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PREPARATION, SEPARATION AND DEGRADATION OF OLIGOURONIDES PRODUCED BY THE POLYGALACTURONIC ACID TRANSELIMINASE OF *BACILLUS PUMILUS*

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SUMMARY

Production of the end products of polygalacturonic acid degradation on a large scale was done by reacting free galacturonic acid with *Bacillus pumilus* polygalacturonic acid transeliminase (PATE, EC 4.2.2.2) to obtain a mixture of the barium salts of several oligouronides. Small amounts of the unsaturated oligouronides were separated by paper chromatography. Large quantities of unsaturated oligouronides were separated on a AG-1-X8 (formate) column by applying a sample of mixed oligouronides and stepwise elution was carried out with sodium formate buffer (pH 4.7). The unsaturated oligouronides were identified on the basis of chromatographic mobilities, Sephadex gel filtration data, COOH/CHO ratio, thiobarbituric acid-reacting material, bromine uptake, and chemical and enzymatic degradation data as unsaturated tri-, tetra-, and hexagalacturonic acids.

The chemical degradation of these unsaturated oligouronides, done with 6 *N* HCl by heating at 100° for 30 min, gave qualitatively identical products of hydrolysis. These products compared with authentic standards, were identified as galacturonic acid, formic acid, 5-formyl-2-furancarboxylic acid, and 2-furancarboxylic acid. Analysis of the enzymatic breakdown products of the higher unsaturated uronides showed that a minimum of four galacturonic acid units was required for the action of purified endo-PATE from *B. pumilus*. The unsaturated trimer was not attacked, thus accounting for its accumulation as the major end product of polygalacturonate degradation by this enzyme.

INTRODUCTION

Since the discovery of pectin transeliminase (PTE) by Albersheim *et al.*[1] in 1960 there has been a considerable advance in our knowledge of transeliminative pectin- and polygalacturonic acid-splitting enzymes. It has been shown that some, but not all, molds produce PTE which is specific for pectin and oligogalacturonide

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methyl esters[2–5], whereas the presently known bacterial transeliminases are most specific for polygalacturonic acid and are quite widely distributed among various genera[6–15]. In contrast, so far as is known, yeasts do not produce such enzymes[16,17] and their production by protozoa[18,19] is, perhaps, conjectural.

As a result of these studies an endo-pectin transeliminase from fungi is known to degrade pectin to unsaturated di-, tri-, tetra-, and pentagalacturonic acids[4,20], whereas the bacterial endo- and exo-polygalacturonic transeliminases preferentially attack pectic acid with the production of large quantities of unsaturated di- or trigalacturonic acids, depending upon the species[9–11,13,14,21].

In elucidating the formation of these primary end products a major problem has been preparation of sufficient attackable substrate of low degree of polymerization (D.P.) to evaluate properly end product production. Various saturated oligouronides produced by hydrolytic cleavage of the polymer by yeast polygalacturonase and unsaturated compounds produced by bacterial pectic acid lyases have been separated by ethanol precipitation of their strontium or barium salts[22,23] and by ion-exchange column chromatography using Dowex-3 resin in the formate form[24], or Dowex-1 column chromatography[7,25,26]. A highly satisfactory method for obtaining more than milligram quantities of end products was sought. The purpose of this paper is to report investigations on the end products produced from polygalacturonic acid by the partially purified polygalacturonic acid transeliminase (PATE) of *B. pumilus* and to firmly establish that unsaturated trigalacturonic acid is the main end product of this degradation.

MATERIALS AND METHODS

The materials and methods employed for the production, assay and purification of the enzyme, as well as the general procedures for paper and column chromatography, have been described earlier by Davé and Vaughn[27]. Several additional materials, along with some analytical methods employed in this study are described in the pertinent sections which follow.

