The degree of specificity inherent in these limited digestions has not been established. However, the distribution of the "additional bases" among the fragments produced by partial digestion of alanine-specific acceptor RNA suggests that some degree of specificity exists. Thus inosinic acid is found predominantly in the peak of small oligonucleotides (IV) in Figure 6; N^2-dimethylguanine, on the other hand, occurs only in the large fragments (I and II), which also contain most of the pseudouridylic acid. The "additional" base N^1-methylguanine appears in both the large and the small fragments, as if incomplete cleavage had occurred at a relatively insensitive internucleotide linkage. However, since only one residue of each of the "additional" nucleotides N^1-methylguanylic acid, N^2-dimethylguanylic, ribothymidylic acid, and inosinic acid is present per molecule of intact alanine-specific acceptor RNA (Ingram and Sjöquist, 1963), a completely specific cleavage should lead to the appearance of each of these components in only one chromatographic peak. With the exception of N^1-methylguanylic acid, this is the case. It may be possible to improve the degree of specificity by modifying the conditions of digestion and to obtain better separation of the large pieces.

Finally, it should be pointed out that these results do not really support the model of acceptor RNA recently proposed by McCully and Cantoni (1962). If the "additional bases" were all concentrated in a nonhelical loop, one would expect, with both mixed and alanine-specific RNA, that the content of such bases would be reduced in the large fragments and enriched in the small fragments resulting from partial ribonuclease digestion. For the most part, our findings do not agree with this prediction.

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Purification and Properties of a Polygalacturonic Acid-*trans*-eliminase Produced by *Clostridium multifermentans**

JAMES D. MACMILLAN AND REESE H. VAUGHN

From the Department of Food Science and Technology, University of California, Davis

Received November 18, 1963

A strain of *Clostridium multifermentans*, although unable to ferment D-galacturonic acid, fermented polygalacturonic acid vigorously with the production of an extracellular pectic enzyme. With polygalacturonic acid as the substrate, this enzyme, with an optimal *p*H of 8.5, produced reaction products which strongly absorbed light at 235 mμ, indicating a *trans*-elimination mechanism. The clostridial enzyme was partially purified and was freed of contaminating pectinesterase. Based on the relationship between increase in absorbancy at 235 mμ and increase in reducing group formation, polygalacturonase appeared to be absent in the crude culture fluid. Activity of the enzyme was dependent on critical levels of certain divalent cations. Calcium, strontium, and manganese showed the greatest stimulation. The major end product from polygalacturonic acid was *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid. Purified enzyme showed no activity on pectin of high methoxyl content (96% esterified) but crude enzyme preparations were able to attack this substrate since they also contained pectinesterase.

A most significant contribution to our knowledge of the enzymic degradation of pectic substances was the discovery by Albersheim, *et al.* (1960) of an enzyme capable of splitting the α-1,4-glycosidic bonds of pectin by a *trans*-elimination mechanism. Prior to this it had been universally assumed that pectinolytic enzymes break these bonds only by hydrolysis. The cleavage of the glycosidic bond of pectin by *trans*-elimination results in the loss of the proton from carbon atom 5 of a methyl galacturonate residue forming an unsaturated bond between carbon atoms 4 and 5. The products absorb light at approximately 235 mμ. There was no evidence of *trans*-elimination when the enzyme was incubated with polygalacturonic acid. This indicated a specific requirement for the presence of carbomethoxy groups rather than free carboxyl

* The studies in this paper were presented at the Symposium on Recent Developments in Pectin Chemistry and Pectin Degradation at the 114th American Chemical Society meeting in Los Angeles, California, March 31 to April 5, 1963.

groups. On the basis of this specificity the enzyme was named pectin-*trans*-eliminase. Purification procedures were reported in a later publication (Albersheim and Killias, 1962).

Edstrom (1962) found a similar enzyme produced by *Aspergillus fonsecaeus* and succeeded in separating it from the enzymes polygalacturonase and pectinesterase which this fungus also produces. He found that the products of the reaction of pectin-*trans*-eliminase on polymethylpolygalacturonate methyl glycoside (Link pectin), were a series of unsaturated methyl oligogalacturonates having chain lengths of two to six units.

Nagel and Vaughn (1961a,b, 1962) found that *Bacillus polymyxa* produces a randomly acting calcium-ion-dependent *trans*-eliminase which attacks polygalacturonic acid rather than pectin. This polygalacturonic acid–*trans*-eliminase (PATE)[1] produced as a major product an unsaturated digalacturonic acid which was identified by Hasegawa and Nagel (1962) as *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid. Starr and Moran (1962) have reported an apparently similar enzyme produced by *Erwinia carotovora*. Preiss and Ashwell (1963a,b) found that an enzyme preparation produced by a pectinolytic pseudomonad degraded polygalacturonic acid by a combination of both elimination and hydrolysis to D-galacturonic acid and 4-deoxy-L-*threo*-5-hexoseuloseuronic acid.

