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Purification and Properties of a Polygalacturonic Acid *Trans*-Eliminase Produced by *Bacillus pumilus*

BHALCHANDRA A. DAVÉ[1] AND REESE H. VAUGHN

Department of Food Science and Technology, University of California, Davis, California 95616

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A strain of *Bacillus pumilus* produced an extracellular pectic enzyme with polygalacturonic acid as the substrate. This enzyme, with optimal activity at *p*H 8.0 to 8.5, produced reaction products that strongly absorbed light at 232 nm, indicating the presence of a pectic acid *trans*-eliminase (PATE). Neither pectin esterase nor polygalacturonase was detected in the cell-free culture fluid. Chromatographic examination of the end products revealed the presence of large quantities of unsaturated oligouronides unlike those found with *B. polymyxa*. It was found that the PATE was produced extracellularly during the negative logarithmic death phase of the organism. The filtrate from sonically treated cells did not show any activity for PATE or hydrolases for lower oligogalacturonides at any time during the growth cycle. The enzyme was inducible. Pectin, National Formulary (NF) was the best inducer, followed by polygalacturonic acid and galacturonic acid. Enzyme activity was markedly stimulated by calcium and other divalent ions. Copper and cobalt ions were inhibitory. The partially purified enzyme showed no significant activity on pectin containing a high methoxyl content (96% esterified). However, pectin NF with a lower methoxyl content (68% esterified) was attacked to a degree by the partially purified and crude enzyme preparations. The initial rate of PATE activity increased up to 60 C, about 16-fold higher than that observed at room temperature. The activation energy was calculated as 12,183 cal/mole. A protective action of calcium chloride against heat inactivation of the PATE was observed. Degradation of polygalacturonic acid by this enzyme produced several unsaturated oligouronides soon after its addition to the substrate. The major endproduct was thought to be different from that of other known PATE enzymes. Paper chromatographic studies and viscosity measurements disclosed the random cleaving nature of the enzyme an endo-PATE.

In the decade since the discovery of pectin *trans*-eliminase (PTE) by Albersheim et al. (3), considerable knowledge has accumulated concerning *trans*-eliminative pectin- and polygalacturonic acid-cleaving enzymes produced by microorganisms.

It has been shown that some, but not all, fungi (molds) produce PTE and that the fungal PTE is specific for pectin and oligogalacturonide methyl esters (5, 6). PTE activity also has been reported in a culture of *Streptomyces viridochromogenes* (2) and in a crude acetone powder extract of a protozoal population associated with retting (1). So far as is known, yeasts do not produce such enzymes (28, 30).

[1] Present address: Pennwalt Corp., Decco Division Research Laboratory, Monrovia, Calif. 91016.

In contrast, the presently known bacterial *trans*-eliminases are specific for polygalacturonic acid. Based on the manner of attack on the polygalacturonic acid moiety, two kinds are known: endo-polygalacturonic acid *trans*-eliminase (endo-PATE) produced by *Bacillus polymyxa* (17, 18), different phytopathogenic bacteria (20, 21-23, 26, 27), and pseudomonads (7, 21, 24, 25) and exo-PATE produced by *Clostridium multifermentans* (13, 14). The major end product of both eliminative enzymes is unsaturated digalacturonic acid, the former cleaving the polymer in a random manner, the latter removing α, β-unsaturated digalacturonic acid from the reducing ends of the polygalacturonate chains. Some of the pseudomonads produce both endo-PATE and the hydrolytic polygalacturonase (18,

22, 23). Before 1960, the latter enzyme was thought to be responsible for degradation of pectic substances by all pectolytic microorganisms.

The present study was undertaken to determine whether the pectolytic enzymes produced in the genus *Bacillus* differed from species to species. During the preliminary screening of pectolytic species, it was observed that a very pectolytic culture of *B. pumilus* (no. 15-A2) originally isolated by Vaughn et al. (29) from a brine of softened cucumbers caused the accumulation of large amounts of what was thought to be unsaturated tri-galacturonic acid when crude, cell-free, culture fluids were reacted with polygalacturonic acid. This account describes the purification and some of the properties of the enzymes causing this reaction. Another paper will substantiate the major end product as being unsaturated tri-galacturonic acid.

MATERIALS AND METHODS

Substrates. Polygalacturonic acid (product 3491) was obtained from Sunkist Growers Inc., Corona, Calif. This material has a moisture content of 5.4% and an ash content of 2.3% (wet weight basis). For most experiments, 1 g of polygalacturonic acid was slowly added to 50 ml of distilled water with rapid stirring. The slurry was dissolved by the addition of suitable amounts of 1 N NaOH to bring the *p*H of the solution to *p*H 8.0. The mixture then was made up to 100 ml with distilled water. A slight turbidity was removed by centrifugation at low speed. The concentration of this stock solution was 0.92% polygalacturonic acid. In experiments concerned with the effects of divalent cations on the activity of the enzyme, a more highly purified sodium polygalacturonate was used. This was prepared by passing the centrifuged stock solution slowly (100 ml/hr) through a column (4 by 15 cm) of Dowex 50 W-X4 (Baker, 100 to 200 mesh, Na^+ form). This treatment effectively removed any contaminating divalent cations.

