POLYGALACTURONATE LYASE ACTIVITY IN *BACTEROIDES* FROM THE HUMAN COLON

by

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INTRODUCTION

Dietary fiber has gained increased nutritional significance as a dietary component due to recent epidemological evidence linking consumption of fiber and decreased incidence of colonic diseases. The general physiological benefits of fiber have been attributed to several of its physical and chemical properties. However, little consideration has been given to alterations of fiber structure and associated properties by the colonic microflora. These alterations in dietary fiber could affect its physiological function(s).

Several species of the genus Bacteroides from the human colon can ferment isolated plant polysaccharides and plant cell walls (dietary fiber). Pectic enzymes produced by these bacteria, particularly the enzyme polygalacturonate lyase, are hypothesized to be instrumental in the degradation of plant cell walls. Bacterial polygalacturonate lyases in other systems are capable of causing extensive degradation of the cell walls of higher plants.

Since species of Bacteroides can ferment plant cell walls and given the information that bacterial polygalacturonate lyase can cause degradation of plant cell walls, the primary question advanced by this research project was: Can species of Bacteroides from the human colon produce a polygalacturonate lyase? The results with four strains of Bacteroides isolated from the human colon demonstrate the presence of polygalacturonate lyase. The lyase has been partially characterized,

including tests of its ability to degrade peanut cell wall, a prototype dietary fiber.

LITERATURE REVIEW

Dietary fiber has been defined as the fraction of plant foodstuffs which is resistant to digestion by the enzymes of the human gastrointestinal tract (Trowell, 1972). Interest in the nutritional aspects of fiber has increased due to epidemological evidence which has linked high dietary intakes of fiber with the decreased incidence of colonic disorders including cancer of the colon and rectum (Burkitt, 1971) and diverticulosis (Painter and Burkitt, 1971). The general physiological benefits of fiber are attributed to its water holding capacity which allows fiber to act as a bulking agent (Huang, et al., 1978) and its cation exchange capacity (McConnell, et al., 1974). However, these characteristics have only been studied in vitro; they do not take into consideration the possible fermentation of dietary fiber by the colonic microflora. Bacterial degradation of fiber could cause chemical and physical alterations in dietary fiber and thus change its physiological function(s). Pectic enzymes produced by certain species of bacteria have been shown to catalyze the degradation of complex polysaccharides such as are found in dietary fiber. These enzymes may contribute significantly to the degradation of dietary fiber in the human colon.

Composition of Dietary Fiber

Dietary fiber is primarily composed of three carbohydrate fractions (cellulose, hemicellulose, and pectin) and one noncarbohydrate fraction

(lignin) (Huang, et al., 1978). Each of these components differs in both composition and chemical structure. Cellulose is a linear glucose polymer with each of its glucose units joined in beta 1,4 - glycosidic linkages. Hemicelluloses are defined chemically as noncellulosic polysaccharides which are readily hydrolyzed with dilute acid or extracted with alkali. Structurally, hemicelluloses consist of beta 1,4 - xylopyranose units with possible arabino-, glyco-, and/or galactopyranoside side chains (Akin and Barton, 1983). Pectic substances are a heterogeneous group of polysaccharides. Pectins consist of a main rhamnogalacturonan chain joined by alpha 1,4 - glycosidic linkages between the galacturonsyl units of the chain with intermittent 2 - linked rhamnosyl residues. The rhamnogalacturonan polymer may be partially methylated at the carboxyl groups of the galacturonan units as well as having side chains of arabinose and galactose (Talmadge, et al., 1973). Lignin, the noncarbohydrate portion of fiber is an irregular polymer of phenylpropanoid units. Its specific structure is not clearly understood (Huang, et al., 1978).

These four components of dietary fiber are also found in the primary and secondary cell walls of all higher plants and function as structural polysaccharides (Talmadge, et al., 1973). Therefore, it may be assumed that dietary fiber is roughly analogous to the cell walls of higher plants. However, "storage polysaccharides" and "gums" which are found in substantial amounts in some plants are also classified as dietary fiber.

The Colonic Microflora and its Possible Role in Degradation of Dietary Fiber

Recently, there has been increased scientific research regarding the composition and metabolism of the microflora of the human colon. This interest may be accounted for by two factors: The development of microbial culturing techniques which permits the study of anaerobes (Hill and Drasar, 1975) and the hypothesis that intestinal bacteria may be etiologically related to noninfective diseases of the colon, specifically cancer of the large bowel (Hill, et al., 1971). Although a thorough understanding of intestinal bacteria has not yet been achieved, studies have shown the colon microflora to be a complex and highly diverse ecosystem.

The colon flora is usually inferred from the bacteria found in the feces. Moore and Holdeman (1975) have found in their studies of human intestinal bacteria that the fecal flora are representative of the bacteria residing in the ascending, transverse, and descending colon and the rectum.

The intestinal bacteria are predominantly anaerobic. Non-sporing, anaerobic, rod-shaped microorganisms account for more than 99% of the total microflora (Hill and Drasar, 1975). The genus Bacteroides accounts for approximately 20% of this total but other species present include Bifidobacterium, Eubacterium, Peptostreptococcus, Lactobacillus, Fusobacterium, Coprococcus, and Ruminococcus. The latter genera all together account for about 50% of the normal flora of the colon

(Salyers, et al., 1977c).

Many of these bacteria are saccharolytic, especially the genus Bacteroides. However, carbohydrate sources available to the colonic microflora would have to be those which are not digested in the upper gastrointestinal tract. Most simple sugars and some polysaccharides such as starch are efficiently degraded and absorbed by the human digestive system. Energy sources for intestinal bacteria could be provided through the carbohydrate moiety of glycoproteins such as the mucins secreted by mucosal cells lining the intestines or the complex plant polysaccharides found in dietary fiber (Salyers, et al., 1977b).

Salyers, et al. (1977b) have demonstrated that many Bacteroides species of the human colon are capable of fermenting isolated plant polysaccharides. However, degradation of these model substrates (i.e. pectin) cannot be an accurate gauge of the extent of degradation of the intact plant cell wall (dietary fiber) by the colonic microflora. Current knowledge of plant cell walls suggest that they present a highly complex substrate to intestinal bacteria. Keegstra, et al. (1973), using degradative enzymes to fragment isolated cell walls of sycamore cell cultures, advanced a model for the molecular structure of primary cell walls. The model projects cellulose and the hemicelluloses as being interconnected by extensive hydrogen bonding. The hemicelluloses are interspersed with a network of pectic polysaccharides which are covalently bound to the hemicelluloses.

The primary cell wall may therefore be considered as one large macromolecule. This complicated mesh of polymers could be resistant to bacterial degradation in the colon.

In vitro fermentation of plant cell walls by intestinal microflora was demonstrated by Dekker and Palmer (1981). A *Bacteroides* isolated from human feces was found to utilize a plant cell wall prepared from peanuts as the sole carbon source in an otherwise nutritionally adequate medium. It may be assumed that since this fermentation can occur *in vitro*, certain species of intestinal bacteria possess the capacity to degrade intact plant cell walls *in vivo*.

Dekker and Palmer (1981) provided some insight into the biochemical mechanisms by which colonic bacterial degradation of the cell wall occurs. Using cultures of *Bacteroides* grown on a peanut cell wall growth media, a crude enzyme solution was prepared from the sonicated bacterial cells. The enzyme preparation had high activity with polygalacturonate as a substrate, evidenced by a large increase in the reducing groups in the reaction mixture. Treatment of peanut cell walls with the crude enzyme gave a predominance of uronic acid residues as the final products. While specific enzymes in these reactions were not elucidated, the results indicated that pectic enzymes were present in the enzyme preparation and could play a key role in initiating cell wall degradation.

Pectic Enzymes

Pectic enzyme may be classified into two general categories: Demethylating enzymes (pectinesterases) and depolymerizing enzymes (polygalacturonases and polygalacturonate lyases). Pectinesterases (PE) de-esterify pectic polysaccharides by splitting off the methyl group from the carboxyl moiety of the galacturonan chain (figure 1). The enzyme demethylates pectin beginning from the reducing end of the molecule or at a point next to a free carboxyl group. It is highly specific for the methyl ester of polygalacturonate. The ethyl ester is attacked but at a much slower rate. Pectinesterases are produced by plants, molds, and some bacteria. Known bacterial PEs have pH optimas near neutrality (7.0) (Pilnik and Rombouts, 1979).

Polygalacturonases (PG) and polygalacturonate lyases (PGAL) both depolymerize the pectin molecule but by different mechanisms. Polygalacturonases split the alpha 1,4 - linkages of polygalacturonic acid by hydrolysis, while lyases split this glycosidic linkage by beta elimination, forming a double bond between carbons 4 and 5 of the galacturonsyl units of the pectic chain (figure 1). Both types of depolymerizing enzymes can only attack the galacturonan polymer if the susceptible alpha 1,4 - glycosidic linkage is next to a free carboxyl group. Therefore, these enzymes preferentially depolymerize substrates with a low methoxy content

