzyme secretion during leaf abscission. Plant Physiol. 43: 1545–1559.
37. NEISH, A. C. 1965. Coumarins, phenylpropanes, and lignin. *In*: J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, Inc., New York. pp. 581–614.
38. REESE, E. T., R. G. H. SIU, AND H. S. LEVINSON. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59: 485–497.
39. SCHWERT, G. W. AND Y. TAKENAKA. 1955. Spectrophotometric determination of trypsin and chymotrypsin. Biochim. Biophys. Acta 26: 570–575.
40. SELBY, K. AND C. C. MAITLAND. 1967. The cellulase of *Trichoderma viride*. Separation of the components involved in the solubilization of cotton. Biochem. J. 104: 716–724.
41. SELBY, K. AND C. C. MAITLAND. 1967. Components of *Trichoderma viride* cellulase. Arch. Biochem. Biophys. 118: 254–257.
42. STOWE, B. B. AND J. B. OBREITER. 1962. Growth promotion in pea stem sections. II. By natural oils and isoprenoid vitamins. Plant Physiol. 37: 158–164.
43. STROMINGER, J. L. AND J. M. GHUYSEN. 1967. Mechanisms of enzymes in determination of wall structure and their role in cell metabolism. Science 156: 213–221.
44. YAGER, R. E. 1960. Possible role of pectic enzymes in abscission. Plant Physiol. 35: 157–162.