Purified galacturonic acid was obtained by repeated acetone crystallization of a concentrated solution of commercial grade galacturonic acid (product 3494) produced by Sunkist Growers, Corona, Calif.

Pectin NF (product 3442) was also obtained from Sunkist Growers, Corona, Calif. Its degree of esterification was 68%.

Polygalacturonic acid D.P.-12 was prepared by James D. Macmillan while a student in this laboratory.

Polymethyl polygalacturonate methyl glucoside (Link pectin) was supplied by H. J. Phaff. Its degree of esterification was about 96%.

Unsaturated digalacturonic acid was prepared by the method of Macmillan and Vaughn (13) using *C. multifermentans* as the enzyme source. Precipitation of the strontium salt of the unsaturated dimer from the reaction mixture was done with 95% ethanol.

Adsorbants. Calcium phosphate gel was prepared by the method of Kunitz (11). The gel was stored at 5 C and remained usable for 4 to 6 months.

Columns of cellulose *N*,*N*-diethylaminoethyl ether powder (DEAE cellulose), Dowex-50H^+, and Dowex-50 Na^+ were prepared in the conventional manner.

Microbiological methods. The culture of *B. pumilus* used in this study (no. 15-A2) was maintained on nutrient agar slants held at 5 C and transferred to fresh slants at monthly intervals.

The medium used for the production of the pectolytic enzymes was composed of yeast extract, 5.0 g; K_2HPO_4, 5.0 g; polygalacturonic acid, 5.0 g; and distilled water to make 1 liter. First, the polygalacturonic acid was dissolved in 500 ml of distilled water, and the *p*H was carefully adjusted to 7.6 with 1 N NaOH. The turbidity of this solution was removed by low-speed centrifugation. The other ingredients were then added, and the volume was made to 1 liter with distilled water. The medium was dispensed in 100-ml amounts in 250-ml Ehrlenmeyer flasks or in 1-liter quantities in 2.5-liter Fernbach flasks. In either case, the sterilization was 15 min at 15 psi of steam pressure. In either quantity, the inoculum consisted of a 24-hr culture of *B. pumilus*. The small flasks received the growth from a 24-hr slant culture; the larger flasks received 10 to 30 ml of growth from a 24-hr shake culture. Incubation in all cases was on a rotary shaker at 30 C for 5 to 7 days.

Enzymatic activities. PATE activity was routinely assayed by the measurement of absorbancy changes at 232 nm in reaction mixtures consisting of 0.46% (dry, ash-free basis) on untreated polygalacturonic acid, 0.001 M $CaCl_2$ and 0.033 M tris(hydroxymethyl)aminomethane (Tris) buffer, adjusted to *p*H 8.5 with HCl, and enzyme. Unless specified otherwise, sodium phosphate buffers were used. All measurements were made at room temperature with a Beckman DB spectrophotometer. One unit of PATE activity was defined as the amount of enzyme which would cause an increase of 0.001 unit in absorbancy at 232 nm in 1 min. To measure end products of acivity at various intervals, it was necessary to inactivate the enzyme. This was accomplished by dilution into 0.1 M acetate buffer at *p*H 3.7.

The search for pectinesterase included two test: the modified cup-plate assay by Macmillan and Vaughn (13) of the hydroxamic acid plate test of McComb and McCready (15) and the titration method involving periodic titration of the liberated carboxyl groups with NaOH (0.02 N).

Analytical methods. Protein was estimated either by the method of Lowry et al. (12) or by the procedure of Warburg and Christian (31). Reducing groups were determined by the Willstätter and Schudel (32) hypoiodite method, as modified by Jansen and MacDonnell (10). One milliequivalent of iodine reduced corresponds to 0.513 mmole of reducing groups.

The unsaturated products formed by the action of PATE on polygalacturonic acid were identified on descending chromatograms developed with ethyl acetate-pyridine-water-acetic acid (5:5:3:1) on Whatman no. 4 paper at room temperature for 12–20 hr by the method of Nagel and Vaughn (17). Unsaturated compounds were detected by the method of Edstrom and Phaff (5). Free aldehyde groups were routinely detected by using the silver nitrate reagent described by

Block et al. (4). Although not as specific as other reagents, the silver nitrate is highly sensitive for the detection of reducing groups, particularly when the quantities are too small to be detected with *m*-phenylenediamine dichloride or *p*-anisidine.