During a taxonomic study of some pectinolytic anaerobic bacteria isolated from soil (Ng and Vaughn, 1963) it was noted that two cultures (*Clostridium multifermentans* and *Clostridium butyricum*) rapidly fermented both pectin and polygalacturonic acid but not galacturonic acid. Furthermore, galacturonic acid did not accumulate in the media after fermentation of the polymers by these two cultures. This suggested that these organisms degraded pectin and polygalacturonic acid to products other than galacturonic acid and that these products were the actual substrates being fermented. The most likely enzymatic process whereby pectin or polygalacturonic acid could be degraded without the production of galacturonic acid was by *trans*-elimination.

We have shown that both organisms produce *trans*-eliminases specific for polygalacturonic acid. This paper deals with the purification and partial characterization of this enzyme from *C. multifermentans*. An accompanying publication is concerned with the action pattern by which this purified enzyme attacks polygalacturonic acid.

Materials and Methods

Substrates.—Polygalacturonic acid (product 3491) was obtained from Sunkist Growers Inc., Corona, Calif. Analysis of this material showed a moisture content of 5.4% and an ash content (wet-weight basis) of 2.3%. For most experiments this substrate was dissolved by neutralization with NaOH and used without additional treatment. In experiments connected with the effects of divalent cations on the activity of PATE a more highly purified sodium polygalacturonate was employed. This was prepared by passing a solution of 1.0% sodium polygalacturonate slowly through a 2.5 × 15–cm column of Dowex 50 W-X 4 (Baker 100–200 mesh Na^+ form). This treatment effectively removed contaminating divalent cations.

[1] Abbreviation used in this work: PATE, polygalacturonic acid–*trans*-eliminase.

Digalacturonic and trigalacturonic acids were donated through the courtesy of Dr. H. J. Phaff. The preparation of these compounds was described by Phaff and Luh (1952). Tetragalacturonic acid was prepared by the procedure of Demain and Phaff (1954). *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid (unsaturated digalacturonic acid) was obtained through the courtesy of Dr. C. W. Nagel and was prepared as reported by Nagel and Vaughn (1961b).

Pectin N.F. was obtained from Exchange Lemon Products Co., Corona, Calif. Its degree of esterification was 68%.

Polymethylpolygalacturonate methyl glycoside (Link pectin) was prepared by the method of Morell and Link (1932) and Morell *et al.* (1934) by refluxing dry polygalacturonic acid with 5% dry HCl in absolute methanol. The degree of esterification of this pectin was about 96%.

Adsorbants.—Calcium phosphate gel was prepared by the method of Kunitz (1952).

Cellulose *N,N*-diethylaminoethyl ether (Whatman-DEAE-cellulose, anion exchanger, powder DE 50) was pretreated as follows: The powdered material was suspended in distilled water, allowed to settle for a few minutes, and the supernatant solution containing the lighter suspended solids was decanted. This procedure was repeated several times and the heavier material obtained was suspended n 1 N NaOH, decanted, and washed with distilled water until the *p*H of the slurry was 7.0. It was finally collected by suction on a filter paper and air dried.

Media and Conditions for Enzyme Production.—The original medium "A" contained 0.46% polygalacturonic acid, 0.5% yeast extract, 0.5% proteose peptone (Difco), 0.5% K_2HPO_4, and 0.05% sodium thioglycolate. Later this medium was modified by deletion of the proteose peptone. This modified medium "B" did not produce as many units of PATE after 3 days' growth of *C. multifermentans*, but since the specific activities of crude dialyzed enzyme preparations were significantly higher its use was warranted.

One-liter Florence flasks were filled with approximately 1 liter of medium and covered with aluminum foil caps. After autoclaving and cooling the flasks were inoculated with 40 ml of an 18 to 24-hour culture of *C. multifermentans*. Sterile medium was then added aseptically until the liquid level was approximately half way up the neck of the inoculated flask and a 1-cm layer of Vaspar (50% vaseline–50% paraffin) was added to prevent oxygen diffusion into the medium. These cultures were incubated for 3 days at room temperature. Higher temperatures (30°, 37°) resulted in the production of considerably less enzyme.

Enzymatic Activities.—PATE activity was routinely assayed by the measurement of absorbancy changes at 235 mμ in reaction mixtures consisting of 0.46% (dry, ash-free basis) of untreated polygalacturonic acid, 0.001 M $CaCl_2$ and 0.033 M Tris buffer (tris-[hydroxymethyl]aminomethane, adjusted to *p*H 8 with HCl). The temperature was maintained at 30° ± 0.5° with a thermostatically controlled cell holder. The unit of PATE activity is the amount of enzyme which will produce 1 μmole of unsaturated digalacturonic acid per minute under the above conditions.