RESULTS AND DISCUSSION

Examination of end products formed by crude and purified PATE

The end products produced by partially purified PATE of *B. pumilus* at various times (5 min to 24 h) had been suspected to be a mixture of unsaturated oligouronides[27]. A comparison was made between the end products elaborated by crude PATE and those evolved by the enzyme preparations at various stages of purification. A reaction mixture containing 0.5% polygalacturonic acid, $5 \cdot 10^{-4}$ *M* $CaCl_2$, and 0.033 *M* Tris–HCl buffer at pH 8.5 was incubated with 5% (v/v) crude enzyme, phosphate gel eluate, or single and double DEAE-cellulose-treated enzyme preparations. All enzyme solutions were diluted suitably to have 1000 units of PATE activity[27]. The results diagrammed to scale in Fig. 1 indicate that the end products produced by the crude enzyme and the various partially purified enzyme preparations were similar. Furthermore, it showed that unsaturated digalacturonic acid was a minor component, and galacturonic acid was not observed among the end products. Four spots with R_F values lower than that of the unsaturated dimer were detected in

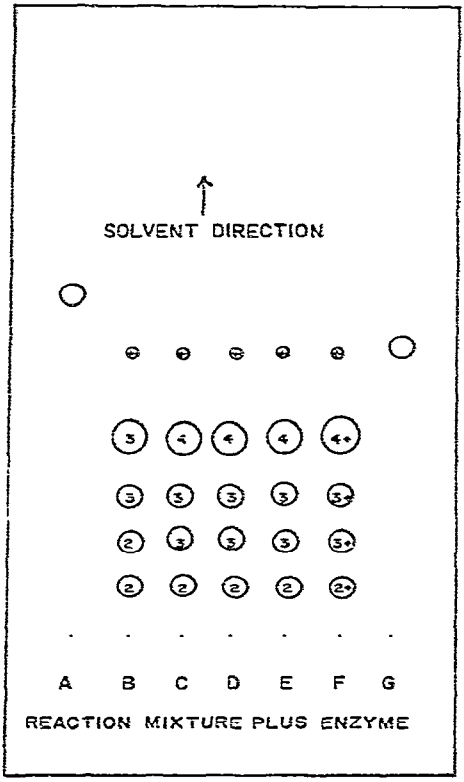

Fig. 1. Reaction mixtures containing polygalacturonic acid and various enzyme preparations at different stages of purification, incubated for 24 h at room temperature. The reaction products were treated with Dowex-50 (H^+) resin to remove Ca^{2+} and chromatographed in pyridine–ethyl acetate–water–acetic acid (5:5:5:5) solvent system on Whatman paper No. 4 at room temperature for 12 h. Silver nitrate reagent was used for detection. A = galacturonic acid (control); B = reaction mixture (Rx) + crude enzyme; C = Rx + phosphate gel eluate; D = Rx + DEAE first passage; E = Rx + DEAE second passage; F = Rx + Sephadex G-100 peak II[27]; G = unsaturated digalacturonic acid (control). Diagram drawn to scale; numbers indicate intensity of spots.

the chromatogram. There may have been some minor components having R_F values very similar to those already observed, which would remain undetected in small-sized sample analysis. In the hope that these same minor components might be detected more readily if a larger quantity of end products could be analyzed, a large-scale production of the end products was attempted.

Dialyzed cell-free culture fluid of *B. pumilus* (1.5 l with a PATE activity of 1500 $A_{232\ nm}$ units/ml) was added to 8.5 l of 1.5% polygalacturonic acid, containing 0.033 *M* $CaCl_2$. Crude enzyme was used in this experiment, since the end products produced by the crude and partially purified enzymes were found to be similar. After 24-h incubation at room temperature, the pH of the solution was adjusted to 5.0 with glacial acetic acid. Solid barium chloride (244 g/10 l) was added, and the mixture was stirred until the barium chloride dissolved. Stirring was continued while 95% ethanol was slowly added until the ethanol concentration had reached 50% (v/v). Preliminary experiments had shown that these concentrations of barium chloride and