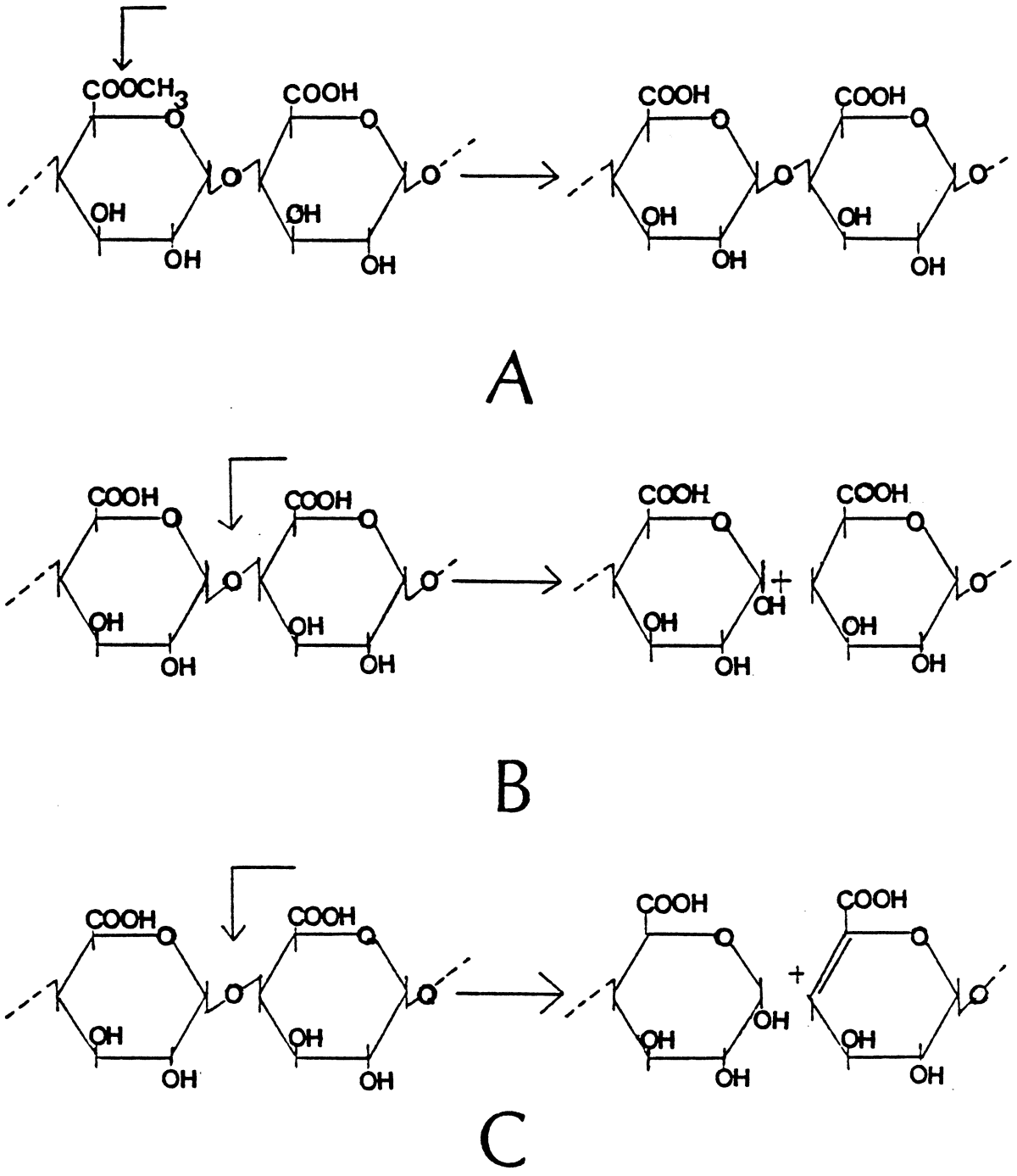

Figure 1. Breakdown of pectic molecule by pectic enzymes. Arrows indicate point of attack. (A) Pectinesterase (B) Polygalacturonase (C) Polygalacturonate lyase.

(Pilnik and Rombouts, 1979).

Polygalacturonases and polygalacturonate lyases can be differentiated on the basis on their sharply different pH optima, cation dependency, and from the products produced in reaction mixtures. PGs have pH optima of 4.0 - 5.5 while PGALs have highly alkaline pH optima (8.0 - 9.5). PGALs require calcium ions for maximal activity and produce unsaturated uronic acid compounds which absorb strongly at 235 nm. PGs have no ion dependence and produce saturated uronide products, which do not absorb in the ultraviolet range (Collimer, et al., 1982).

Although PGs are produced by some bacteria PGALs are considered to be the major pectic enzyme produced by pectinolytic bacteria. Polygalacturonate lyases are widespread among bacteria, having been found in members of the genera Clostridium (MacMillan and Vaughn, 1964), Bacillus (Kelly and Fogarty, 1975; Kurowski and Dunleavy, 1976), Erwinia (Moran, et al., 1968; Stack, et al., 1980), Pseudomonas (Zucker and Hankin, 1971; Hagar and McIntyre, 1972), Azospirillum (Tien, et al., 1981), Bacteroides (Wojciechowicz, 1971),Yersinia and Klebsiella (Chatterjee, et al., 1979). Most of the PGALs produced by these genera attack polygalacturonic acid in a random mode; however, Clostridium multifermentans produces a PGAL which degrades polygalacturonate from the reducing end producing uronic acid dimers (0 - (4 - deoxy - beta- threo - hexopyranose 4 - enyluronic acid) - (1,4) - D-galacturonic acid) (MacMillan, et al., 1964). In

all cases, the PGALs had an alkaline pH optima and activity was enhanced by the presence of calcium ions.

PGAL appears to be primarily an inducible enzyme. Constitutive PGAL activity has been observed in Bacillus sp RK 9 where PGAL activity was greatest when bacterial cells were cultured in media containing sucrose as the sole carbon source (Kelly and Fogarty, 1975). However, the majority of pectinolytic bacteria require carbon sources such as polygalacturonic acid or pectin before appreciable PGAL synthesis can occur. Rate of PGAL synthesis depends upon the bacterial source of the enzyme. The maximum rate of PGAL production can occur in Erwinia chrysanthemi after 2 hours of incubation using polygalacturonate as the inducing carbohydrate whereas maximum PGAL synthesis in Yersinia enterocolitica occurs after 36 hours of incubation with polygalacturonate (Chatterjee, et al., 1979).

Rolé of Polygalacturonate Lyase in Cell Wall Degradation

Bacterial PGALs have been observed to degrade the cell walls of higher plants. These PGALs are produced by several bacteria, but perhaps the most intensely studied enzymes are those synthesized by the genera Pseudomonas and Erwinia.

Hagar and McIntyre (1972) isolated two PGALs from potato tuber tissue inoculated with Pseudomonas fluorescens. These purified PGALs could produce tissue maceration in 400 µm sections of potato tissue (pH 8.5) in 60 minutes or less. The loss of cohesion in the potato tissue is attributable to PGAL breakdown of the pectic

fraction of the potato cell wall.

An endo-PGAL purified from culture filtrates of Erwinia chrysanthemi produced extensive degradation of cell walls prepared from suspension cultures of beans and rice (Baker, et al., 1980). Twenty-seven percent of the noncellulosic wall carbohydrate was released from bean cells after one hour of treatment with PGAL. This corresponded to 50% of the PGAL susceptible carbohydrate in the bean cell wall. Three percent of the noncellulosic carbohydrates, accounting for 60% of the enzyme susceptible carbohydrate fraction of the rice cell wall, was released after one hour of treatment with PGAL. Also, PGAL degraded cell walls of beans and rice showed structural differences including swollen cell walls and wall separations. These changes in the cell walls point to the significance of PGALs in cell wall degradation.

The above two studies suggest that breakdown of the pectic network in the plant cell walls is a key reaction in cell wall degradation. One of the biochemical mechanisms by which intestinal bacteria degrade plant cell walls may be by PGALs. The possible role of pectic enzymes such as PGAL in the breakdown of plant polysaccharides as found in dietary fiber should not be ignored. Breakdown of the cell wall by pectic enzymes may make the substrate more accessible for further bacterial degradation. Further knowledge concerning pectic enzymes and the sequence in which they degrade dietary fiber could provide important information regarding the degradation of fiber

in the colon.

MATERIALS AND METHODS

A. Peanut Cell Wall Preparation

Peanuts were chosen as the source for cell walls since they are composed primarily of parenchymal cells and thus provide a relatively homogenous substrate for enzymatic assays. Peanuts are also a normal constituent of the human diet; therefore, peanut cell walls would not be an unlikely carbohydrate source for intestinal bacteria.

1. Preliminary Processing

Peanuts were purchased from a local supermarket. The peanuts were processed as described by Dekker (1979). Peanuts were shelled, de-germed, chopped into small pieces, and then were dried at 100°C for 4 hours in a forced convection oven. The dried peanuts were extracted for 8 hours in a Soxhlet appartus with a 50:50 mixture of petroleum ether and anhydrous ether. The defatted peanuts were air dried under a hood and then ground to a fine powder using a mortar and pestle. The processed peanuts were stored in a dessicator over a saturated solution of $CaCl_2$ in order to maintain a constant, low moisture content (about 7.0%) of the peanut powder.

2. Enzymatic Digestion

A cell wall preparation was needed that would parallel or resemble plant cell walls as they would enter the colon. The method of Hellendoorn, et al. (1975) as modified by Dekker (1979) was chosen since it involves digestion of the peanuts with enzymes and under

conditions which are similar to those of the human digestive system. A 4.0 gram sample of ground, defatted peanuts was mixed with 200 mg of pepsin (Sigma Chemical Co., St. Louis, MO), a few crystals of thymol, and 200 ml of 0.1 N HCl. The mixture was incubated for 9 hours at 40°C in an agitating water bath (Fermentation Design, Inc., Allentown, PA). After incubation was complete, the pH of the reaction mixture was adjusted to 6.8 with 4 N NaOH. Then 200 mg of pancreatin (Sigma Chemical Co., St. Louis, MO), 0.6 g of sodium dodecyl sulfate (to help solubilze the pancreatin), and 100 ml of 0.067 M phosphate buffer (pH 6.8) were added to the reaction mixture. The mixture was incubated for 9 more hours at 40°C in an agitating water bath. The enzymatic reaction was terminated by acidifying the reaction mixture to a pH of 4 - 5 with 4 N HCl. The suspension was centrifuged at 23,000 x g for 15 minutes and 4°C. The supernatant fluid was decanted and discarded. The digested residue was washed with distilled water and recentrifuged as above. The water wash was repeated twice and was followed by three acetone washings. The residue was allowed to air dry under a hood. The digestion procedure was repeated with additional 4 gram samples of defatted peanut powder until a total of 10 grams of cell wall material was obtained. The 10 gram pooled cell wall sample was washed with acetone and centrifuged as described previously. The acetone wash was repeated twice. These acetone washes mixed the pooled samples to give a more homogeneous cell wall material. The peanut cell walls were dried under a stream of N_2 gas and stored

in a dessicator over a saturated solution of $CaCl_2$.