The reaction mixture for pectinesterase assays contained 0.5% (w/v) of an aqueous solution of pectin N.F. adjusted to *p*H 7.0 w th dilute NaOH and containing 10% (v/v) of a suitably diluted enzyme solution. Activity was determined at 30° by periodically titrating the liberated acid to *p*H 7.0 with 0.02

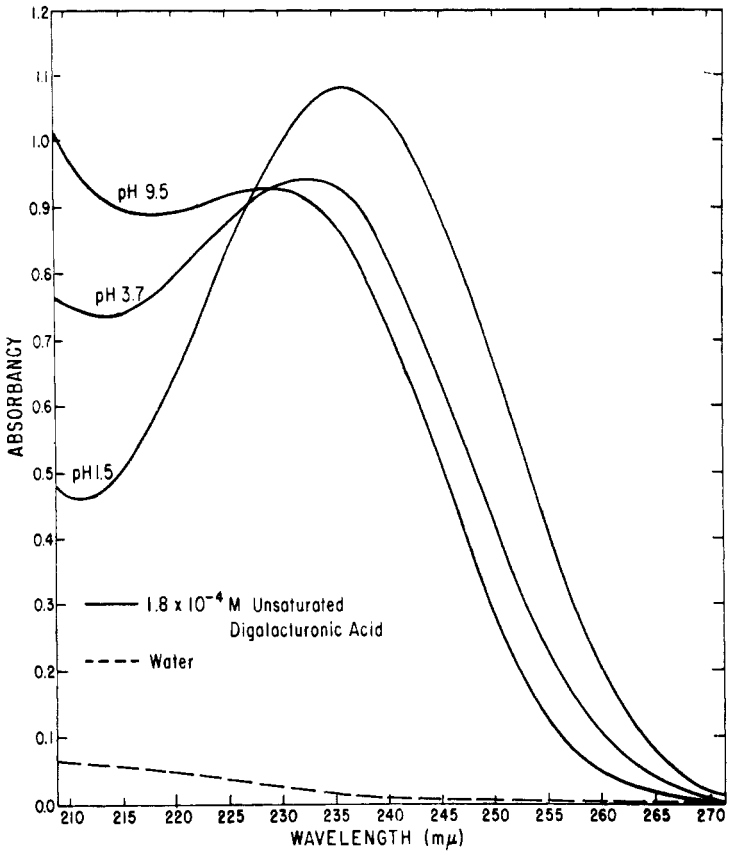

FIG. 1.—Ultraviolet-absorption spectra of *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid at various *p*H values.

N NaOH. The *p*H was measured by microelectrodes immersed in the reaction mixture. The total volume of NaOH added during a reaction never exceeded 10% of the volume of the mixture. Thus errors caused by dilution of the substrate and enzyme were not excessive. The unit of pectinesterase activity is the amount of enzyme which will cause the release of 1 μmole of carboxyl groups per minute under the above conditions. During certain phases of this work the hydroxamic acid plate test of McComb and McCready (1958) was employed as a qualitative test for pectinesterase activity. This method was modified in that enzyme solutions were placed in holes made in the solidified agar medium with a number 2 cork borer rather than added to the surface on filter paper disks.

Analytical Methods.—Reducing groups were determined by the Jansen and MacDonnell (1945) modification of the Willstätter and Schudel (1918) hypoiodite method. Protein was estimated either by the colorimetric method of Lowry *et al.* (1951) or by comparison of absorption at 280 and 260 mμ by the method of Warburg and Christian (1942).

The products formed by the action of PATE on polygalacturonate 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 hours. Nagel and Vaughn (1961b) found this solvent mixture suitable for separating unsaturated digalacturonic acid from the normal lower oligouronides. Spots on the developed chromatograms were located by spraying with *m*-phenylenediamine dihydrochloride followed by heating at 100° for 5 minutes (Chernick *et al.*, 1951). Aliquots from enzymic reaction mixtures were inactivated by adding the samples to an equal volume of 0.1 M acetate buffer at *p*H 3.7. The amounts chromatographed varied, but generally 0.1-ml portions were spotted.

RESULTS

Preliminary Observations.—Cell-free dialyzed broth from 3-day-old cultures of *C. multifermentans* was found to produce the following effects when incubated at 30° in the presence of 0.46% polygalacturonate: (a) The viscosity decreased; (b) the concentration of reducing groups increased; and (c) the absorbancy at 235 mμ increased. None of these changes took place with samples of culture broth which had previously been boiled for 10 minutes. The first two phenomena are known to occur in polygalacturonase–polygalacturonic acid mixtures. The ability to increase the absorbancy at 235 mμ, however, implied that an enzymatic *trans*-elimination reaction was involved in the degradation of the polygalacturonic acid.

In addition to PATE, crude enzyme solutions also contained appreciable pectinesterase. Furthermore, when crude enzyme solutions were incubated with 0.5% pectin the absorbancy at 235 mμ increased at a rate approximately half of that found with 0.5% polygalacturonate. Initially, it was not known whether this apparent *trans*-eliminase activity on pectin was due to attack by PATE on the nonesterified portion of the pectin molecule (augmented by the action of pectinesterase) or to the presence of a third enzyme, pectin *trans*-eliminase, capable of attacking the esterified port on of the pectin molecule. It was also not known whether crude PATE preparations also exerted hydrolytic action on polygalacturonic acid. Poly-

galacturonase cannot be assayed by direct methods in the presence of PATE since both enzymes produce aldehyde groups and decrease the viscosity of solutions of polygalacturonic acid. These are the only criteria presently used for determining polygalacturonase activity. Later in this paper an indirect method will be described for detecting polygalacturonase activity in the presence of PATE. Since pectinesterase was the only known contaminating pectic enzyme, purification was directed toward its removal in addition to increasing the specific activity of PATE.