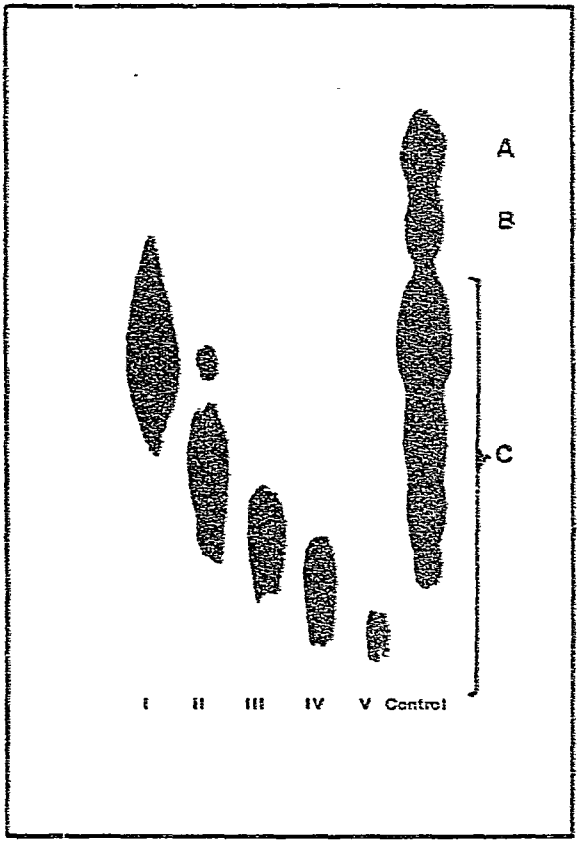

Fig. 2. Chromatogram showing eluted bands I–V obtained by preliminary PC separation of the end products of polygalacturonic acid degradation by *B. pumilus* PATE. A mixture of unsaturated oligouronides (800 mg) in 0.8 ml of distilled water spotted on eight Whatman 3MM papers (18 × 22 in.) developed in descending solvent system pyridine–ethyl acetate–water–acetic acid (5:5:5:5) at room temperature for 24 h. Vertical end-strips cut from each dried paper chromatogram and checked by silver nitrate reagent for location of oligouronides. Respective zones from the remaining papers were cut horizontally and eluted in distilled water. The eluates were concentrated by lyophilization and checked for purity. Spots I–V were five components separated as above. Control spots were: A, galacturonic acid; B, unsaturated digalacturonic acid; C, the mixture of some of the higher unsaturated uronides produced by *B. pumilus* PATE.

ethanol were enough to precipitate almost all of the oligouronides present in the reaction mixture. A fluffy white precipitate was removed by filtration, and washed several times with 50% ethanol, once with 95% ethanol, and finally with diethyl ether. The filtrate had only trace amounts of unsaturated digalacturonic acid. The precipitate was thoroughly dried in a vacuum desiccator. By this method 137 g of a mixture of oligouronides were obtained as barium salts and were converted to the free acids by treatment with Dowex-50 (H^+) resin. The aqueous solution thus obtained was lyophilized for use in subsequent experiments.

Preliminary separation of various components

Preparative paper chromatography (PC) was done to separate the various

components of the end products of polygalacturonic acid degradation. Fig. 2 is a chromatogram of the eluates from band I through band V obtained by preparative PC. The eluted bands show identical absorption spectra in the ultraviolet (UV) region with maximum absorptions around 230–235 nm. The possible presence of higher unsaturated oligouronides was indicated by their R_F values which were relatively lower than those of galacturonic acid and unsaturated digalacturonic acid.