RESULTS AND DISCUSSION

Comparisons of B. pumilus and B. polymyxa. Cell-free dialyzed broths from 5-day-old cultures of *B. pumilus* and *B. polymyxa* were compared. Figure 1 presents the differences in behavior of the crude enzymes of the two bacteria with respect to absorbancy changes at 232 nm. It was found that the crude *B. pumilus* enzyme caused the greater change in absorbancy during the first 8 hr of reaction time. This indicated a larger accumulation of unsaturated products by the *B. pumilus* enzyme system.

Paper chromatographic analysis of the reaction products obtained by action of the crude enzyme preparations on polygalacturonic acid are shown in Fig. 2A, B, C, and D. It is apparent that the reaction products of the two different crude enzymes are not the same. No unsaturated digalacturonic acid was observed with the culture fluid from *B. pumilus* (Fig. 2A). Longer reaction times did not change the picture (Fig. 2B). Better resolution of the reaction products of the *B. pumilus* enzyme was obtained when the developing time of the chromatograph was increased from 16 to 40 hr (Fig. 2C). A modified solvent system (pyridine-ethyl acetate-water-acetic acid, 5:5:5:5) gave better resolution of the reaction products in a shorter time and also retained the monomer control on the paper (Fig. 2D). It is also seen that the saturated di-, tri- and tetragalacturonic acids used as controls were not produced by the crude enzyme of *B. pumilus*. This solvent combination (pyridine-ethyl acetate-water-acetic acid, 5:5:5:5) was then routinely used in all subsequent paper chromatography experiments. It was concluded that the PATE produced was different; thus, a detailed study of the *B. pumilus* enzyme was initiated.

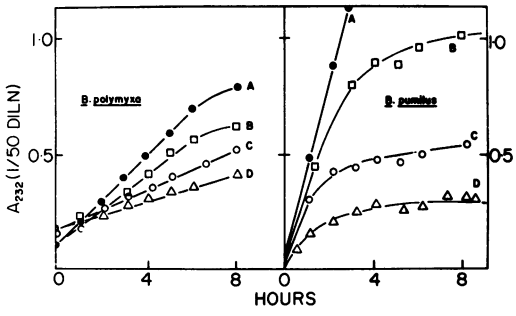

FIG. 1. *Absorbancy changes caused by crude enzymes of B. polymyxa and B. pumilus prepared under identical conditions. Absorbancy of reaction mixtures was measured at 232 nm after 1:50 dilution with 0.1 M acetate buffer (pH 3.7). Polygalacturonic acid concentrations in the reaction mixtures were as follows: A, 2.0%; B, 1.5%; C, 1.0%; and D, 0.5%. All reaction mixtures had 0.033 M Tris-hydrochloride buffer at pH 8.5 and 0.001 M $CaCl_2$. The enzyme solution was 1.0 ml in a total volume of 20 ml.*

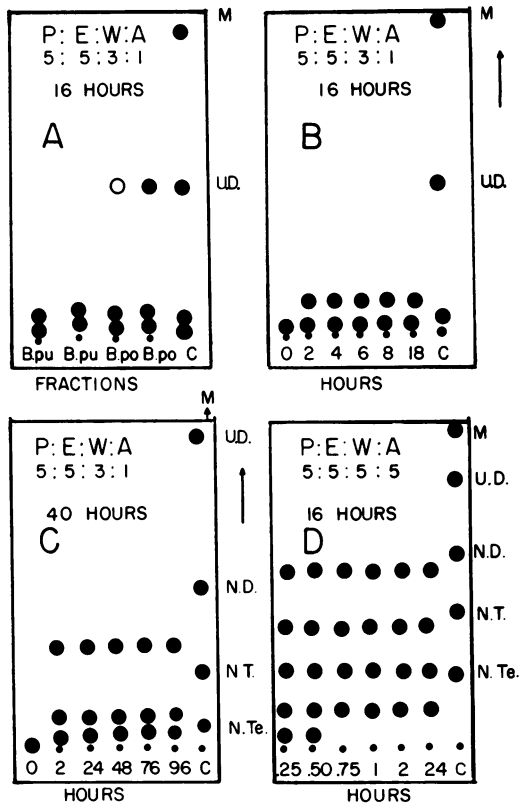

FIG. 2. *Chromatographic analysis of endproducts of polygalacturonic acid degradation by crude cell-free enzymes of B. pumilus and B. polymyxa. Descending chromatography was done on Whatman paper no. 4, with pyridine-ethyl acetate-water-acetic acid solvent mixture, in the ratio shown for the time indicated. The arrows indicate solvent direction. Abbreviations were: M, galacturonic acid; U.D. = unsaturated digalacturonic acid; N.D., N.T., and N.Te., normal di-, tri-, and tetragalacturonic acids; B.po, B. polymyxa; B.pu, B. pumilus; C, standards. Symbols: O, a weak reaction; ●, strong reactions with silver nitrate (4) and quinoline sulfate reagent (5). (A) B. pumilus and B. polymyxa reaction products at completion, barium salts precipitated with ethanol. Chromatograph developed for 16 hr. (B) B. pumilus reaction products at various times. Chromatograph developed for 16 hr. (C) B. pumilus reaction products at various times. Chromatograph developed for 40 hr. (D) B. pumilus reaction products at various times. Chromatograph developed in modified solvent system for 16 hr.*