Purification.—The source of crude PATE consisted of culture fluid which had been centrifuged for 10 minutes at 8000 × *g* to remove the cells and dialyzed for 24 hours against tap water at 4°.

The PATE in crude enzyme solutions produced in medium A was readily precipitated with ammonium sulfate at 3°. The precipitate obtained at 45% saturation showed the greatest increase in specific activity. Under these conditions 75% of a total activity of 90 PATE units in 500 ml of crude enzyme solution was recovered after the precipitate had been redissolved in 25 ml of distilled H_2O and dialyzed overnight against distilled H_2O. The specific activity after this treatment had increased 10-fold on the basis of protein determined by the method of Warburg and Christian (1942). A qualitative test for pectinesterase showed that this enzyme was still present in the partially purified preparation.

TABLE I

RECOVERY OF PATE BY AMMONIUM SULFATE AND CALCIUM PHOSPHATE GEL FRACTIONATION OF DIALYZED CULTURE FLUID FROM MEDIUM A

Purification Step	Volume (ml)	Total PATE (units)	Specific Activity[a] (PATE units/mg protein)	Recovery (%)
Original, dialyzed culture fluid	500	96	0.08	(100)
$(NH_4)_2SO_4$ precipitate[b] (45% saturation)	34	73	0.73	76
Calcium phosphate gel eluate[c] (10% gel v/v)	20	35	1.19	36

[a] Protein determined by the method of Warburg and Christian (1942). [b] Redissolved in 20 ml 0.1 M Tris buffer, *p*H 8.0, and dialyzed overnight against distilled water. [c] Eluted with 0.1 M phosphate buffer, *p*H 8.0, and dialyzed overnight against distilled water.

The PATE activity in either crude dialyzed culture fluid or in preparations which had been previously partially purified by precipitation with $(NH_4)_2SO_4$ was easily adsorbed on 10% (v/v) calcium phosphate gel at *p*H 8.0. Elution was effected with 0.1 M phosphate buffer at *p*H 8.0. The results of purification of dialyzed medium A by treatment with ammonium sulfate followed by calcium phosphate–gel adsorption and elution are shown in Table I. This purification produced a 15-fold overall increase in specific activity, but pectinesterase was still present in the eluate from calcium phosphate gel.

Removal of pectinesterase was finally accomplished in crude enzyme preparations produced on medium B. With this enzyme source ammonium sulfate did not precipitate either PATE or inactive protein at any level of saturation. This implied that the presence of proteose peptone in medium A was a factor in precipitation with ammonium sulfate. PATE from medium B, however, was readily adsorbed and eluted from 5% (v/v) calcium phosphate gel. Once PATE was eluted from the phosphate gel and dialyzed against distilled water, only a very small fraction could be readsorbed on the gel, even when its concentration was increased. The specific activity of this unadsorbed fraction, however, was increased 3-fold over that of the original eluate from 5% phosphate gel showing that extraneous protein was being adsorbed by the second treatment with phosphate gel. A summary of this purification is shown in Table II. The overall purification was 18-fold over crude dialyzed enzyme on the basis of protein determined by the optical density method of Warburg and Christian (1942) or 48-fold on the basis of protein determination by the method of Lowry *et al.* (1951).

Other attempts to further increase the purity of the enzyme were not successful. For example, PATE from either crude dialyzed culture fluid (medium A or B) or eluates from calcium phosphate gel were easily adsorbed on DEAE-cellulose at *p*H 8.0. Once adsorbed, however, the enzyme was difficult to elute. No combination of changes in *p*H or ionic strength gave satisfactory elution in either batch or column experiments. The final purification procedure which was adopted for routine use, therefore, was the one shown in Table II. Purified enzyme produced in this manner was studied in some detail and the results are reported in the remainder of this work.

Reaction Product.—A number of samples of reaction mixtures were examined by paper chromatography for identification of compounds produced during reactions of PATE on polygalacturonic acid. Re-

TABLE II

SUMMARY OF THE STEPS APPLIED IN THE PURIFICATION OF POLYGALACTURONIC ACID-*trans*-ELIMINASE PRODUCED BY *Clostridium multifermentans* ON MEDIUM B

Purification Step	Volume after Dialysis (ml)	Total PATE (units)	Recovery (%)	Specific Activity (PATE units/mg protein)		Pectinesterase (units/ml)
				a	*b*	
Dialyzed 24 hr against tap H_2O at 4°	3000	470	(100)	0.141	0.194	0.078
Adsorbed on 5% (v/v) phosphate gel; eluted 3 × with 100, 50, 50 ml 0.1 M phosphate buffer, *p*H 8.0; eluates combined and dialyzed 24 hr against distilled water	204	296	63	0.79	3.0	nil[c]
Added 50% (v/v) phosphate gel; centrifuged to remove gel; supernatant dialyzed 24 hr against distilled water	366	256	55	2.5	9.4	nil[c]

[a] Protein determined by the method of Warburg and Christian (1942). [b] Protein determined by the method of Lowry *et al.* (1951). [c] No change in titratable acidity during a 10-hr reaction period with 0.5% pectin N.F.