B. Cell Wall Composition

1. Starch Determination

Starch in the human digestive system is degraded and the products absorbed in the upper gastrointestinal tract. Any starch present in the cell wall preparation must be considered a contaminant since it is possible that colon bacteria would utilize starch in preference to the cell wall as a source of fermentable carbohydrate. Therefore, absence or a low concentration of starch in the cell wall preparation is highly desirable. The presence of starch was determined using a amyloglucosidase test (Thivend, et al., 1972). Amyloglucosidase (Sigma Chemical Co., St. Louis, MO) was mixed in cold 0.10 M sodium acetate buffer (pH 4.5) to give a final concentration of 15 mg/ml, 30 - 50 units/ml. Units were defined as mg of glucose released from starch in 3 minutes at pH 4.5 and 25°C. The enzyme solution was stirred for 10 minutes at 4°C and then centrifuged for 15 minutes at 15,000 x g and 4°C. The supernatant fluid was decanted and stored under refrigerated conditions.

A 100 mg sample of cell wall was suspended in 3.0 ml of 0.10 M sodium acetate buffer (pH 4.5), and placed in a boiling water bath for 5 minutes. The heat treatment gelatinizes any starch present in the cell wall material. Amyloglucosidase (2.0 ml) was added to the cooled cell wall suspension and the mixture incubated for one hour at room temperature with occasional stirring. The reaction mixture was

centrifuged for 10 minutes at 15,000 x g and 4°C. The supernatant fluid was collected, deionized using AG 501-X8 hydrogen-hydroxide resin (Biorad Labs, Richmond, CA), and filtered through a 0.45 μm filter (Gelman, Ann Arbor, MI). The filtered supernatant was analyzed for glucose by HPLC (high performance liquid chromotography), essentially as described by Palmer (1975). Sugars were separated on a μBondapak Carbohydrate column (Waters Associates, Milford, MA) at a flow rate of 1.5 ml/minute. The solvent system employed was water and acetonitrile (CH_3CN) mixed in a 25:75 ratio and detection was via refractive index. The HPLC equipment consisted of a M6000 pump, U6K injector, and Model # R401 refractive index detector (Waters Associates, Milford, MA).

2. Moisture Determination

Samples of 2.000 grams of cell wall material were dried in a vacuum oven for 16 hours at 60°C, cooled in a dessicator, reweighed, and % moisture calculated from the loss in weight.

3. Uronic Acid Determination

The uronic acid content of the cell walls was determined with the colorimetric assay of Ahmed and Labavitch (1977). Samples (5.0 mg) of cell wall material were dissolved in cold, concentrated H_2SO_4. The samples were then treated with sodium tetraborate reagent, boiled, and reacted with m-hydroxydiphenyl. The resulting color was measured in a Bausch and Lomb 710 spectrophotometer at 520 nm. D-galacturonate (Sigma Chemical, St. Louis, MO) was used

in preparation of a standard curve.

4. Cellulose Determination

The cellulose content of the cell wall material was analyzed by the method of Updegraff (1969). Samples (75 mg) of dried cell walls from the moisture determination (Materials and Methods, section B-2) were mixed in an acetic:nitric acid reagent and placed in a boiling water bath for 30 minutes. Treatment with acetic:nitric reagent solubilizes the lignin, hemicelluloses, and xylans present in the cell walls. The residue was washed with water and dissolved in 67% H_2SO_4. Glucose from the hydrolyzed cellulose was reacted with anthrone and cellulose content determined by the increase in absorbance at 620 nm in a Bausch and Lomb 710 spectrophotometer. A standard curve was prepared using vacuun dried BW-40 cellulose (Brown Co., Berlin, NH).

5. Noncellulosic Neutral Sugar Determination

The noncellulosic neutral sugars were obtained by hydrolzying the cell wall by a modification of the method of Saeman, et al. (1963). Cell wall material (10 mg) was weighed into four reacti-vials (Pierce Chemical Co., Rockford, ILL). Then 0.1 ml aliquots of 72% H_2SO_4 were added to each reacti-vial, and the mixture incubated in tightly capped vials with stirring for one hour at room temperature. At the end of this incubation, 2.8 ml of water were added to each vial and the mixture stirred for 10 minutes to insure adequate mixing. All four vials were tightly capped, placed on a

heating block, and incubated at 135°C. Since different sugars could have different rates of hydrolysis from the cell wall, a vial was removed from the heating block every 2 hours. The sample was cooled and a 0.1 ml aliquot was removed. The aliquot was mixed with 0.05 ml of a 5:1 H_2O:NH_4OH reagent and 0.85 ml distilled water. Samples were deionized with 0.1 ml AG 501-X8 hydrogen and hydroxide resin (Biorad Labs, Richmond, CA), filtered through a 0.45 μm filter (Gelman, Ann Arbor, MI), and analyzed by HPLC.

HPLC analysis of the hydrolyzed cell walls employed a 30 cm x 0.6 cm column of Ca^{++} form Aminex Q-15-S (Biorad Labs, Richmond, CA) opearated at 85°C (Conrad and Palmer, 1976). Sugars were eluted from the column with distilled water at a flow rate of 0.4 ml/minute. Sugars were detected after elution from the column by mixing the eluent with 2,2' bicinchoninate reagent, prepared as described by McFeeters (1980), which was also flowing at a rate of 0.4 ml/minute. The bicinchoninate reagent reacts with reducing sugars to give a peak absorbance at 560 nm. Increase in absorbance was measured at 546 nm using a Model 440 detector (Waters Associates, Milford, MA). Standard solutions of glucose, xylose, galactose, rhamnose, mannose, arabinose, and fucose at a concentration of 0.1 mg/ml of each sugar were analyzed to determine retention times and response factors for neutral sugars present in the prepared sample.

C. Bacterial Cultures

1. Bacterial Strains

Cultures were obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (VPI). The four *Bacteroides* strains were classified in the DNA homology group 3452A and were VPI strain numbers C14-3, C10-2, C7-8, and B6-11. These cultures were maintained in chopped meat broth (CMB) at room temperature. Master cultures were transferred every 3 months and working stock cultures were prepared as needed. Periodically, cultures were inoculated into brain heart infusion (BHI) broth and Gram stained to check for culture contaimination. All transfers were made using the VPI Anaerobic Culture System (Bellco, Inc., Vineland, NJ).

2. Growth Media

Growth media were prepared using the basal medium described by Varel and Bryant (1974) for *Bacteroides*. Composition of the medium is given in Table 1. Four different carbohydrates were tested as PGAL inducing substrates: glucose, D-galacturonate, polygalacturonic acid, or peanut cell wall. All carbohydrates with the exception of peanut cell wall were purchased from Sigma Chemical Co. (St. Louis, MO).

Carbohydrates, except peanut cell walls, were added to the other dry ingredients (excluding cysteine hydrochloride), the

Table 1. Composition of Growth Media

Component	Amount/Liter
K_2HPO_4	2.26 gm
KH_2PO_4	0.90 gm
NaCl	0.90 gm
$CaCl_2 \cdot 2H_2O$	27 mg
$CoCl_2.6H_2O$	10 mg
$FeSO_4.7H_2O$	4 mg
$MgCl_2.6H_2O$	20 mg
$MnCl_2.4H_2O$	10 mg
$Na_2CO_3.H_2O$	0.50 gm
$(NH_4)_2SO_4$	1.00 gm
Vitamin B_{12}	5 ug
Hemin	5 mg
Cysteine Hydrochloride	0.05 gm
*Carbohydrate	5.00 gm

*Glucose, D-galacturonate, polygalacturonic acid, or peanut cell wall.

mixture dissolved in distilled water, and then boiled under reflux for 10 minutes to reduce O_2 tension. Because of its insolubility, the peanut cell wall was weighed into individual test tubes and then suspended in an appropriate volume of carbohydrate free media so that final cell wall concentration was 0.5%. Growth media was prepared under the guidelines set for preparation of pre-reduced media by the Anaerobe Laboratory Manual, 4th ed. (1977), except that no resazurin was added and pH adjustment was performed under O_2-free N_2 gas instead of CO_2. The media was anaerobically dispensed into test tubes. The tubes were placed in a press clamp and autoclaved at 121°C and 15 psi for 15 minutes. Final pH of the sterile media was between 6.9 and 7.1.

D. Enzyme Preparation

The procedure used for preparing a crude enzyme fraction was based on the method described by Dekker (1979).

A 0.1 ml aliquot of working stock culture was diluted 1:100 in 0.1% peptone dilution blanks and 0.1 ml of the dilution blank was transferred into 7 ml of CMB. CMB cultures were incubated at 37°C for 16 hours. Aliquots from the CMB cultures were inoculated at a 5% level into growth media containing one of the carbohydrates listed in Materials and Methods, section C-2. Unless otherwise stated, inoculated growth media were incubated for 16 hours at 37°C.

After incubation, the cultures were centrifuged for 15 minutes at 15,000 x g and 4°C. The supernatant fluid was decanted and stored

at 4°C. This supernatant fluid constituted the extracellular enzyme fraction (ECF). A volume equal to the original supernatant volume of cold 0.05 M phosphate buffer (pH 6.8) was added to the pellet and the pellet material resuspended by gentle stirring with a sterile glass rod. The suspension was centrifuged as above and the buffer wash decanted and discarded. The pellet was resuspended in an equal volume of cold 1.0 mM dithiothrietol in phosphate buffer (pH 6.8). Cells in the suspension were sonicated using a Branson Sonifier Cell Disruptor (Danbury, CT). Cells were sonicated (35 W) for 2 minute intervals interspersed with 2 minute cooling periods for a total sonication time of 6 minutes. The cell suspension was kept in an ice bath (4°C) throughout the sonication procedure. The sonicated suspension was centrifuged for 15 minutes at 30,000 x g and 4°C. The supernatant fluid, which represented the intracellular fraction (ICF), was decanted and stored at 4°C. A flow chart of the basic procedure for enzyme preparation is given in figure 2. Both the ECF and ICF were layered with a few drops of tolulene to prevent microbial contamination and returned to storage at 4°C until ready for use. No enzyme preparation over 24 hours old was used.