Growth and enzyme production. It was found that PATE production increased during the logarithmic death phase, just before sporulation of the culture (Fig. 3). It also was observed that the centrifuged, dialyzed extract of sonically treated cells washed in buffer at *p*H 7.0 contained no PATE activity with any cell samples collected at intervals for 144 hr.

Only one enzyme was detected in the cell-free culture fluids of *B. pumilus*. This enzyme was a PATE. No others were detected. Only pectin NF and polygalacturonic acid were assimilated by cultures of *B. pumilus*. The culture could not use any of the lower oligouronides. In contrast, *B. polymyxa*, in addition to producing four pectic acid lyases (17–19), is known to form pectin esterase and a digalacturonic acid hydrolase (17, 18), and the unidentified bacillus has been shown to produce hydrolases for both saturated oligogalacturonides (9, 16).

The rate of degradation of polygalacturonic acid by the crude PATE was studied by comparing the reducing groups formed and the change in absorbancy at 232 nm against time. Linear relationships were observed between the reaction time and the change in absorbancy or formation of reducing groups during the initial stages of the reaction (Fig. 4). However, after about 1 hr, the linearity was lost. The initial linearity suggested that the liberation of the reducing groups was caused by a *trans*-eliminase. This supposition was strengthened by the observation that the crude enzyme produced only a very small amount of reducing groups at *p*H 5.2, thus supporting the suggestion of the absence of polygalacturonase (PG) which has an optimum activity around *p*H 5.0.

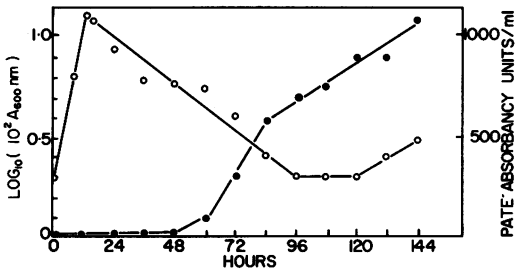

FIG. 3. *Growth and PATE production by B. pumilus (15-A2). Growth was followed by reading absorbancy of a 1/20 dilution of washed cells in 0.05 M phosphate buffer (pH 7.0) at 600 nm. Dialyzed culture fluid was assayed for PATE activity with a reaction mixture containing 0.5% polygalacturonic acid, 0.033 M Tris-hydrochloride buffer (pH 8.5), and 0.001 M $CaCl_2$. The total volume was 2.0 ml and 0.001 unit of absorbancy at 232 nm per min = 1 absorbancy unit of PATE activity. Symbols: O, growth; ●, PATE activity.*

PATE production in various media. Three basal media were used: (i) 3.3 g of Difco yeast nitrogen base (YNB) in 250 ml of distilled water, (ii) 2.5 g each of Difco yeast extract and K_2HPO_4 in 250 ml of distilled water, and (iii) 250 ml of double-strength Difco nutrient broth. Each medium, adjusted to *p*H 7.6, was dispensed in 50-ml portions into five 250-ml flasks. Fifty milliliters each of 1% pectin NF, 1% polygalacturonic acid, 1% galacturonic acid (all three adjusted to *p*H 7.0 with NaOH), 1% glucose solution, or distilled water was added to the three basal media. The media containing pectin NF were filter sterilized. The rest were autoclaved for 15 min at 15 psi. After inoculation with strain 15-A2, the flasks were incubated on a rotary shaker at 30 C. Growth of the organism and PATE production were followed at 24-hr intervals for 6 days. It was found that nutrient broth and YNB were not useful as basal media, and glucose and galacturonic acid were not suitable carbon sources for PATE production under the conditions of this experiment. Pectin NF and polygalacturonic acid induced PATE production in the yeast extract basal medium, as did galacturonic acid, although to a markedly lesser degree. Although pectin NF sometimes yielded a higher enzyme activity per milliliter, the results were not always reproducible. Therefore, because the yeast extract-polygalacturonic acid medium consistently gave the same activity, it was employed as the standard for enzyme production in