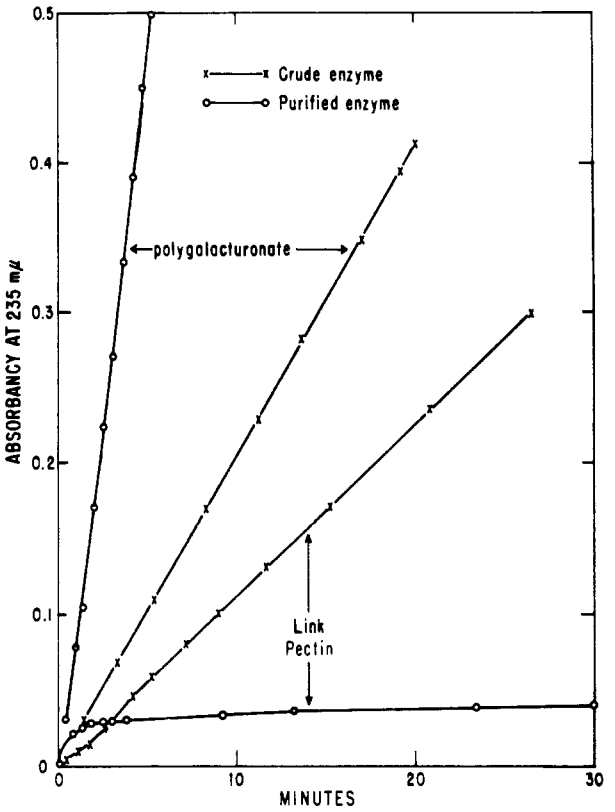

FIG. 2.—Comparison of polygalacturonic acid and polymethylpolygalacturonate methyl glycoside (Link pectin) as substrates for crude and purified PATE. Reaction mixtures contained 0.5% substrate, 0.033 M Tris buffer at *p*H 7.0, and 0.001 M $CaCl_2$. The reaction mixtures containing the crude enzyme preparation had 0.01 unit/ml and the ones containing the purified enzyme preparation had 0.05 unit/ml. Temperature, 30°.

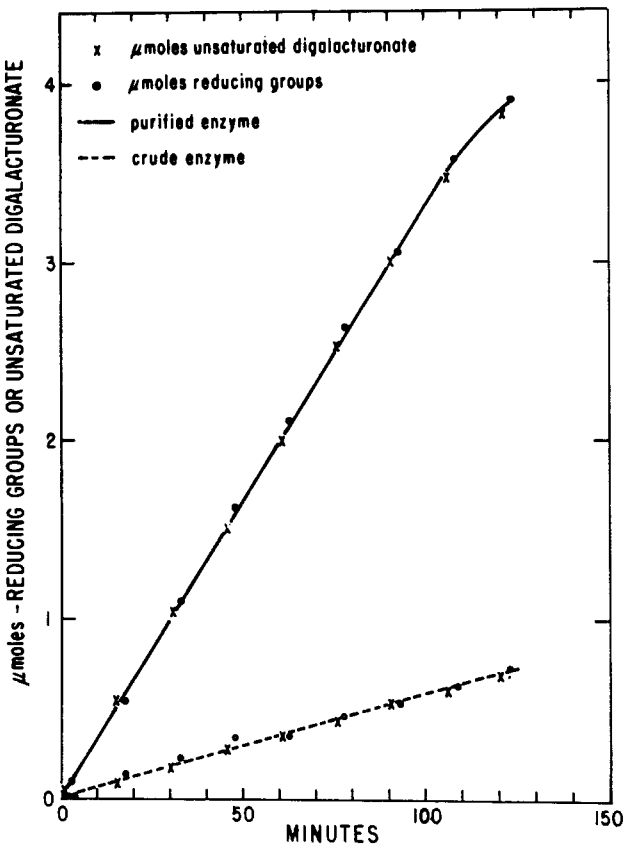

FIG. 3.—Production of unsaturated digalacturonic acid and reducing groups by the action of PATE on polygalacturonic acid. Reaction mixtures contained 0.46% substrate, 0.033 M Tris buffer at *p*H 8.0, and 0.001 M $CaCl_2$. The reaction mixture containing the crude enzyme preparation had 0.0054 unit/ml and the one containing the purified enzyme preparation had 0.031 unit/ml. The concentration of unsaturated digalacturonic acid was calculated from the absorbancy at 235 mμ based on a molar extinction coefficient of 4800. Temperature, 30°.

gardless of the length of reaction time or the strength of the enzyme solution employed, the only product detected had the same mobility as authentic unsaturated digalacturonic acid. This was true even in samples in which the absorbancy at 235 mμ had ceased to change.

Unsaturated digalacturonic acid was precipitated and purified by the procedure of Nagel and Vaughn (1961b) from 100 ml of a reaction mixture which had been incubated for 24 hours with 10 units of PATE. Figure 1 shows ultraviolet-absorption spectra of the free acid at three *p*H values. Evidently the *p*H influences not only the lateral displacement of the absorption peak but also the maximum absorption value.