Separation of oligouronides by anion-exchange column chromatography

The anion-exchange resin AG-1-X8 (Bio-Rad Labs., Richmond, Calif., U.S.A.) available in the formate form, was washed several times with distilled water, followed by 0.5 *M* sodium formate buffer (pH 4.7). Finally it was suspended in a formate buffer (pH 4.7) of a suitable strength and poured into a 4 × 200 cm long column, and washed for 20–24 h with the same buffer. A 5-g sample of a mixture of oligouronides was applied, and stepwise elution carried out with 4 l each of 0.57, 0.60, 0.70, and 0.80 *M* sodium formate buffer (pH 4.7). The flow-rate was 60–80 ml/h. The fraction size was 20 ml per tube. Every fifth tube was used for PC and absorbance measurements at 232 nm. After PC the fraction tubes with similar material were pooled as shown in Fig. 3. The four components with the $R_{\text{galacturonic acid}}$ (R_{gal}) as 0.59, 0.36, 0.23, and 0.14 correspond to the four peaks observed for absorbance at 232 nm. The individual components were recovered from the buffer solutions as ethanol precipitable barium salts, converted to free acids by treatment with Dowex 50 (H^+), and then lyophilized. Under the above conditions yields as high as 80% were obtained, and the four components were checked for their final purity by PC.

An anion-exchange resin in the acetate form had been used by Hasegawa and Nagel[7] to separate the saturated oligouronides obtained on a large scale by the action of yeast endo-polygalacturonase (YPG) on pectic acid. Use of the AG-1-X8 (formate) column for separation of unsaturated uronides was highly successful in this study. Preliminary experiments indicated that a Dowex-1 (formate) or a similar column might be useful in separation of a mixture of saturated and unsaturated oligouronides[28].

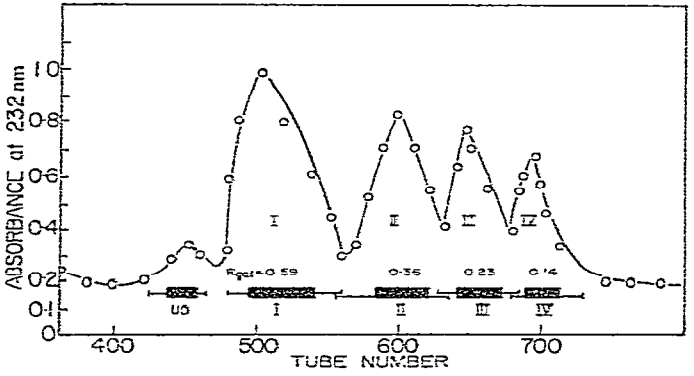

Fig. 3. Column chromatography of oligouronides on AG-1-X8 (formate) anion-exchange resin. A 4 × 200 cm long column of AG-1-X8 (formate) was equilibrated with 0.57 *M* sodium formate buffer (pH 4.7). Each component was recovered from the pooled material as the barium salt and converted to free acid by Dowex-50 (H^+) resin. UD = unsaturated dimer; I–IV represent various higher oligouronides.

Shortly thereafter Nagel and Wilson[26] described the separation of such mixtures by ion-exchange (Dowex-1-X8) column chromatography, thus substantiating our speculations. However, the separation is dependent not only on the amount and type of the material applied to the column, but also on the relative concentrations of the compounds present in the mixture as well as the concentrations of buffer used for their elution.

Characterization of the various unsaturated oligouronides

Gel filtration on Sephadex G-15 and G-25. Whitaker[29] used Sephadex gel to show the linear relationship between the logarithm of molecular weight of a protein and the ratio of its elution volume (V) to the void volume (V_0) of the Sephadex column used. If the converse is true, *i.e.*, if we assume the molecular weights of a series of compounds, and after determining the eluting volumes plot the molecular weights *versus* V/V_0, the linear relationship should be shown. This converse method is useful in determining molecular weights of a homologous series of compounds, such as the homologous series of unsaturated oligouronides produced by PATE of *B. pumilus*. The retaining volumes or eluting volumes (V) were calculated on the basis of peaks observed at 232 nm. Unsaturated digalacturonic acid and galacturonic acid were used as reference compounds wherever possible. The fractions were checked for purity by PC for their R_{gal} values. In Fig. 4 data obtained by gel filtration of oligouronides on Sephadex columns are presented. A linear relationship of the ratio V/V_0 *versus* the ^{10}log of the assumed molecular weights of the uronides was observed in all three cases shown. Also, a similar type of linear relationship between the PC mobilities and the degree of polymerization was observed. When individual components were applied to the column (as in Fig. 4, C), the eluates collected in the peak zone (of absorbance measurements) were pooled together and lyophilized for further analysis.