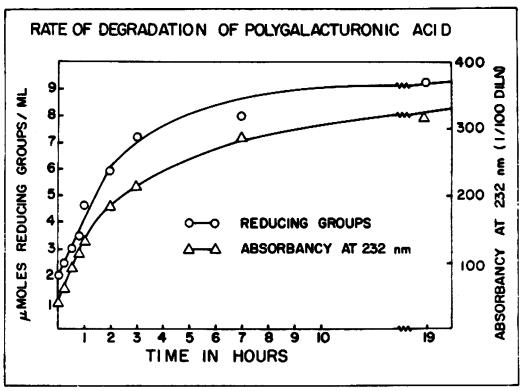

FIG. 4. *Rate of degradation of polygalacturonic acid as determined by comparison of reducing groups formed and by increase in absorbancy at 232 nm against time (in the initial reaction period). The reaction mixture contained 0.5% polygalacturonic acid, 0.033 M Tris-hydrochloride buffer (pH 8.5), and 0.001 M $CaCl_2$. Reducing groups were determined by the hypoiodite method. One milliequivalent of iodine reduced corresponds to 0.513 mmole of reducing groups. Absorbancy at 232 nm was measured in 0.1 M acetate buffer (pH 3.7).*

all subsequent experiments.

Purification of B. pumilus PATE. Preliminary experiments showed some *trans*-eliminase activity in the crude culture fluid with pectin NF as a substrate. This activity could have been caused by the action of pectin esterase (PE) together with PATE during the reaction, by the action of the PATE alone on the non-esterified portions of the pectin NF polymer, or PTE present in the cell-free culture fluid. No measurable PE, PG, or PTE activity was found in the crude enzyme; thus, the parameters for purification were only the PATE activity itself, its specific activity, and the end products it formed.

Scheme I: purification of PATE by ammonium sulfate precipitation and DEAE cellulose column chromatography. The PATE in crude cell-free enzyme solutions was readily precipitated with ammonium sulfate at 0 C. The precipitate obtained at 85 to 90% saturation (64 to 68% ammonium sulfate, w/v) showed the greatest increase in specific activity. Under these conditions, the specific activity of the redissolved, dialyzed precipitate increased two to five times that of the original crude enzyme with recoveries ranging from 50 to 80%.

The redissolved precipitate (5 ml) obtained by ammonium sulfate treatment (65%, w/v) of 200 ml of the crude enzyme as just described was further purified by passage through a DEAE cellulose column (1.5 by 30 cm) equilibrated with 0.01 M Tris-hydrochloride buffer at *p*H 7.6. The enzyme was eluted with a linear gradient of 200 ml of 0.2 M Tris-hydrochloride buffer at *p*H 7.6 and 200 ml of distilled water with the flow rate adjusted to 40 ml/hr, and 4-ml fractions were collected. Four protein peaks were observed for absorbancy at 280 nm, and the PATE activity was localized in the area of the first peak (fractions 10 to 13). The enzyme was purified 16-fold by these two maneuvers, as the other 3 peaks at 280 nm were found to contain no PATE activity (Table 1).

Scheme II: purification of PATE by calcium phosphate gel adsorption and DEAE cellulose and Sephadex column chromatography. For this experiment, 150 ml of calcium phosphate gel was added to 1,500 ml of crude enzyme solution, stirred for 2 hr at room temperature, and centrifuged. The packed gel was eluted twice with 200 ml of 0.1 M phosphate buffer at *p*H 8.0. The two eluates were combined, dialyzed against distilled water overnight at 5 C, and then lyophilized. The resultant 0.68 g of fluffy white powder was dissolved in 5 ml of 0.02 M Tris-hydrochloride buffer (*p*H 7.6) and applied to a DEAE cellulose column (3.5 by 35 cm). Elution was carried out stepwise by using 500 ml each of 0.02, 0.2, and 1.0 M Tris-hydrochloride buffers (*p*H 7.6) in that order. Three protein peaks were observed at 280 nm, and the PATE activity was largely localized in the first protein peak area (fractions 8 to 12). The eluate from the five tubes localized under the first protein peak was pooled (100 ml), dialyzed overnight against distilled water at 5 C, and lyophilized. The resultant fluffy white powder was dissolved in 2.5 ml of 0.02 M Tris-hydrochloride buffer (*p*H 7.6) and again applied to the DEAE cellulose column. This time, the elution was restricted to 500 ml of *p*H 7.6 0.02 M Tris-hydrochloride buffer. Only a single protein peak was observed at 280 nm, and it corresponded to the single peak of PATE activity. A 74% recovery was obtained with a 19-fold increase in specific activity of the PATE at this stage of purification. The eluates from this single peak were pooled, dialyzed overnight in the usual manner, and lyophilized. The resultant 80 mg of fluffy white powder was used for other experiments.