The molar extinction coefficient (ϵ) at 235 mμ was determined with a sample of unsaturated digalacturonic acid which had been dried to constant weight in a vacuum oven at 70°. The value calculated from absorbancy measurements of various dilutions (1–3 $\times 10^{-4}$ M) of free acid in distilled water was 4800 M^{-1} cm^{-1}. The *p*H of these aqueous solutions was 3.7. The same extinction value was obtained when the solvent was 0.1 M acetate buffer at *p*H 3.7. Thus, the concentration of unsaturated digalacturonic acid in enzymic reaction mixtures was readily calculated from absorbancy measurements of samples appropriately diluted in acetate buffer. The absorbancy of samples so diluted did not change over a period of 10 hours since PATE was inactivated at *p*H values below 5.0 (cf. Fig. 4).

Substrate Specificity.—Purified PATE preparations still exhibited activity on pectin N.F. even though pectinesterase was no longer present. Next, reaction mixtures were prepared with highly esterified pectin as the substrate (Link pectin). A *p*H of 7.0 was selected to avoid alkaline de-esterification of the Link pectin. Both crude and purified PATE preparations were employed and the results were compared with polygalacturonic acid as a substrate. Figure 2 shows the results. Evidently Link pectin is not a substrate for purified PATE since, in spite of the appreciably higher concentration of PATE in this reaction mixture, the absorbancy at 235 mμ increased very slightly and only during the first few minutes of the reaction. This small initial rise in absorbancy corresponded to about 0.1% cleavage of the substrate and is undoubtedly due to the presence of some free carboxyl groups in the Link pectin. Nevertheless, the implication was quite clear that PATE was specific for the unesterified compound. Crude PATE attacked Link pectin readily and the marked difference in activity between crude and purified enzyme was attributed to the lack of pectinesterase in the latter.

In view of the results of Edstrom (1962), who found that pectin-*trans*-eliminase from *Aspergillus fonsecaeus* exhibited high activity with Link pectin at *p*H 6.8 and under conditions similar to those used here, it may be concluded that pectin-*trans*-eliminase was

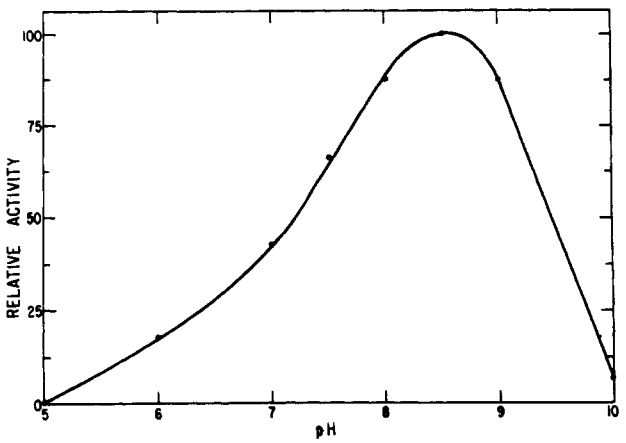

FIG. 4.—*p*H-activity curve of PATE acting on 0.46% polygalacturonic acid in 0.1 M Tris buffers containing 0.001 M $CaCl_2$. Temperature, 30°.

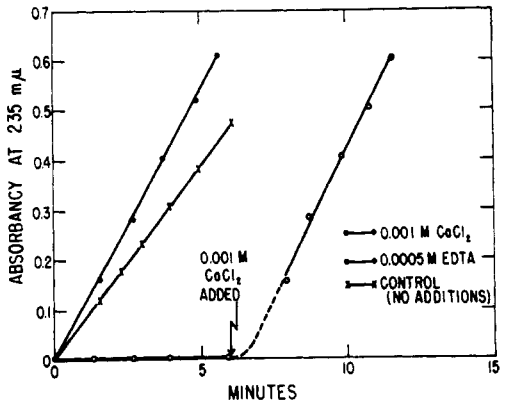

FIG. 5.—The effect of calcium chloride and sodium ethylenediaminetetraacetate (EDTA) on the activity of PATE. Reaction mixtures contained 0.46% polygalacturonic acid, 0.033 M Tris buffer at *p*H 8.0, and 0.023 unit of PATE per ml.

not present in purified PATE. It was not possible, however, to determine if there was pectin-*trans*-eliminase activity in the crude enzyme since pectinesterase would complicate its detection in the presence of PATE.

Polygalacturonase in PATE Preparations.—If PATE is the only enzyme produced by *C. multifermentans* which is capable of cleaving the glycosidic linkages of polygalacturonic acid, then for every molecule of unsaturated digalacturonic acid formed during a reaction one reducing group should be liberated. If more than one reducing group is produced per molecule of unsaturated dimer, it is likely that hydrolysis due to polygalacturonase is occurring in addition to the *trans*-elimination by PATE. The following experiment was designed to compare the number of moles of unsaturated digalacturonic acid formed (based on its molar extinction coefficient of 4800) with the number of moles of aldehyde groups produced during reactions of either crude or purified PATE on polygalacturonate.