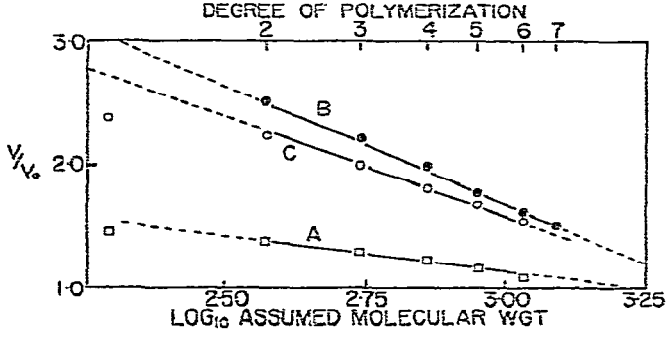

Fig. 4. Gel filtration of oligouronides on Sephadex G-15 and G-25. A sample containing a 10-mg mixture of oligouronides or 2 mg of a single oligouronide in 0.2 ml distilled water applied to Sephadex column G-15 (2 × 200 cm) or G-25 (1.5 × 60 cm). Elution in all cases was with distilled water. The flow-rate was 1–2 ml/h and the fraction size was 1–2 ml per tube, depending on the column used. $A_{232\ nm}$ was measured with a Beckman DB spectrophotometer and fractions were checked by PC for homogeneity of the component. Unsaturated digalacturonic acid and galacturonic acid were used as controls. Void volume (V_0) was determined using blue dextran solution; V = elution volume. A, mixture of oligouronides on Sephadex G-15, average of two experiments; B, mixture of oligouronides on Sephadex G-25, average of two experiments; C, individual components from AG-1-X8 (formate) column on Sephadex G-25.

Characterization of oligouronides by chemical and analytical methods. After separating the unsaturated oligouronides as described above, their characterization and identification were undertaken by examining various properties of the compounds. Methods used for this purpose included the ratio of carboxyl to aldehyde groups (COOH/CHO ratio), reaction with thiobarbituric acid (TBA), bromine uptake, UV absorption and by assaying for galacturonic acid after hydrolysis of the different oligouronides.

The COOH/CHO ratio was determined by analyzing for free carboxyl and reducing groups with the methods used by Davé and Vaughn[27]. The UV spectra of aqueous solutions of each of the uronides (1 mg per 10 ml) were determined. All spectra showed a maximum absorption at 232 nm. From this absorbance and the concentration of the uronide and its assumed molecular weight, molar absorptivities were calculated. The TBA reaction was carried out according to the method described by Albersheim *et al.*[1]. Two concentrations of each compound were tested: 10 mg per ml of distilled water and 1 ml of a 0.01 *M* solution of each uronide. Bromine uptake by the oligouronides in question was determined by the method described by Linker *et al.*[30]. Micromole bromine uptake per micromole of oligouronide was calculated using bromine blank titres. Galacturonic acid assay was determined after acid hydrolysis of the oligouronides by use of the naphthoresorcinol method of Rahman and Joslyn[31]. In order to assay within the sensitivity of the method, 0.2-ml samples of the hydrolysate were suitably diluted before the test was made. In all cases, the known unsaturated digalacturonic acid was used as a reference compound.

The results of these various tests and calculations designed to help identify the different compounds are shown in Table I. The homologous nature of the series of compounds in question had already been suggested by the results of gel filtration and PC. All of the criteria applied show clearly that the compounds are such a series of unsaturated oligouronides. Thus, on the basis of absorbance at 232 nm, molar absorptivities and bromine uptake it is certain that the four compounds are unsaturated and that there is a single unsaturated component present in each. The TBA reactions with equimolar concentrations of the compounds also are supporting evidence for single unsaturation. The COOH/CHO ratios indicate that the compounds are unsaturated trimer, tetramer, pentamer and hexamer. Convincing proof of the identity of these compounds was obtained by hydrolysis and estimation of the units of galacturonic acid released from each oligouronide. On a molar concentration basis, galacturonic acid in amounts 2, 3, 4, and 5 times that expected from unsaturated dimer was obtained on hydrolysis of the compounds under consideration.