A column (1.5 by 190 cm) of Sephadex G-100, prepared and calibrated by using proteins of known molecular weights (including cytochrome *c*, α-chymotrypsin, trypsin and pepsin) which permitted development of a standard curve, was used for final purification of the *B. pumilus* PATE. We are grateful to John Whitaker and

TABLE 1. *Purification of PATE by ammonium sulfate and DEAE cellulose*

Enzyme preparation	Protein[a] (mg/ml)	PATE (units/ml)	Specific activity (units/mg of protein)	Vol (ml)	Recovery (%)	Purification
Crude enzyme	2.5	1400	560	300	100	1×
$(NH_4)_2SO_4$-method	8.5	22,000	2,600	5	40	5×
DEAE cellulose						
Peak I[b]	0.48	4,500	10,000	20	32	16×
Peak II	0.12	180		12		
Peak III	0.02	20		16		
Peak IV	0.01	50		14		

[a] Protein concentration measured by Lowry's method (12).
[b] Peaks as observed by measuring absorbancy at 280 nm.

Don Williams of this Department for permission to use the column and standard curve. A 0.5-ml sample containing 20 mg of the partially purified enzyme from the experiment above was applied to the column. Elution was carried out with 0.02 M Tris-hydrochloride buffer containing 0.4 M NaCl at *p*H 7.6. Two-milliliter fractions were collected, and measurements of absorbancy at 280 nm and PATE activity at 232 nm were made. Three protein peaks were observed. The first one included fractions 70 to 80; the second, fractions 88 to 102; and the third, fractions 135 to 145 (Fig. 5). The majority of the PATE activity was found in the fractions comprising peak 2. Only a very small amount of enzyme activity was found in peak 1, and none at all was found in peak 3. The eluant volumes of the three peaks were 150, 190, and 290 ml, respectively. The void volume for the Sephadex G-100 column was 95.5 ml using Blue Dextran solution. From these data and from the standard curve already available, the molecular weight of the PATE protein peak was calculated as 20,000 daltons. The overall purification by this scheme is presented in Table 2.

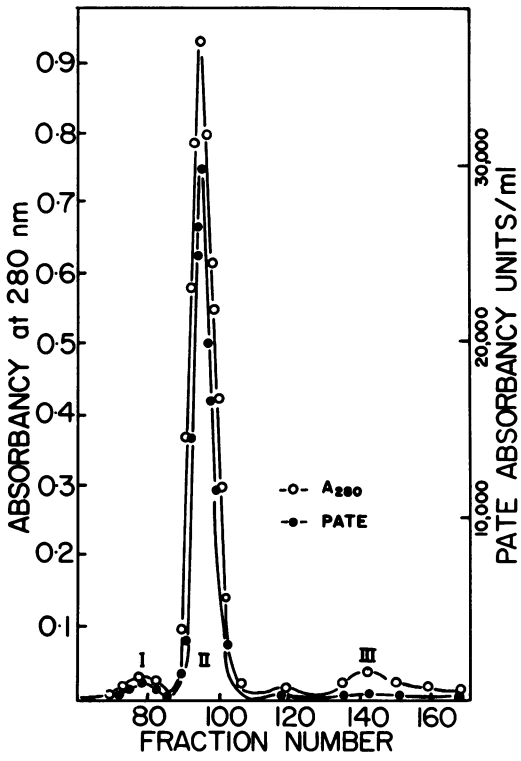

FIG. 5. *Purification of PATE of B. pumilus on Sephadex G-100. A Sephadex G-100 column (1.5 by 190 cm) was equilibrated with 0.02 M Tris-hydrochloride buffer (pH 7.6) containing 0.4 M NaCl. A 0.5-ml sample containing 20 mg of PATE partially purified by DEAE cellulose was applied to the column. Elution was carried out with the same buffer. The flow rate was 20 ml/hr. The fraction size was 2 ml per tube.*

Substrate specificity. Partially purified PATE preparations (specific activity, 20,000 units/mg) degraded polygalacturonic acid DP-12 most rapidly and polygalacturonic acid at a somewhat slower rate. Partially esterified (68%) pectin NF was attacked at a relatively low velocity, and highly esterified (96%) polymethyl polygalacturonate methyl glycoside (Link pectin) was hardly attacked at all (Fig. 6), again substantiating the absence of PE from the pectolytic enzyme complex of *B. pumilus*, either in crude or partially purified systems.

The initial rate of PATE activity with various concentrations of polygalacturonic acid was determined in the presence of 5×10^{-4} M $CaCl_2$, 1000 units of PATE per ml, and 0.033 M Tris-hydrochloride buffer at *p*H 8.5. It was found that the use of approximately 0.5% substrate in the standard PATE assays was justified since the initial rate of the reaction was not changed significantly in this region of concentration. Similar results were found with the PATE of *C. multifermentans* (13). The V_{max} and K_m values for the *B. pumilus* enzyme reaction system were calculated as 20.0 optical density (OD) units per min per mg of protein and 0.137% polygalacturonic acid (dry, ash-free basis), respectively.