E. Substrate Preparation

1. Polygalacturonic Acid

The polygalacturonic acid substrate solution was prepared according to MacMillan and Phaff (1966). A measured amount of

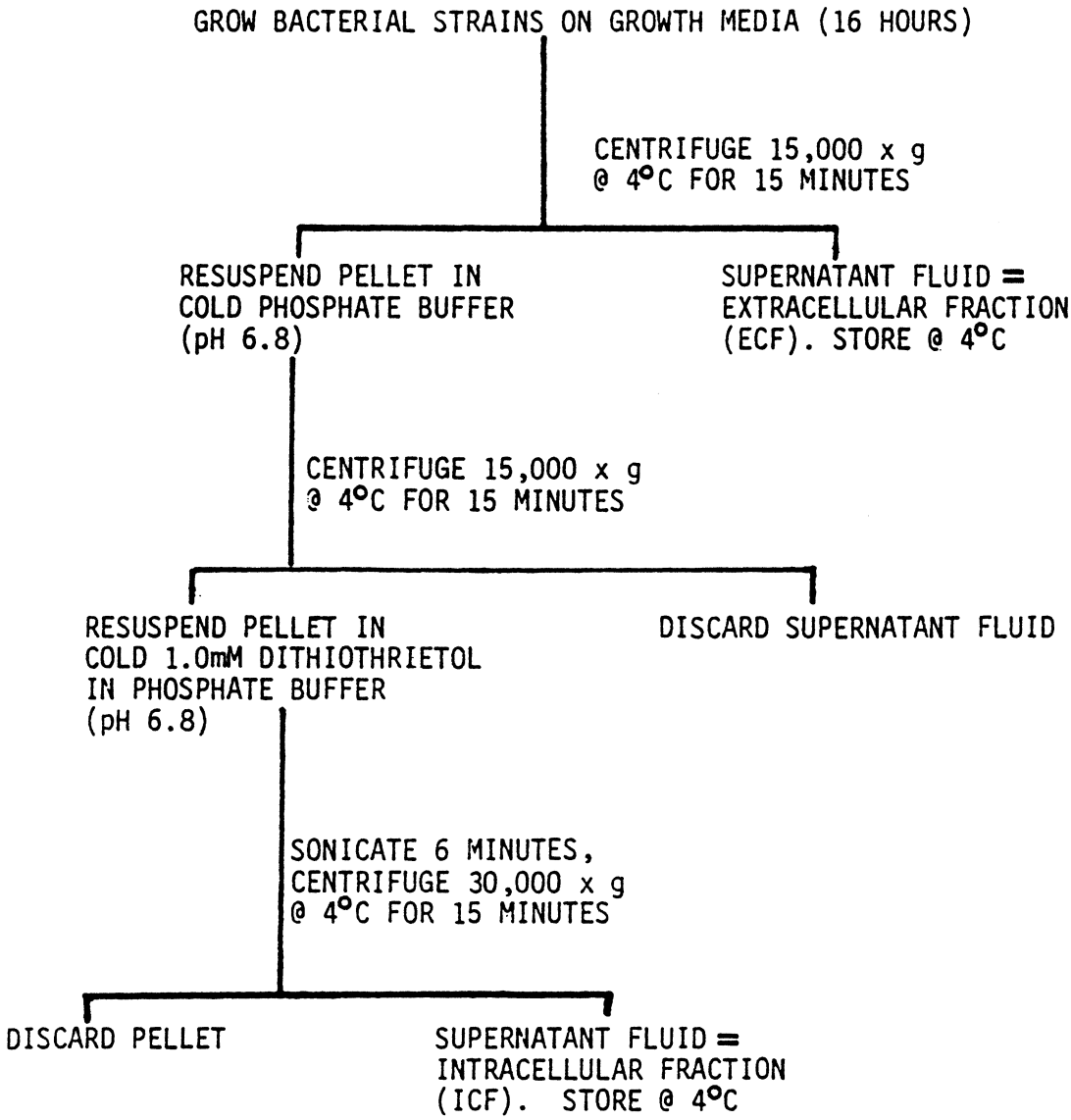

Figure 2. Basic procedure for the preparation of an enzyme solution from bacterial strains grown on growth media.

polygalacturonic acid was suspended in distilled water and dissolved by gradual addition of 1.0 N NaOH. The pH of the polygalacturonic acid solution was adjusted to 8.0 by dropwise addition of 0.3 N NaOH. A stock solution of substrate was prepared by mixing the above polygalacturonic acid solution, 0.05 M tris buffer (pH 8.0), and 0.005 M $CaCl_2$ in a 5:3:1 ratio. Unless otherwise stated, final polygalacturonic acid concentration of the substrate solution was 1.0%. Substrate not used immediately was stored frozen at 0°C. Prior to use in enzymatic assays, the substrate solution was filtered through a 0.45 μm filter (Gelman, Ann Arbor, MI) to remove small amounts of insoluble residue.

2. Pectin

Pectin (Sigma Chemical Co., St Louis, MO) used for substrate preparation had a methoxy content of 7.4%, which corresponds to a degree of esterification of approximately 68%. Pectin (1.8 gm) was suspended in 50 ml of distilled water. The suspension was heated gently with stirring until the pectin dissolved. The pH of the pectin solution was brought to 7.0 with 0.3 N NaOH and diluted to 100 ml with distilled water. Final substrate solution was prepared by mixing 100 ml of pectin solution, 60 ml of 0.02 M phosphate buffer (pH 7.0), and 20 ml of 0.01 M $CaCl_2$. This gave a final pectin concentration of 1.0% in the substrate solution. The substrate solution was filtered through a 0.45 μm membrane filter to clarify the solution. Substrates containing the pectin solution

were prepared immediately prior to use to avoid possible de-esterification of the pectin at neutral pH.

3. Peanut Cell Walls

A 50 mg sample of cell wall material was mixed throughly with 4.5 ml of 0.05 M tris buffer (pH 8.5) and 0.5 ml of 0.01 M $CaCl_2$. Cell wall substrate solutions were prepared immediately prior to use in enzyme assays.

F. Assays for Polygalacturonate Lyase

1. Enzymatic Assays Using Polygalacturonic Acid or Pectin as Substrates

a. Assays Using the Crude Enzyme Preparation from Bacterial Cells Grown on Glucose, D-galacturonate, or Polygalacturonic Acid

Polygalacturonate lyase (PGAL) activity was monitored by following the rate of increase of absorbance at 235 nm. This absorbance results from formation of the double bond at positions 4 and 5 in the substrate molecule. A 1.5 ml aliquot of substrate solution, containing either pectin or polygalacturonic acid, was warmed to 37°C. An equal volume of enzyme preparation (prewarmed to 25°C) was mixed well with the substrate solution and this incubation mixture transferred to a quartz cuvette in a thermostatically controlled cell holder at 37°C. The increase in absorbance was measured in a Lambda 3 System spectrophotometer (Perkin Elmer, Norwalk, CT). Reaction mixtures were read against a blank containing a 1:1 mixture of substrate solution and 1.0 mM dithiothrietol

in phosphate buffer. Controls were prepared in an identical manner except the enzyme solution was autoclaved (121°C and 15 psi for 5 minutes) to inactivate PGAL before addition to the substrate solution.

b. Assays Using the Crude Enzyme Preparation from Bacterial Cells Grown on Peanut Cell Wall

PGAL activity was monitored as described in the preceding section except that the crude enzyme preparation was filtered through a 0.22 μm membrane filter (Gelman, Ann Arbor MI), prior to addition to the substrate solution. Filtration removed the fine suspended material created by sonication of the peanut cell wall residue present after bacterial growth. Controls were prepared using autoclaved enzyme solutions.

2. Enzymatic Assays Using Peanut Cell Wall as Substrate

A 5.0 ml aliquot of enzyme solution was added to 5 ml of substrate, prepared as described in Materials and Methods, section E-3, and the mixture incubated at 37°C for 24 hours in an agitating water bath (GCA/Precision Scientific, Chicago, ILL). Blanks contained 5.0 ml of 0.05 M tris buffer (pH 8.5) instead of enzyme solution and controls were prepared using autoclaved enzyme solutions. Reactions were terminated by adding 10 ml of HCl - potassium chloride buffer (pH 2.0) to the reaction mixtures. The mixtures were centrifuged for 15 minutes at 15,000 x g and 4°C. The supernatant fluid was decanted and analyzed at 235 nm for increase

in absorbance when corrected for the absorbance values of the supernatant fluids from blanks and controls. The cell wall pellet was washed by stirring with 10 ml of distilled water and recentrifuged as above. This water wash was repeated twice followed by three washings with acetone. The residue was transferred to a drying pan and dried in a forced convection oven at 60°C for 2 hours. The uronic acid content of the residue was determined using the method of Ahmed and Labavitch (1977) (Materials and Methods, section B-3).

G. Characterization of Polygalacturonate Lyase

1. Determination of Limiting Substrate Concentration

Polygalacturonic acid substrate was prepared as described in Materials and Methods, section E-3 so that the final concentration of polygalacturonic acid in the substrate solution was 0.5%, 1.0%, or 1.5%. Enzyme assays with these three differenct substrate concentrations were performed as previously described (Materials and Methods, section D-1a.)

2. Polygalacturonate Lyase Activity with Variation in Induction Time

Bacteriodes cultures were grown for 4, 8, 12, 16, and 20 hours at 37°C in the basal growth medium containing polygalacturonic acid. Enzyme preparations for each of these induction times were prepared as described in Materials and Methods, section D. Enzyme assays with these enzyme preparations were performed using 1.0%

polygalacturonic acid substrate and following the increase in absorbance at 235 nm (Materials and Methods, section F-1a).

3. Polygalacturonate Lyase Activity with Variations in pH

The effect of pH on PGAL activity in the range of 3 - 10 was determined. Polygalacturonic acid substrate was prepared as described previously (Materials and Methods, section E-1) except that in place of tris buffer (pH 8.0) the following buffers were used: 0.02 M sodium citrate buffer (pH values 3.0, 4.0, 5.0, 6.0), 0.05 M tris buffer (pH values 7.0, 8.0, 8.5, and 9.0), and 0.05 M sodium carbonate buffer (pH 10.0). Enzyme assays using these substrate solutions were performed as described in Materials and Methods, section F-1a.

4. Polygalacturonate Lyase Activity with Different Concentrations of $CaCl_2$

Polygalacturonic acid substrate solution (Materials and Methods section E-1) were prepared to contain the following final concentrations of $CaCl_2$: 10^{-5} M, 5×10^{-5} M, 10^{-4} M, 5×10^{-4} M, 10^{-3} M, 5×10^{-3} M, and 10^{-2} M. A control substrate containing no added $CaCl_2$ was also tested to determine in PGAL activity was enhanced by calcium ions. Enzyme assays were performed as described in section F-1a of Materials and Methods.