Chemical degradation of unsaturated galacturonides

Infrared (IR) spectrum analysis and mass spectrometry generally are used to supplement data obtained by other methods to augment determination of the structure of a compound. These methods were unsatisfactory. Interpretation of the data obtained in IR analysis was difficult because no known reference compound other than unsaturated dimer was available. Mass spectrometry was unsuccessful because the samples charred at the temperature of analysis and the compounds may have been degraded. Therefore acid hydrolysis was resorted to in an attempt to obtain further confirmatory evidence for the structure of the unknown oligouronides.

Methods described by Hasegawa and Nagel[32], and Okamoto *et al.*[33], were

TABLE I

ANALYTICAL DATA ON UNSATURATED OLIGOURONIDES

Compound	*Absorbance at 232 nm (0.1 mg/ml)*	*Molar absorptivity of aqueous solution* ε_{232nm}*	*COOH/CHO ratio*	*TBA reaction absorbance at 547 nm (10 mg/ml 0.01 M soln.)*		*Bromine uptake (μmole per μmole of compound)*	*Galacturonic acid assay (after hydrolysis**) by naphthoresorcinol method*	
							Observed (μg/0.1 ml)	*Calculated (μg/0.1 ml)*
I	0.870	4750	2.90	0.97	0.51	1.36	370	388
II	0.550	3971	3.75	0.75	0.55	1.29	555	582
III	0.430	3861	4.70	0.69	0.58	1.10	740	776
IV	0.330	3544	5.80	0.55	0.56	1.05	900	970
Unsaturated dimer	1.300	4870	1.90	1.40	0.60	1.40	190	194
Galacturonic acid	0.000	—	0.95	0.080	0.025	0.00	180	194

* Data based on assumed or known molecular weights of the compounds.

** Hydrolysis of 0.1 ml of 0.01 *M* solutions of uronides by 6 *N* HCl at 100° for 30 min.

TABLE II

COMPARISON OF HYDROLYSATES OF UNSATURATED OLIGOURONIDES

Abbreviations: PEWA = pyridine–ethyl acetate–water–acetic acid; BAW = *n*-butanol–acetic acid–water; BPB = bromophenol blue.

Reference	*Oligouronides used*	*Concentration (mg/ml)*	*Degradation conditions*	*PC solvent system*	*Detection of products formed*	*Spots (R_F or R_{gal})*
Hasegawa and Nagel[32]	Unsaturated dimer	2	Mild, 1 *N* HCl, 100°, 30 min	PEWA (5:5:3:1), 6–8 h	$AgNO_3$, 2 spots	(1) R_F similar to that of galacturonic acid, identified; (2) $R_F = 1.6 \times R_F$ of galacturonic acid, compound unidentified
Okamoto *et al.*[33]	Unsaturated dimer	30 (0.08 *M*, pH 3.0)	Thermal degradation, 120°, 30 min	BAW (4:1:2), 6–8 h	Aniline chloride, 4 spots	(1) $R_F = 0.30$ (galacturonic acid); (2) $R_F = 0.74$ (5-formyl-2-furancarboxylic acid; (3) $R_F = 0.89$ (2-furancarboxylic acid) (4) formic acid, identified chemically
Modified method	Unsaturated trimer*	5	Strong, 6 *N* HCl, 100°, 30 min	PEWA (1:1:1:1), 6–8 h	$AgNO_3$, 3 spots; BPB, 3 spots	(I) $R_{gal} = 1.0$; (Ia) $R_{gal} = 1.2$; (II) $R_{gal} = 1.7$ (later identified in BAW (4:1:2) as 4 spots as in Okamoto *et al.*[33]

* Unsaturated dimer and all the higher unsaturated uronides yielded apparently identical products.

tried and were found to give incomplete hydrolysis of the unsaturated oligouronides. Hence a modification of the method of Hasegawa and Nagel was attempted by increasing the concentration of HCl from 1 *N* to 6 *N*. With this modification, complete hydrolysis of uronides was observed within 30 min at 100°.