Pattern of action. Comparison of reducing group formation, changes in absorbancy at 232 nm, decrease in viscosity, and paper chromatographic studies were used to determine the pattern of action of *B. pumilus* PATE. Both reducing groups and absorbancy increased rapidly during the first hour of reaction. The viscosity decreased markedly during the first 1,000 sec of reaction (Fig. 7). These observations all suggest that polygalacturonate molecule is attacked in a random manner. Chromatograms of the reaction products at various time intervals show the presence of several oligouronides (Fig. 8). The products formed are all similar in nature and increase in concentration only during the earlier part of the reaction (1 to 3 hr). This also is interpreted as confirming the random splitting nature of the enzyme.

pH optimum. The activity of the partially purified enzyme was determined in 0.033 M Tris-hydrochloride buffer at various *p*H values. The results showed that optimal activity occurred at *p*H 8.0 to 8.5. This range is close to that reported for the PATE of *C. multifermentans* (13), but is somewhat lower than the optimal range *p*H 8.9 to 9.4 reported for the enzyme of *B. polymyxa* (17) or the optimal range *p*H 9.3 to 9.7

TABLE 2. *Overall purification of PATE of Bacillus pumilus*

Enzyme preparation	Protein[a] (mg/ml)	PATE (units/ml)	Specific activity (units/mg)	Vol (ml)	Total units	Recovery (%)	Purification
Crude enzyme	1.000	1,200	1,200	5,000	6.0×10^6	100	1×
Phosphate gel eluate	0.900	3,000	3,333	1,040	3.12×10^6	52	3×
Single DEAE cellulose passage	160	2.0×10^6	12,500	1.6[b]	3.2×10^6	53	10.5×
Double DEAE cellulose passage	40	8.0×10^5	20,000	4.0[b]	3.2×10^6	53	16×
Sephadex G-100, peak II	0.200	10,000	50,000	30	2.4×10^6	40[c]	41×

[a] Protein concentration measured by Lowry's method (12).

[b] Volumes represent the volumes of buffer used to dissolve the dialyzed and lyophilized material before going to the next step.

[c] Recovery data based on material applied to the Sephadex column (only 0.5 ml was applied as a sample from a 4-ml total obtained in a previous step).

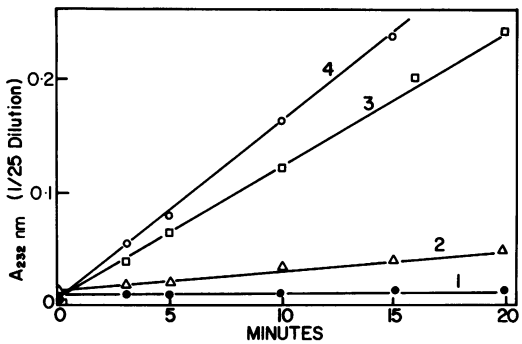

FIG. 6. *Substrate specificity of B. pumilus PATE. For determining the substrate specificity the following compounds were used: (1) polymethyl polygalacturonate methyl glycoside (96% esterified), (2) pectin NF (68% esterified), (3) sodium polygalacturonate, and (4) polygalacturonic acid DP-12. The reaction mixture contained 0.5% substrate, 0.033 M Tris-hydrochloride buffer (pH 8.5) and 5 × 10⁻⁴ M $CaCl_2$. Partially purified enzyme (500 units/ml) was added to the reaction mixture and incubated at room temperature. Absorbancy at 232 nm was measured in 1/25 dilution with 0.1 M acetate buffer at pH 3.7 at appropriate times to inactivate the enzyme.*

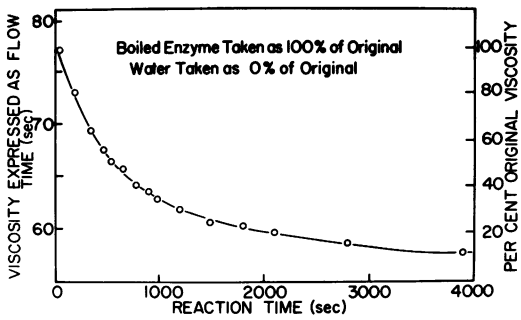

FIG. 7. *Pattern of action of PATE as determined by viscosimetry. One milliliter of enzyme solution (166 units with a specific activity of 20,000 units/mg of protein) was added to an Oswald-Cannon-Fenske capillary viscosimeter, containing 9 ml of standard reaction mixture. Flow times were determined at various reaction times. Per cent of the original viscosity was calculated at various intervals. Viscosity of various control solutions expressed as flow times were: water, 54.8 sec; 9 ml of reaction mixture + 1 ml of boiled enzyme, 77.3 sec; 9 ml of reaction mixture + 1 ml of water, 79.4 sec.*

reported for the unidentified bacillus (8). The optimal *p*H for formation of reducing groups and the rate of change in absorbancy at 232 nm paralleled each other rather closely. The optimal *p*H for reducing group formation by the PATE was 8.0. The parameter of PG activity is reducing group formation. Since most polygalacturonases described thus far have a low *p*H optimum, these data also indicate that the enzyme preparation contains no PG.