5. Polygalacturonate Lyase Activity in the Presence of Ethylenediaminetetraacetic Acid (EDTA)

EDTA is a chelating agent which can bind cations in solution. To determine if cations are necessary for PGAL activity, EDTA was added to the reaction mixutre. A 0.1 ml aliquot of 0.6% EDTA was added to 1.4 ml of 1.0% polygalacturonic acid substrate at pH 8.5 and 37°C. An equal volume of enzyme solution was added to the substrate and increase in absorbance at 235 nm and 37°C was monitored in a Lambda 3 System spectrophotometer (Perkin Elmer, Norwalk, CT) for 5 minutes. At the end of this period, 0.2 ml of 0.01 M $CaCl_2$ was added to the reaction mixture and the rate of double bond formation was followed at 235 nm and 37°C for a further 5 minutes.

RESULTS AND DISCUSSION

A. Cell Wall Preparation

The yield of cell wall from the enzymatic digestion procedure of Hellendoorn, et al. (1975) was approximately 6% of the weight of the defatted peanut material. This low yield and the lenghty digestion and isolation procedure were not conducive to producing large quantities of cell wall material. A total of 10 grams of peanut cell wall was prepared for this study.

B. Cell Wall Compostion

Composition of the peanut cell wall is given in Table 2. Despite the extensive enzymatic digestion, the cell wall was not entirely starch free. Starch is potentially a more fermentable carbohydrate for bacterial growth than the peanut cell wall itself. At 2.7% starch in the cell wall, and at 0.5 gram/100 ml of cell wall in the growth medium, the starch concentration in the medium would only be approximately 0.01%. Even assuming all the starch was readily available for fermentation, this starch concentration would only support limited bacterial growth. Any growth observed in the growth medium containing peanut cell wall can be assumed to represent growth from fermentation of the peanut cell wall only.

The uronic acid, cellulose, and noncellulosic neutral sugar contents of the cell wall were analyzed by independent methods. These methods were selected for the following reasons: The component

Table 2. Compostion of the Peanut Cell Wall

Component	Percent by Weight
Starch	2.7 ± 0.2%*
Uronic Acid	14.0 ± 0.0%
Cellulose	24.2 ± 0.9%
Noncellulosic Neutral Sugars	38.4 + 0.4%
Moisture	7.4 ± 0.2%
Undetermined	13.3%

*Mean ± standard deviation (n=3)

**Obtained by subtracting the sum of the determined components from 100. This fraction has traditionally been termed as lignin but may also contain protein, Maillard Browning products, and ash.

assayed could be analyzed without excessive background interference from other materials present and only small sample sizes (mg) of cell wall were required for analysis.

The uronic acid content (14.0%) of the cell wall represents mainly the galacturonan backbone of the pectic polysaccharides. Since this uronide polymer is a substrate for PGAL, 14.0% of the cell wall is theoretically susceptible to degradation by PGAL.

The noncellulosic neutral sugar content of the cell wall was 38.4%. Although no attempt was made to calculate the concentrations of individual sugars because of poor resolution between sugar peaks, a value for the total neutral sugar content could be calculated. It was clear from the chromatograms that xylose, arabinose, galactose, and glucose (noncellulosic) were the major neutral sugars present in the peanut cell wall in agreement with the results of Dekker and Palmer (1981). This fraction of the cell wall represents the neutral sugars of the hemicelluloses and any side chain components of the pectic polysaccharides.

The undetermined fraction was obtained by subtracting the sum of the determined components from 100. Traditionally this fraction has been termed lignin, but it may also contain protein, Maillard Browning products, and ash.

C. Constitutive Versus Induced Polygalacturonate Lyase Activity in Bacteroides Strains

The four Bacteroides strains used in this study were selected

on the basis of their ability to ferment pectin and polygalacturonic acid (Salyers, et al., 1977b). All four strains were grown on glucose and D-galacturonate as non-inducing carbohydrate sources and also on peanut cell wall and polygalacturonic acid as potential inducing carbohydrate sources. Both the intracellular fraction (ICF) and the extracellular fraction (ECF) of all four strains were tested for PGAL activity on 1.0% polygalacturonic acid substrate (pH 8.0), by monitoring the rate of double bond formation at 235 nm and 37°C. This increase in absorbance at 235 nm, with this substrate and at this alkaline pH, is highly specific for and characteristic of PGAL. No PGAL activity was observed in enzyme preparations from bacterial cells grown on glucose and D-galacturonate. PGAL activity was present in all four Bacteroides grown on polygalacturonic acid or cell wall media.

These results indicate that PGAL is an induced rather than a constitutive enzyme in these bacterial strains.

D. Localization of Polygalacturonate Lyase Activity

No PGAL activity was observed in the ECF of any of the four Bacteroides strains tested. All activity occurred in the ICF, which suggested that PGAL was a cell-associated ("intracellular") enzyme in these four bacterial strains. This was not unexpected considering the complex and highly competitive environment of the human colon. Cell-associated enzymes confer a distinct advantage by insuring that breakdown products are metabolized by the same bacterial cell that synthesized the enzymes (Salyers, et al., 1977a). The ICF obtained

from each of the Bacteroides strains was used in all subsequent experiments as the source of PGAL.

E. Properties of Polygalacturonate Lyase From Polygalacturonic Acid Induced Bacterial Cells

1. Characterization of Polygalacturonate Lyase

For many pectinolytic bacteria, polygalacturonic acid is considered an optimal substrate for inducing PGAL synthesis and for measuring the induced activity. Therefore, experiments to characterize the properties of the crude PGAL enzyme were performed using polygalacturonic acid as inducer and substrate.

a. Determination of Limiting Substrate Concentration

The limiting substrate concentration for PGAL activity was determined using final substrate concentrations of 0.5%, 1.0%, and 1.5% polygalacturonic acid substrate (pH 8.0) (figure 3). Maximum activity (increase in absorbance per minute at 235 nm) was reached at the 1.0% level with Bacteroides strains B6-11 and C10-2. Activity decreased slightly at the 1.5% level. PGAL activity increased only slightly between substrate concentrations 1.0% and 1.5% with strains C14-3 and C7-8. A final polygalacturonic acid concentration of 1.0% was used in all subsequent assays requiring polygalacturonic acid substrate.

b. Effect of Induction Time on Polygalacturonate Lyase Activity

Bacteroides cultures were grown on polygalacturonic acid growth medium for 4, 8, 12, 16, and 20 hours at 37°C. Prior to enzyme

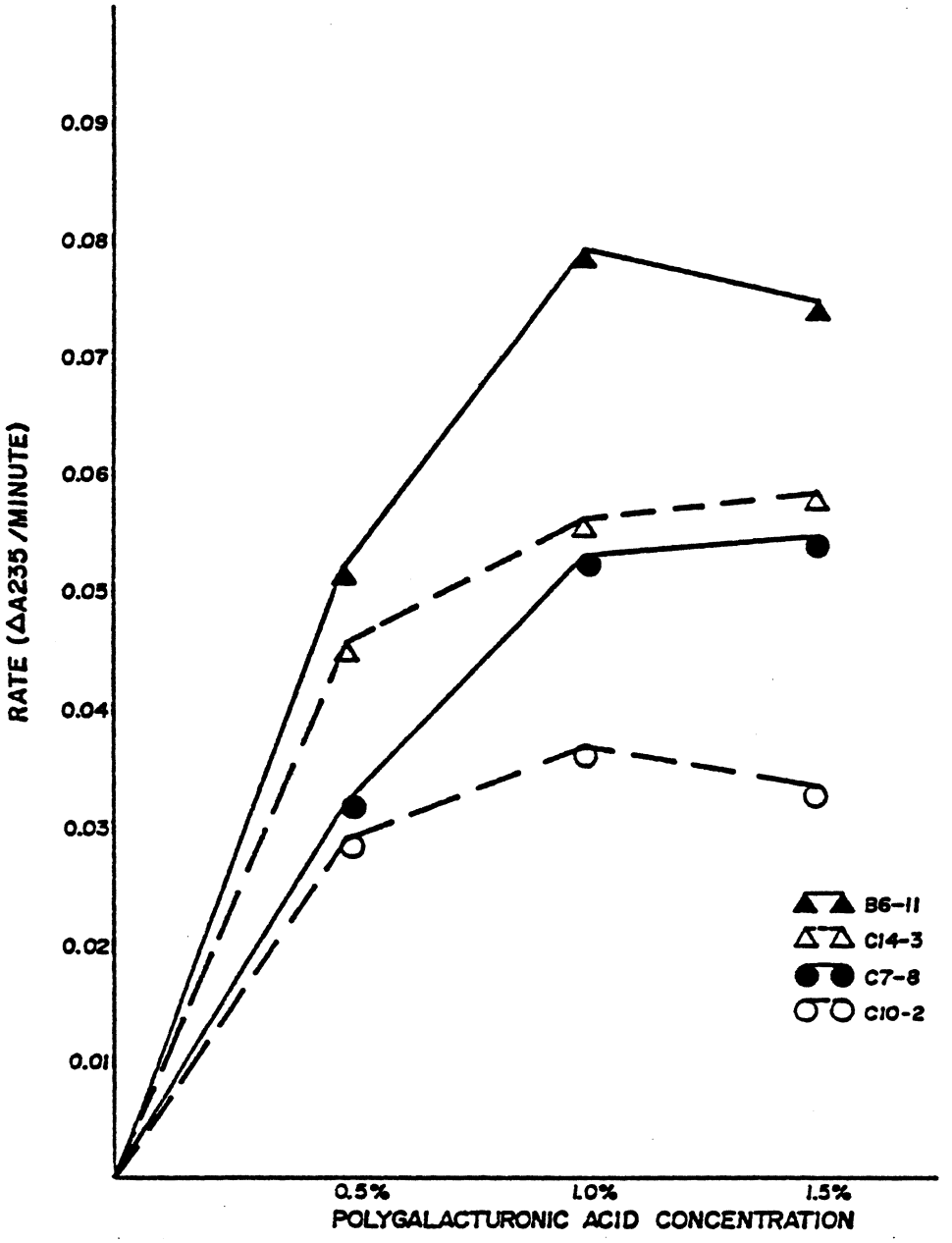

Figure 3. Effect of substrate concentration on PGAL activity. Reaction mixtures contained one of the above concentrations of polygalacturonic acid, 0.05 M tris buffer (pH 8.0), 0.0005 M $CaCl_2$ and enzyme solution. Reaction temperature was 37°C.

preparation, each culture was checked for optical density at 600 nm in a Bausch and Lomb Spectronic 20 spectrophotometer in order to follow the rate of bacterial growth. The PGAL activity curve (figure 4 A) and the growth curve (figure 4 B) followed the same general pattern for each Bacteroides strain. The greatest increase in enzyme synthesis and culture growth occurred between 4 and 8 hours for strains B6-11, C14-3, and C10-2 and between 8 and 12 hours for C7-8. Because of these trends, it can be assumed that enzyme production in these strains is directly related to cellular density in the cultures. No further increase in growth or PGAL activity was evident after 16 hours. The induction time for all polygalacturonic acid induced cultures was standardized at 16 hours for subsequent assays.

c. Effect of pH on Polygalacturonate Lyase Activity

The pH activity profile for 1.0% polygalacturonic acid substrates incubated with the PGAL enzyme is given in figure 5. The enzymes from all four Bacteroides yielded essentially identical profiles, with optimal activity at pH 8.5. In order to rule out a specific inhibition or stimulation effect for particular buffers, the effect of pH 7.0 was tested with both 0.02 M phosphate buffer and 0.05M tris buffer and pH 4.0 was tested with both 0.02 M acetate and 0.02 M citrate buffers. No effect of different buffers at the same pH values on PGAL activity was observed.