The data presented in Table II, showing the comparison of the degradation of unsaturated oligouronides using the above mentioned three different methods, showed serious descrepancies. However, it was determined that the discrepancy was due to the solvent used for chromatography and the method of preparation of the compounds for chromatography. In actuality, all the methods appear to form the same four compounds (Table II). According to the method of Okamoto *et al.*[33], utilizing PC with the solvent system *n*-butanol–acetic acid–water (BAW) (4:1:2), the compounds detected were D-galacturonic acid, 5-formyl-2-furancarboxylic acid, 2-furan carboxylic acid, and formic acid. While using the method of Hasegawa and Nagel, the hydrolysate was evaporated to dryness, and thus formic acid, a volatile compound, might have

TABLE III

PAPER CHROMATOGRAPHIC CHARACTERISTICS OF THE HYDROLYTIC PRODUCTS OF UNSATURATED TRIGALACTURONIC ACID BY 6 *N* HCl AT 100° FOR 30 min

Abbreviations: PEWA = pyridine–ethyl acetate–water–acetic acid; BAW = *n*-butanol–acetic acid–water; BPB = bromo phenol blue. +, ++, +++, and ++++ indicate relative intensities of spots; — = not observed.

Compound	R_F *in BAW (4:1:2)*	R_F *in PEWA (5:5:5:5)*	*$AgNO_3$ spray*	*BPB*	*Aniline·HCl*	*Special character*	*Identification*
1	0.30	0.45	++++	++	Reddish brown	Gives pink color with naphthoresorcinol	Galacturonic acid
2	0.40	0.53	+*	+*	Light brown	$\lambda_{max.}$ (210 nm)	Formic acid
3	0.80	0.80	+	+	Light yellow	$\lambda_{max.}$ (285 nm)	5-Formyl-2-furancarboxylic acid**
4	0.93	0.80 (not seen as a separate spot)	—	++—	No color or very light yellow	$\lambda_{max.}$ (245 nm)	2-Furancarboxylic acid***

* Variable color reaction depending on amount present.

** Reference compound was prepared according to Stutz and Deuel[34].

*** Synonym: 2-furoic acid; compound obtained (practical grade) from Eastman, Rochester, New York.

been lost. When the hydrolysate, obtained by our modified method, was checked by PC in the BAW (4:1:2) solvent system, the hydrolysate showed four spots. Two distinct spots for 5-formyl-2-furancarboxylic acid and 2-furancarboxylic acid appeared in the BAW (4:1:2) solvent system instead of one fused spot observed in the pyridine–ethyl acetate–water–acetic acid (PEWA) (5:5:5:5) solvent system. Table III summarizes the identity of the individual components of the hydrolysate of the unsaturated trimer.

Enzymatic degradation of the unsaturated oligouronides

The purified enzyme PATE from *B. pumilus*[27] was used for degradation of the unsaturated uronides and for establishing its minimum chain length requirement. Two millilitres of reaction mixture containing 0.5% of a single unsaturated uronide in a solution of 0.033 *M* Tris–HCl buffer (pH 8.5), $5 \cdot 10^{-4}$ *M* $CaCl_2$, and 1000 units of purified PATE, were incubated at room temperature. Various unsaturated uronides ranging from di- to hexagalacturonides were used as substrates. At various time intervals, samples were taken and chromatographed at room temperature for 8 h on Whatman paper No. 4 using PEWA (5:5:5:5) as the solvent system. From the results obtained shown in Table IV, it was observed that unsaturated di- and trigalacturonides were not attacked for 96 h. Hexagalacturonide was attacked rapidly and it completely disappeared in about 6 h with the production of a single end product, unsaturated trigalacturonide. Unsaturated pentagalacturonide was attacked at a lower rate, and in 48 h it had disappeared giving rise to unsaturated dimer and trimer as the end products. Unsaturated tetragalacturonide was attacked at such a slow rate that its reaction products appeared only after 24 h or more, and even after 96 h a slight amount of unsaturated tetragalacturonide remained. The reaction products were limited to