Ninety ml of each reaction mixture was prepared. Five-ml aliquots were removed at intervals and the increase in aldehyde groups was determined by oxidation with hypoiodite. At the same time 2-ml aliquots were removed and diluted with 48 ml of 0.1 M acetate buffer, *p*H 3.7, for absorbancy measurements at 235 mμ. The results (Fig. 3), plotted in terms of μmoles

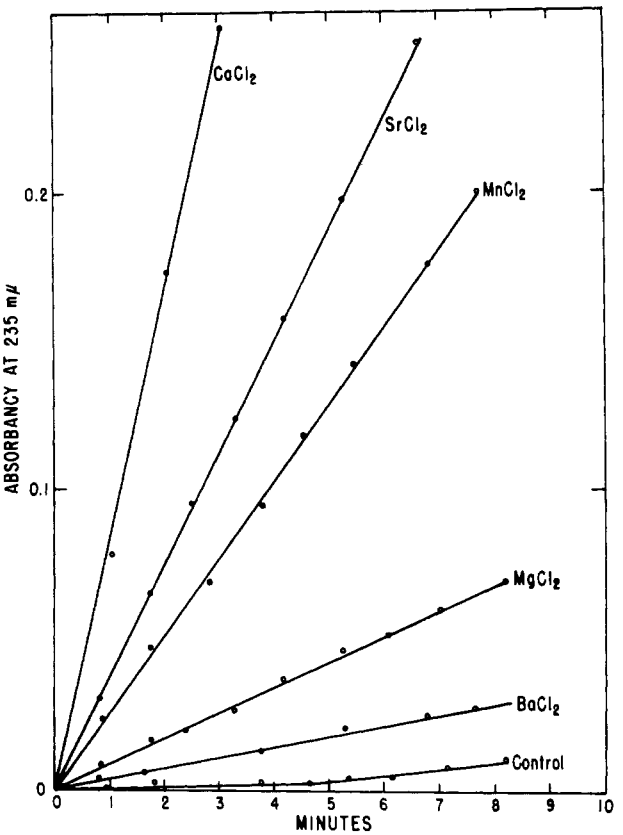

FIG. 6.—Stimulation of PATE activity by various divalent cations. The substrate was purified sodium polygalacturonate (0.46% on the basis of dry, ash-free polygalacturonic acid) in 0.033 M Tris buffer at *p*H 8.0. The cationic concentration was 0.001 M and each reaction mixture contained 0.023 unit of PATE per ml. Temperature, 30°.

of reducing groups or unsaturated dimer per ml of reaction mixture at various times, show conclusively that under the reaction conditions no hydrolysis due to polygalacturonase could be detected in reaction mixtures using either crude or purified PATE. At 123 minutes 31% of the substrate was converted to unsaturated digalacturonic acid by the purified PATE and 6% by the crude enzyme.

*p*H *Optimum.*—The activities of purified enzyme were determined at various *p*H values. The results in Figure 4 show that optimal activity occurs at *p*H 8.5. This relatively high value is in good agreement with the *p*H optimum found by Nagel and Vaughn (1961a) for the PATE from *Bacillus polymyxa* and is also consistant with the high optima of other bacterial pectic enzymes reported in the literature prior to the discovery of *trans*-eliminases (Nortje and Vaughn, 1953; Wood, 1955; Smith, 1958).

Effect of Divalent Cations on the Activity of PATE.—Exploratory experiments with crude dialyzed culture fluid of *C. multifermentans* showed that the rate of absorbancy change at 235 mμ was markedly stimulated by the addition of 0.001 M $CaCl_2$ to a reaction mixture containing 0.46% polygalacturonic acid. Addition of 0.0005 M sodium ethylenediaminetetraacetate (EDTA) to such a reaction mixture not containing $CaCl_2$ completely stopped the reaction, whereas the addition of 0.001 M $CaCl_2$ to the EDTA-polygalacturonate-broth mixture restored PATE activity (Fig. 5). Attempts to determine whether other divalent cations could replace calcium were unsuccessful with polygalacturonate-PATE mixtures without EDTA.

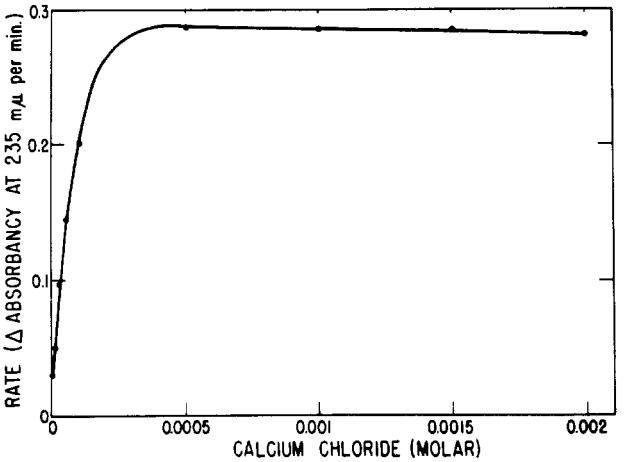

FIG. 7.—The effect of various concentrations of calcium chloride on the initial rate of PATE activity. Reaction mixtures contained purified sodium polygalacturonate (0.46% on the basis of dry, ash-free polygalacturonic acid) in 0.033 M Tris buffer at *p*H 8.0. Enzyme concentration was 0.058 unit of PATE per ml of reaction mixture. Temperature 30°.