It is of interest to note that PGAL from all four Bacteroides strains still retained about 60% of the optimum activity at pH 7.0,

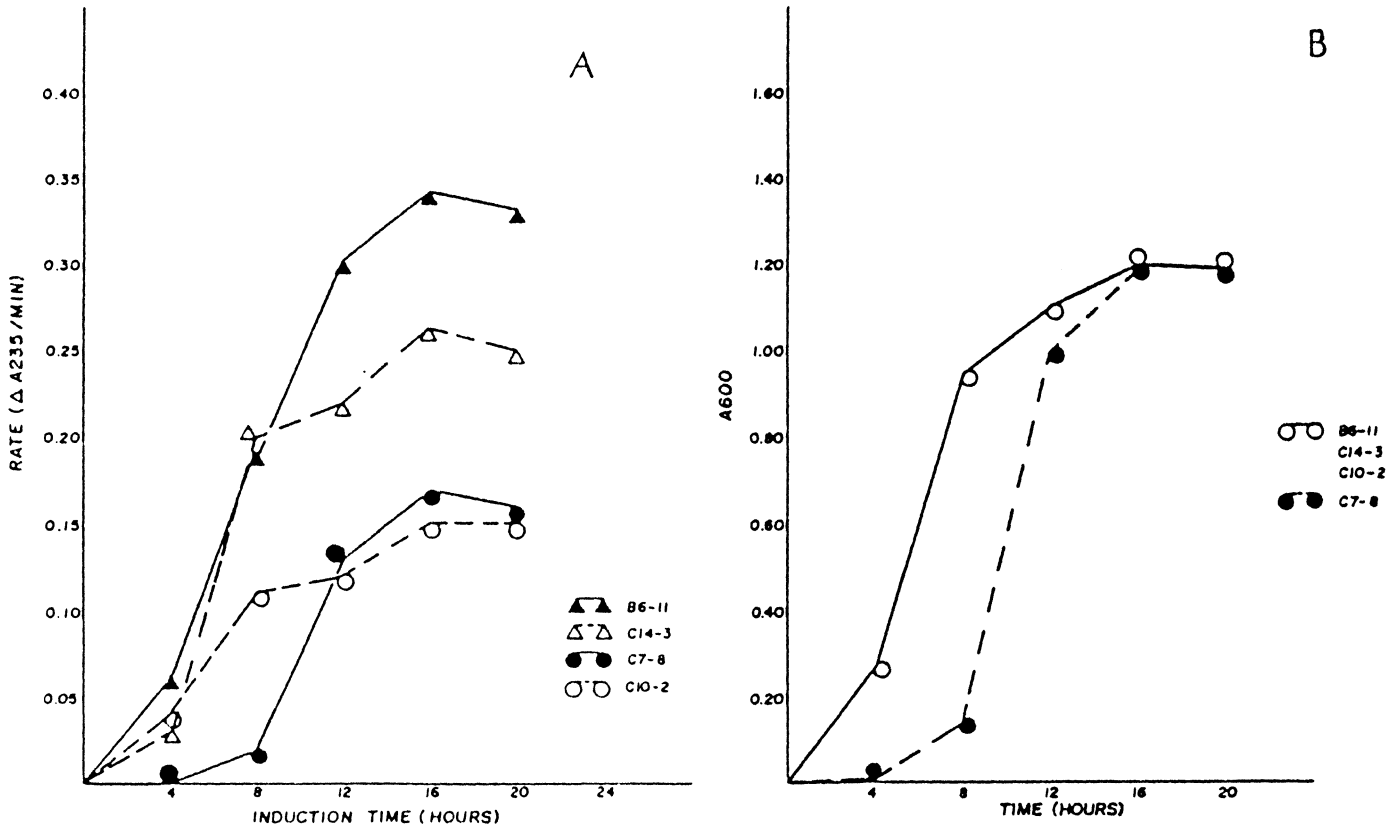

Figure 4. Effect of variations in induction time on PGAL activity. (A) PGAL activity versus induction times. Reaction mixtures: enzyme, 1.0% polygalacturonic acid substrate, 0.05 M tris (pH 8.0), and 0.005 M $CaCl_2$ at 37°C. (B) Growth curve of the four Bacteroides strains incubated at 37°C for the indicated time in polygalacturonic acid growth medium.

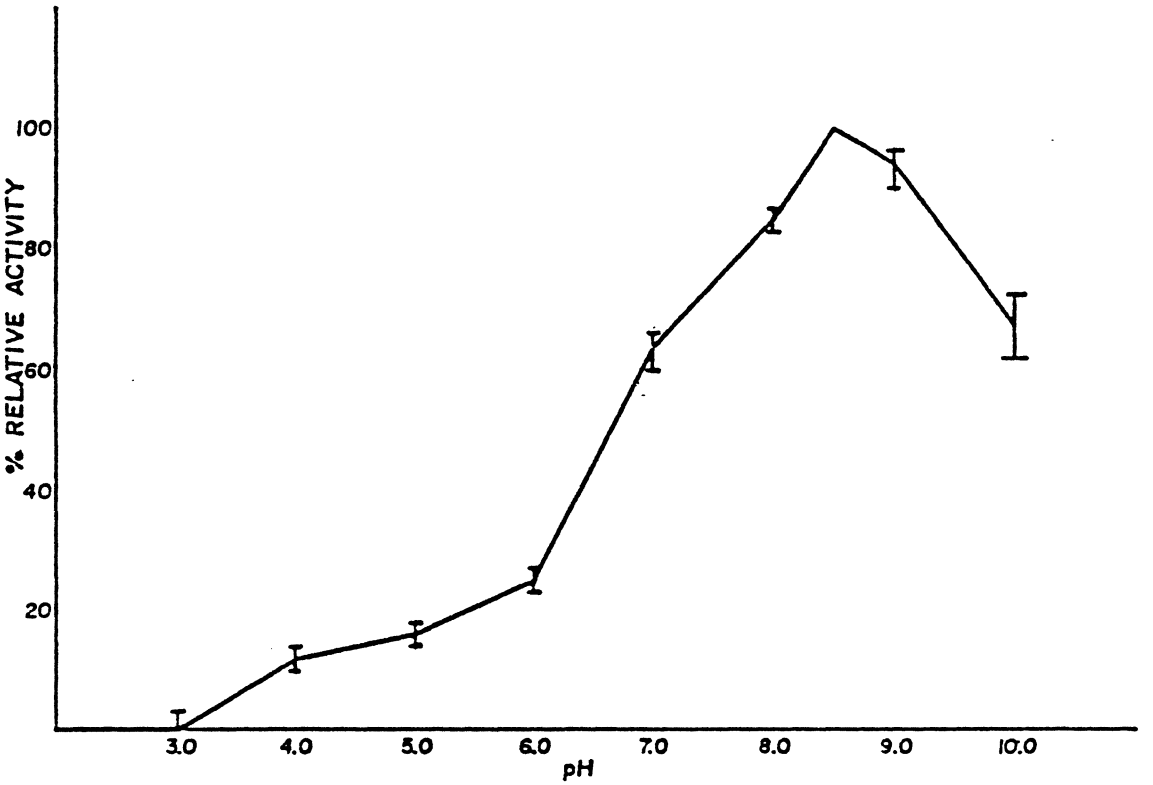

Figure 5. Activity of PGAL from C14-3, C10-2, C7-8, and B6-11 at different pH values. Bars indicate the variation between individual Bacteroides strains. Reaction mixtures contianed 1.0% polygalacturonic acid, 0.0005 M $CaCl_2$, buffer at one of the indicated pH values, and enzyme solution. Reaction temperature was 37°C.

Which is close to the pH of the colon. Therefore, it is possible that in the colon there is appreciable PGAL activity from these bacteria, if pectic substances are available for degradation. In all four strains, enzymatic activity decreased sharply as the pH dropped below 7.0. Activity above 8.5 was only slightly decreased at pH 9.0 and began a downward trend at pH 10.0. A pH of 8.5 was used in all subsequent assays.

d. Effect of $CaCl_2$ Concentration on Polygalacturonate Lyase Activity

Calcium ions have been reported to have a stimulatory effect on PGAL activity. To determine the optimal concentration of calcium for PGAL activity, 1.0% polygalacturonic acid substrates (pH 8.5) were prepared as described in Materials and Methods, section E-1, but adding $CaCl_2$, so that the final $CaCl_2$ concentration ranged from 10^{-5} M to 10^{-2} M. A polygalacturonic acid substrate with no added calcium was also prepared. PGAL activity was evident on substrates containing no added calcium for all the Bacteroides strains (Table 3). However, this activity was doubled when 10^{-5} M $CaCl_2$ was present in the reaction mixture. PGAL activity in C14-3, C10-2, B6-11, and C7-8 showed an optimum at 10^{-3} M $CaCl_2$ where the activity was approximately 12 times that with calcium free substrate. Gelling of the substrate occurred when 5×10^{-3} M and 10^{-2} M $CaCl_2$ were present. This was presumably caused by the formation of calcium bridges between the carboxyl groups of the pectic polymer. Since there was no increase in

Table 3. Effect of $CaCl_2$ Concentration on Polygalacturonate Lyase Activity

Bacterial Strain	Rate (ΔA_{235} Per Minute)							
	Molar Concentration of $CaCl_2$ in Reaction Mixtures							
	0.00	$1x10^{-5}$	$5x10^{-5}$	$1x10^{-4}$	$5x10^{-4}$	$1x10^{-3}$	$5x10^{-3}$**	$1x10^{-2}$**
C14-3	0.010	0.025	0.026	0.044	0.068	0.120	0.121	0.119
C10-2	0.007	0.014	0.016	0.028	0.044	0.094	0.080	0.079
C7-8	0.011	0.025	0.026	0.044	0.070	0.114	0.112	0.102
B6-11	0.012	0.027	0.030	0.054	0.093	0.202	0.200	0.182

*Substrate contained 1.0% polygalacturonic acid, 0.05 M tris buffer (pH 8.5), and the above concentrations of $CaCl_2$. Temperature of reaction was 37°C.