TABLE IV

PRODUCTS OF ENZYMATIC DEGRADATION OF UNSATURATED OLIGOURONIDES

Abbreviations: M′ = probably unsaturated monomer; U. di., U. tri., U. tetra., U. penta., and U′ hexa. are all unsaturated oligouronides increasing D.P. from 2 to 6. − = not observed; ± = doubtful; +, ++, +++, and ++++ indicate relative intensities of spots. As shown earlier[27], the enzyme reacted with polygalacturonic acid rapidly, and in less than one hour the spot for polymer had disappeared with the formation of U. tri., U. tetra., U. penta., and U. hexa. When U. tri. and U. di. were incubated with the enzyme, no change was observed for as long as 96 h.

Reaction time (h)	*Substrate U. tetra.*	*Products*		*Substrate U. penta.*	*Products*		*Substrate U. hexa.*	*Product U. tri.*
		U. tri.	*M′*		*U. tri.*	*U. di.*		
0.5	± + + +			+ + + ±			+ + +	+
1.0	+ + + +			+ + + +			+ +	+ +
6.0	+ + + +			− + + +			−	+ + + +
9.0	+ + + +			− ± +	+	+	No further change	
12	+ + + +			+ +	+ +	+ +		
24	+ + +	+		+	± + +	+ + +		
48	+ +	+ +	±	−	± ± + +	+ + + +		
96	+	+ + +	+	−	+ + + +	+ ± + +		

unsaturated trigalacturonide and a compound having a R_F higher than galacturonic acid, possibly a mono-unsaturated galacturonic acid.

The production of various end products described above is further evidence for the structure of the uronides used as substrates. A decrease in the reaction rates, as the chain-length of the substrate decreases is clearly observed (see Table IV), indicating that a certain minimum size of the chain-length is required by the PATE of *B. pumilus* for its action on the uronides. Furthermore, since unsaturated trimer is not attacked, it appears that the minimum chain-length required for the action of *B. pumilus* PATE enzyme corresponds to a D.P. of 4.0. Thus, the endo-PATE from *B. pumilus* differed from that of *B. polymyxa*[12] which produced unsaturated digalacturonic acid as the major end product of polygalacturonic acid degradation. Nevertheless, in some respects, it was similar to the endo-PATE produced by the thermophilic *B. stearothermophilus*[9] which produced unsaturated trigalacturonic acid as the major end product. However, the differences between the endo-PATE of *B. pumilus* and that of the thermophilic *Bacillus* may or may not be significant. The later enzyme had a higher molecular weight (24,000 daltons) and an optimum activity at 70° in the presence of Ni^{2+}, whereas the former was lighter (molecular weight 20,000 daltons) and had optimum activity at 60° in the presence of Ca^{2+}.

Another species of *Bacillus* isolated from soil is known to produce unsaturated trigalacturonic acid[7]. Unfortunately, the culture was not described in detail enough to compare it with the *B. pumilus* used here. There are some similarities between the two PATE enzymes. Both are endo-polygalacturonic acid *trans*-eliminases which cause the accumulation of the unsaturated trimer as the major end product. Both require Ca^{2+} for maximum activity. The *B. pumilus* enzyme showed optimal activity at pH 8.0–8.5. This range is somewhat lower than the optimal range pH 8.9–9.4 reported for the enzyme of *B. polymyxa*[12] or the optimum of pH 9.3–9.7 reported for the unidentified *Bacillus*[7]. Other possible pertinent comparisons such as molecular weight and optimum temperature cannot be made as such information is lacking for the unnamed soil *Bacillus*.

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