Effect of divalent cations on PATE activity. The known bacterial PATE enzymes require divalent cations for maximum activity, calcium ions being the most stimulatory. Exploratory experiments with crude cell-free dialyzed culture fluid of *B. pumilus* showed that the rate of absorbancy change at 232 nm was markedly stimulated by the addition of 3×10^{-4} M $CaCl_2$ to a reaction mixture containing 0.46% polygalacturonic acid. Addition of 3×10^{-4} M sodium ethylenediaminetetraacetate (EDTA) to such a reaction mixture not containing added $CaCl_2$ completely stopped the reaction, whereas the addition of 6×10^{-4} M $CaCl_2$ to the EDTA treated mixture completely restored the PATE activity from any time between 5 minutes and 4 hours, the longest period tested. Similar results had already been observed with *B. polymyxa* and *C. multifermentans* in this laboratory (13, 16).

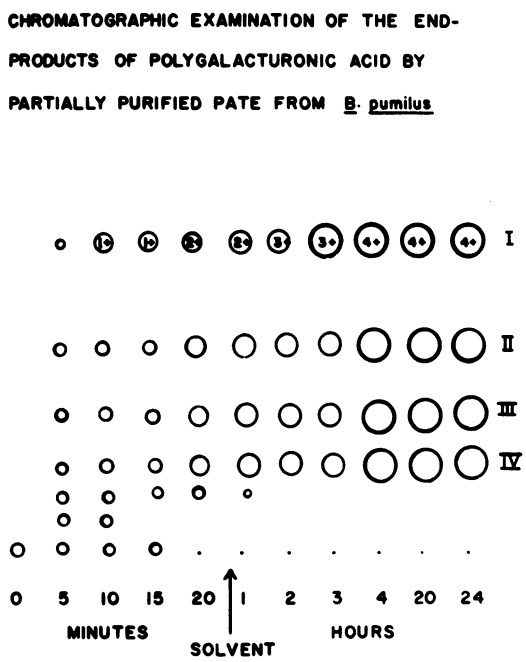

FIG. 8. *Chromatographic examination of the end products of polygalacturonic acid by partially purified PATE from B. pumilus. Reaction mixtures taken at various time intervals and chromatographed on Whatman paper no. 4 (after Dowex-50 H^+ treatment) in pyridine-ethyl acetate-water-acetic acid, 5:5:5:5, with a descending solvent system at room temperature for 16 hr. Silver nitrate reagent was used for detection of the oligouronides. The diagram is drawn to scale. Spots I, II, III, and IV with R_{gal} values of 0.59, 0.36, 0.23, and 0.14, respectively, indicate various unsaturated oligogalacturonides, DP 3 - DP 6 (Davé and Vaughn, manuscript in preparation). D-Galacturonic acid and unsaturated digalacturonic acid used as controls are not shown in the diagram.*

Effect of temperature. Reaction mixtures containing polygalacturonic acid (0.46%) and purified PATE were incubated at various temperatures from 20 to 60 C, and changes in absorbancy were measured at 232 nm at regular intervals. The rate of reaction increased with temperature changes up to 60 C. At 75 C, 90% of the enzyme was inactivated in less than 2 min. The final absorbancies at 232 nm of the undiluted reaction mixtures after 24 hr were 24.5 at 20 C; 27.5 at 25.5 C, 29.0 at 30 C, 31.0 at 37 C, 30.5 at 45 C, and 31.5 at 54 C. A final absorbancy of 31.0 was observed at 60 C in less than 2.5 hr.

Unfortunately, the optimal temperature for activity of the PATE of the unidentified bacillus (8) was not reported; thus, no comparison is possible with the unusually high temperature observed for the *B. pumilus* enzyme. However, the optimal temperature for activity of the PATE of *B. polymyxa* was around 45 C (17) as compared to 60 C for the one of *B. pumilus*.

Initial reaction rates calculated from the data were used to construct an Arrhenius plot. The plot was linear from 25 to 60 C. Activation energy for *B. pumilus* PATE as calculated from the plot was 12,183 cal/mole.

Heat inactivation of PATE was studied both in the presence and in the absence of 0.01 M $CaCl_2$ at 45, 60, and 75 C. It was found that $CaCl_2$ has some kind of protective effect on PATE during heat inactivation, and this effect diminishes as the temperature increases.

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