**Gelling of the substrate occurred at these concentrations of $CaCl_2$.

activity above 10^{-3} M $CaCl_2$ (perhaps in part related to the gel formation at higher calcium concentrations), this $CaCl_2$ concentration was used in all subsequent assays.

e. Effect of EDTA on Polygalacturonate Lyase Activity

EDTA, a chelating agent, was added to reaction mixtures to further test the calcium requirement of the PGAL (figure 6). No PGAL activity was observed in reaction mixtures containing 1.0% polygalacturonic acid substrate (pH 8.5), PGAL enzyme and 0.6% EDTA (a concentration sufficient to bind all calcium ions present in solution, in a 1:1 molar ratio). Complete activity was restored upon addition of excess 0.01 M $CaCl_2$ to the reaction mixtures. These results indicate that the PGALs from all four Bacteroides strains required calcium ions (or possibly other divalent cations) for activity.

f. Substrate Specificity of Polygalacturonate Lyase

Substrate preference for the PGALs was tested using polygalacturonic acid and pectin. Substrates at 1.0% final concentration were prepared in 0.02 M phosphate buffer (pH 7.0) containing 10^{-3} M $CaCl_2$. A reaction pH of 7.0 was chosen to avoid possible alkaline de-esterification of the pectin. Enzyme assays were performed by measuring the increase in absorbance at 235 nm and 37°C. PGAL activity was 5 times greater on polygalacturonic acid than on pectin for PGAL preparations from B6-11, C14-3, and C7-8 (Table 4). PGAL enzyme from C10-2 demonstrated 4 times more activity on polygalacturonic acid. Although PGALs

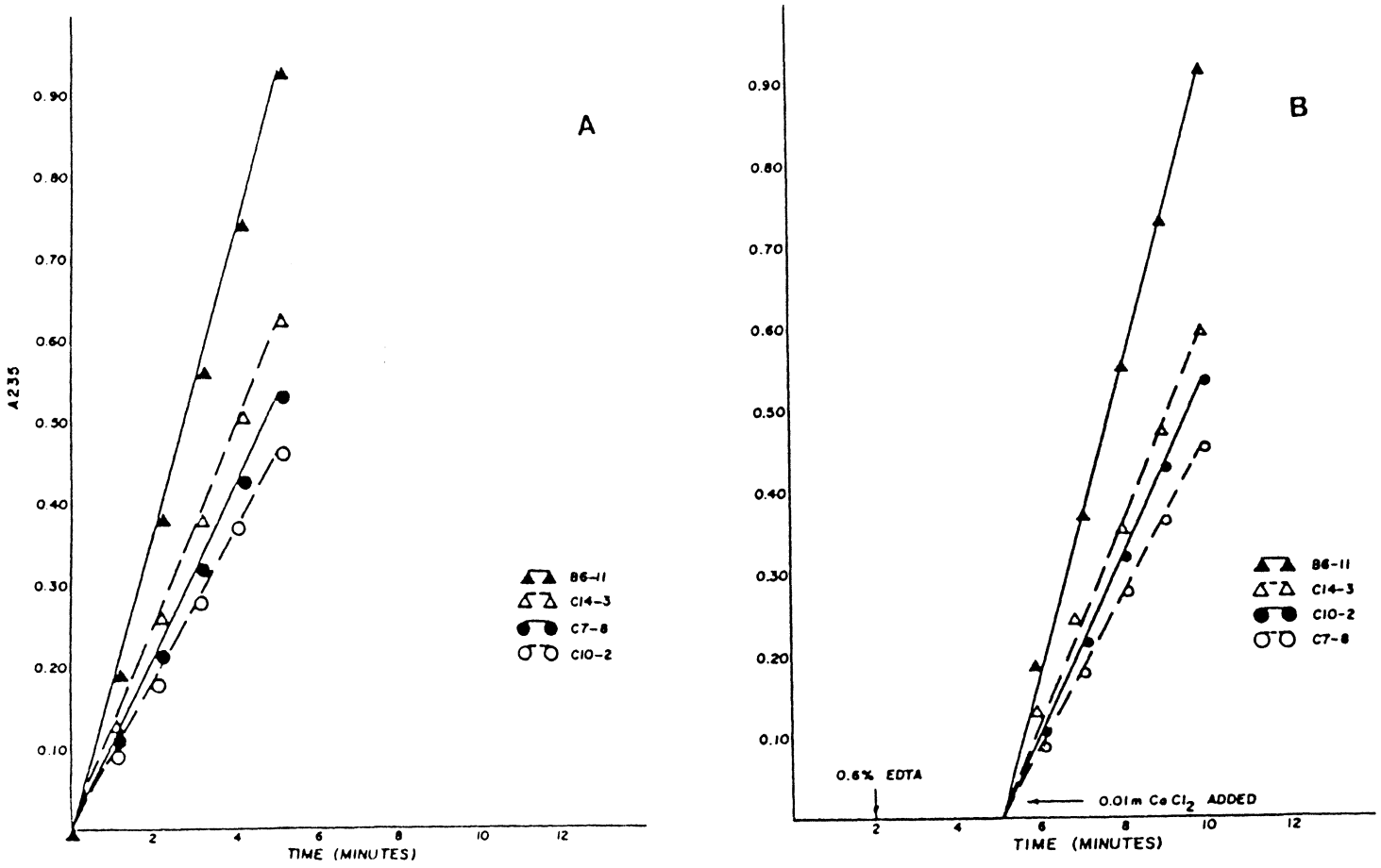

Figure 6. Effect of EDTA on PGAL activity. (A) PGAL activity with no added EDTA. (B) PGAL activity in the presence of 0.6% EDTA. Complete activity was restored upon addition of excess 0.01 M $CaCl_2$.

Table 4. Comparison of Polygalacturonic Acid and Pectin as Substrates for Polygalacturonate Lyase

Bacterial Strain	Rate (ΔA_{235} Per Minute)	
	Polygalacturonic Acid*	Pectin*
C14-3	0.073	0.015
C10-2	0.055	0.015
C7-8	0.060	0.011
B6-11	0.103	0.019

*Reaction mixtures contained 1.0% polygalacturonic acid or pectin, 0.02 M phosphate buffer (pH 7.0), 0.001 M $CaCl_2$, and enzyme solution. Temperature of reaction was 37°C.

from these strains preferentially catalyzed degradation of polygalacturonic acid, pectin was degraded at a significant rate. In view of the fact that PGAL can only attack uronide chains adjacent to a free carboxyl group, the degradation of pectin may be explained in two ways. First, the PGAL might have catalyzed chain breakage at unesterified regions of the pectin molecule (degree of esterification = 68%). Secondly, there may have been a pectinesterase present in the crude enzyme preparation. The pectinesterase would demethylate the pectin molecule leaving free carboxyl groups and making the pectin molecule more accessible to attack by PGAL. Pectinesterases are not usually found in bacteria but have been reported to be present in Clostridium multifermentans (MacMillan and Vaughn, 1964) and Bacteroides ruminicola (Wojciechowicz, 1971) along with PGALs. Pectinesterase activity in the enzyme preparations from the four Bacteroides strains was not determined in the present study.

2. Activity of Polygalacturonate Lyase on Peanut Cell Wall Substrate

The PGAL enzyme preparations from the Bacteroides strains were incubated at 37°C with 1.0% peanut cell wall substrate for 24 hours. The reaction mixtures were acidified with HCl - potassium chloride buffer (pH 2.0) to inactivate PGAL, centrifuged, and the supernatant fluid examined for increase in absorbance at 235 nm. Also the cell wall residue was analyzed for its uronic acid content (Table 5). To

Table 5. Polygalacturonate Lyase Activity on Peanut Cell Wall Substrate

Bacterial Strain	*Rate	% Cell Wall Degraded	% Total Uronides Degraded
C14-3	3.1×10^{-2}	2.8	20.0
C10-2	2.7×10^{-2}	3.1	22.1
C7-8	1.6×10^{-2}	3.2	22.9
B6-11	1.7×10^{-2}	3.0	21.4

*Rate = μmoles of unsaturated uronides per hour.

Reaction mixtures contained 1.0% peanut cell wall, 0.05 M tris buffer (pH 8.5), 0.001 M $CaCl_2$, and enzyme solution. Temperature of reaction was 37°C.

determine the rate of reaction (μmoles of unsaturated uronides per hour), a molar extinction coefficient of 4800 $M^{-1}cm^{-1}$ for unsaturated uronides was used (MacMillan and Vaughn, 1964).

Cell wall degradation (based on the decrease in uronic acid content) by the four Bacteroides PGALs was all similar, being in a range of 2.8 - 3.1%. However, relating the rate of reaction for the PGALs and the amount of cell wall degraded presents some problems. In order to correlate the rate of reaction and % cell wall degradation for C14-3 and C10-2, the smallest possible unsaturated uronide produced after incubation would have a degree of polymerization (DP) of 10. This may be illustrated using the results of cell wall degradation by the PGAL from C14-3. The μmoles of unsaturated uronides produced per hour for C14-3 is 3.1×10^{-2}. Thus after the 24 hour incubation period, a total of 0.74 μmoles of unsaturated uronides were released from the cell wall. One μmole of an unsaturated galacturonide with a DP of 10 has a formula weight of 1988 μg and 0.74 μmoles would therefore be equal to 1471 μg unsaturated uronides. This 1471 μg represents a 2.9% loss of the original 50,000 μg sample of peanut cell wall analyzed. From the independent data on the uronide content of the cell wall residue, it was calculated that 20% of the uronides were lost, which in turn represents a 2.8% degradation of the original cell wall (Table 5). Thus if it is assumed that PGAL splits off uronide oligomers with a mean chain length of DP 10, the enzyme assays and uronide content determinations would be correlated.

Using a similar calculation to correlate these values for B6-11 and C7-8 would require that the smallest possible unsaturated uronide formed would have a DP of 20. This is unlikely since bacterial PGALs have been shown to degrade uronide polymers with DP's as low as 3 (Nagel and Anderson, 1965; Wojciechowicz, 1972).

Presence of a hydrolase (polygalacturonase) in the enzyme preparation could help to explain this discrepancy. Polygalacturonase could degrade the uronide portion of the cell wall producing saturated uronides which would not absorb at 235 nm. Therefore, the unsaturated uronides produced (measured by absorbance at 235 nm) would represent only part of the uronides lost from the cell wall during enzyme action. Polygalacturonase activity was not determined in the present study.

Because of the long incubation period, another possibility lies in the nature of the final products produced by PGAL attack. Unsaturated monomer (D-galacturonate) can rearrange by enol-keto shift to form the deoxyketouronic acid, 4 - deoxy - L - threo - 5 - hexulose uronic acid. This deoxyketouronic acid does not absorb at 235 nm and hence would not be detected in the enzyme assay. Thus, there would be a decrease in the uronic acid content of the cell wall without corresponding production of the unsaturated uronides.

F. Polygalacturonate Lyase Activity of Peanut Cell Wall Induced Bacterial Cells

A spectrophotometric assay of Bacteroides growth in peanut cell wall medium was impossible due to interference from the insoluble cell

wall material. Growth was therefore monitored by visual comparison to growth of cultures in polygalacturonic acid medium.

The four Bacteroides cultures grew moderately well on the cell wall, but obviously more slowly than on polygalacturonic acid growth media. Therefore, all cultures were grown on cell wall growth medium for 48 hours. The ICF fraction was prepared as previously described (Materials and Method, section D).

Bacteroides cell wall induced enzyme preparations were assayed using 1.0% polygalacturonic acid substrate (pH 8.5) containing 10^{-3} M $CaCl_2$, reaction conditions determined to provide optimal PGAL activity using polygalacturonic acid induced bacterial enzyme preparations. PGAL activity was similar for C14-3, C10-2, C7-8, and B6-11 (Table 6). Activity of the cell wall induced PGALs on polygalacturonic acid substrate was approximately 40 - 60 times less than the activity of PGALs induced on polygalacturonic acid and then assayed on polygalacturonate. This may have been due in part to the complex nature of the cell wall. The cell wall presents a more difficult substance to degrade than polygalacturonic acid. The cell wall contains carbohydrates other than the pectic polysaccharides, no doubt causing other enzymes to be induced as well as pectic enzymes. Polygalacturonic acid is composed entirely of alpha 1,4 - linked galacturonide units; a greater quantity of pectic enzymes would be synthesized to utilize this carbon source. Peanut cell wall presents a more heterogeneous carbon source for the growth of Bacteroides cultures whereas polygalacturonic acid

Table 6. Polygalacturonate Lyase Activity of Peanut Cell Wall Induced Bacterial Cells

Bacterial Strain	Rate (ΔA_{235} Per Minute)
C14-3	0.0033
C10-2	0.0026
C7-8	0.0024
B6-11	0.0031

Reaction mixtures contained 1.0% polygalacturonic acid, 0.05 M tris buffer (pH 8.5), 0.001 M $CaCl_2$, and enzyme solution. Temperature of reaction was 37°C.

is a more homogeneous carbon source and thus provides a more homogeneous source of enzymes (i.e. pectic enzymes).

Because of the low activity of the cell wall induced PGALs on polygalacturonic acid substrate, no assay of the cell wall induced PGALs on peanut cell wall substrate was performed.

CONCLUSIONS

The results of this research indicated that the four Bacteroides strains studied produce inducible, cell-associated PGALs. The enzymes were characterized as PGALs based on similarities to the properties of other bacterial PGALs: An alkaline pH optima (8.5), a requirement for calcium ions for activity, preference of polygalacturonic acid as a substrate over pectin, and accumulation of unsaturated products absorbing at 235 nm.

Variations existed among the different Bacteroides strains with respect to polygalacturonic acid induced PGAL activity. With optimal substrate concentration, pH, and $CaCl_2$ levels, the PGAL activity from cells induced for 16 hours on polygalacturonic acid and assayed on polygalacturonic acid substrate was as follows: B6-11 PGAL activity > C14-3 > C7-8 > C10-2. In terms of relative activity, this relationship may be expressed as 100:59:56:47, respectively. It appears that B6-11 cells can synthesize considerably more PGAL when induced on polygalacturonic acid than the other three Bacteroides strains.

The bacterial strains were induced for 48 hours on peanut cell wall growth media because of the relatively slow growth of the cultures on this insoluble carbohydrate (compared to growth on soluble polygalacturonic acid) and because of low PGAL activity detected after 16 hours of induction in preliminary experiments. The average transit time in the human colon of a Western individual is approximately 48

hours. Considering this, the 48 hour induction period is appropriate when relating it to possible degradation of cell wall during passage through the colon.

There were also variations among the bacterial strains in the PGAL activity induced by growth on peanut cell wall media. With polygalacturonic acid substrate and optimum reaction conditions, the PGAL of C14-3 exhibited the highest activity. The PGAL from B6-11, C10-2, and C7-8 were 94%, 79%, and 73%, respectively of the activity of the C14-3 PGAL.

Examining cell wall induced versus polygalacturonic acid induced PGALs raises some questions regarding the accessibility of these two carbohydrates to bacterial degradation. PGAL from B6-11 exhibited a clearly higher activity than the other three Bacteroides strains when induced on polygalacturonic acid. However, when induced on peanut cell wall growth media, B6-11 PGAL activity is lower than that of C14-3 and only somewhat higher than the activity of C10-2 and C7-8 PGALs on polygalacturonic acid substrate. This significant activity difference of induced B6-11 PGAL may result from the complex construction of the peanut cell wall. Whereas B6-11 cells can readily degrade polygalacturonic acid, an isolated and soluble plant polysaccharide, these bacterial cells cannot degrade the plant cell wall as easily. The plant cell wall contains many polysaccharides other than pectin which could limit the accessibility of pectic polysaccharides to fermentation by the B6-11 strain. A more thorough understanding of the detailed

structure of the peanut cell wall is needed in order to further elucidate the mechanisms of enzyme action. Although cell wall composition was determined, the macromolecular structure of the cell wall is unknown. Hemicelluloses or lignin may limit enzymatic degradation by PGAL by ensheathing or surrounding the pectic polymers. The induced PGALs may encounter steric hindrance from netural sugar side chains of the pectic fraction of the peanut cell wall. A cell wall preparation with a known molecular structure and chemical composition would represent the ideal carbohydrate source for cell wall induction studies.

A purified PGAL preparation is needed to examine the degradation of plant cell walls. The possible presence of other uronide degrading enzymes complicated the assay of crude bacterial PGAL on peanut cell wall substrate. Although evidence was obtained that the peanut cell wall is susceptible to attack by a PGAL (increase in absorbance at 235 nm), the percentage of cell wall degraded (based on loss of uronides) did not agree with the amount of unsaturated uronides actually released by PGAL action. One probable reason for this desagreement is that the enzyme preparation from the Bacteroides strains contained other enzymes capable of degrading uronides. Use of a purified PGAL enzyme would remedy the problem of enzymes such as hydrolases degrading the cell wall by mechanisms other than beta elimination.

Mode of attack by the PGAL should be studied further. PGAL degradation by endo- mechanisms (random cleavage) results in a relatively rapid and drastic shortening of the pectic chain. Exo-type PGALs tend

to split off unsaturated dimers (or other short chain oligomers) from the pectic chain, leaving the main pectic chain relatively intact. Thus, endo-PGALs have a more drastic effect on breaking down cell wall structure.

The sequence of enzyme degradation of the cell wall should be examined in future studies. A synergistic effect of the colon bacteria may be essential for breaking down plant cell walls. Certain bacterial species may produce enzymes which degrade only one fraction of the cell wall, rendering the cell wall more accessible to degradation by other colon bacteria. There is evidence from studies with plant pathogens that pectic enzymes, particularly PGALs, could intiate this series of enzyme reations. Whether the pectic enzymes from Bacteroides strains are the initiators of dietary fiber degradation in the human colon remains to be determined.

In vitro, the four Bacteroides strains studied produce PGALs capable of at least parial degradation of prototype dietary fiber, the peanut cell wall. The extent to which PGAL action occurs in the colon is unknown. Future research efforts in this field should include use of a purified PGAL to examine the amount of cell wall metabolized, determination of the major final products produced by this reaction, and the effect of the enzyme action on cell wall structure. The chemical and physical changes caused by PGAL action on the cell wall or other dietary fiber fractions could have significance in understanding the beneficial physiological effect of dietary fiber.

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POLYGALACTURONATE LYASE ACTIVITY IN *BACTEROIDES* FROM THE HUMAN COLON

by

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(ABSTRACT)

In the course of studies on degradation of dietary fiber by the human colon bacteria, a number of *Bacteroides* strains from the human colon have been found to degrade polygalacturonic acid and/or pectin. Four of these strains were examined for presence of polygalacturonate lyase (PGAL). The *Bacteroides* strains studied synthesized inducible, cell-associated PGALs when the bacteria were grown on media containing polygalacturonic acid or on peanut cell wall (a prototype dietary fiber with a uronide content of 14.0%) as the sole carbon source. No PGAL activity could be detected in cultures grown on media containing glucose or D-galacturonate. The PGALs produced by the four *Bacteroides* strains had properties similar to other bacterial PGALs: An alkaline pH optima (8.5), stimulation of activity by calcium ions. complete inhibition by ethylenediaminetetraacetic acid (EDTA), preference for polygalacturonic acid as a substrate over pectin, and accumulation of unsaturated products which absorb at 235 nm. The *Bacteroides* PGALs catalyzed partial degradation of the uronides of peanut cell wall substrate, but at a slower rate compared to their action on polygalacturonic acid substrate.