This was attributed to a masking effect due to contaminating cations in the polygalacturonic acid. The polygalacturonic acid used as the substrate in these experiments had an ash content of 2.3% and thus was likely to contain considerable calcium or other cations. The reaction rate of PATE in controls not containing added calcium or other divalent cations could be markedly lowered by using sodium polygalacturonate which had been passed through a column of Dowex 50 (Na^+ form) as described earlier. With this substrate the following chloride salts were tested for their ability to stimulate PATE: calcium, strontium, barium, magnesium, manganese, and zinc. Calcium showed the highest stimulation (Fig. 6). Zinc was the only cation which failed to exhibit any stimulation over the control which contained no added divalent cations. This experiment demonstrated the relatively unspecific nature of the dependence of PATE on the presence of divalent cations. That monovalent cations were not stimulating was suggested by the low activity in the control, which contained a high concentration of sodium ions from the substrate.

TABLE III

FERMENTATION OF VARIOUS SUBSTRATES BY *Clostridium multifermentans* AND SUBSEQUENT PRODUCTION OF PATE

Substrate[a] (0.2%)	Fermentation[b]	PATE units/ml (after 3 days)
Galacturonic acid	−	nil
Digalacturonic acid	+	0.016
Unsaturated digalacturonic acid	+	0.016
Trigalacturonic acid	+	0.034
Tetragalacturonic acid	+	0.092
Polygalacturonic acid	+	0.096
Pectin N.F.	+	0.032
Polymethylpolygalacturonate methyl glycoside (Link pectin)	+	0.046
Glucose	+	nil
Galactose	+	nil
Glucuronic acid	−	nil
Glucose and polygalacturonic acid	+	nil

[a] The basal medium contained 0.5% yeast extract, 0.1% K_2HPO_4, and 0.05% sodium thioglycolate. The *p*H was 7.3 prior to inoculation. [b] Ability to ferment the various substrates was determined on the basis of gas production upon incubation for 3 days at 30°. Gas was collected in Durham tubes inverted in the culture tubes which contained 10 ml of medium. −, no gas produced; +, at least half the Durham tube filled with gas.

The effect of various concentrations of calcium on the initial rate of PATE activity is shown in Figure 7. A maximum rate was evidently established at about 0.0005 M calcium. The Michaelis constant (K_m) was 0.00006 M calcium chloride. In this experiment the time during which absorbancy changes were measured for the various mixtures ranged from 3 to 8 minutes depending on the rate. The total change in absorbancy was only 1–2% of the total change possible with 0.46% polygalacturonate as the substrate. Therefore the data reported represent only the initial reaction rates, which were linear in every instance. This is particularly significant in view of the data reported below, in which a number of reaction mixtures containing various concentrations of calcium chloride were followed until absorbancy at 235 mμ ceased to increase. Since very high absorbancy values were expected, samples of the reaction mixtures were diluted 50-fold with 0.1 M acetate buffer, *p*H 3.7, which served also to stop the action of the enzyme. The absorbancy of these diluted samples was determined at 235 mμ and the percentage of degradation of the polygalacturonic acid was calculated on the basis of the molar extinction coefficient of 4800 for unsaturated digalacturonic acid. Complete conversion of the substrate to unsaturated digalacturonic acid would mean 100% degradation.

The results are shown in Figure 8. The initial reaction rates for the various levels of calcium chloride agree well with the data reported in Figure 7. It can be seen, however, that concentrations of calcium higher than 0.0005 M profoundly influenced the reaction during the later stages. With 0.002 M calcium the reaction did not proceed to completion even after 20 hours. Although the initial rate was lower, the reaction with 0.0001 M calcium progressed to completion sooner than the others and was essentially complete after 10 hours.

Dependence of PATE on Substrate Concentration.—The initial rate of PATE activity on various concentrations of purified sodium polygalacturonate was determined in the presence of 0.0005 M $CaCl_2$ in 0.033 M Tris buffer at *p*H 8.0 with 0.056 unit of PATE per ml. Under these conditions the reaction rate of PATE was progressively higher with increasing concentration as measured in the range of 0.03–0.4% polygalacturonic acid. No significant difference in rate was detected in the range of 0.4–0.9% polygalacturonic acid, showing that the use of 0.46% substrate in standard assays for the activity of PATE was justified since slight variation in substrate concentration would not affect initial rates of reaction. The values for V_{max} and K_m were calculated as 0.37 OD unit/min and 0.114% polygalacturonic acid (dry, ash-free basis).

Induction of PATE.—Clostridium multifermentans was examined for its ability to produce PATE during the fermentation of a number of different substrates. The results shown in Table III confirm the original observation of Ng and Vaughn (1963), that galacturonic acid cannot be fermented. All of the oligogalacturonides tested were capable of being fermented by *C. multifermentans*. The fermentation of unsaturated digalacturonic acid is significant in that this compound, produced in cultures by the action of PATE,

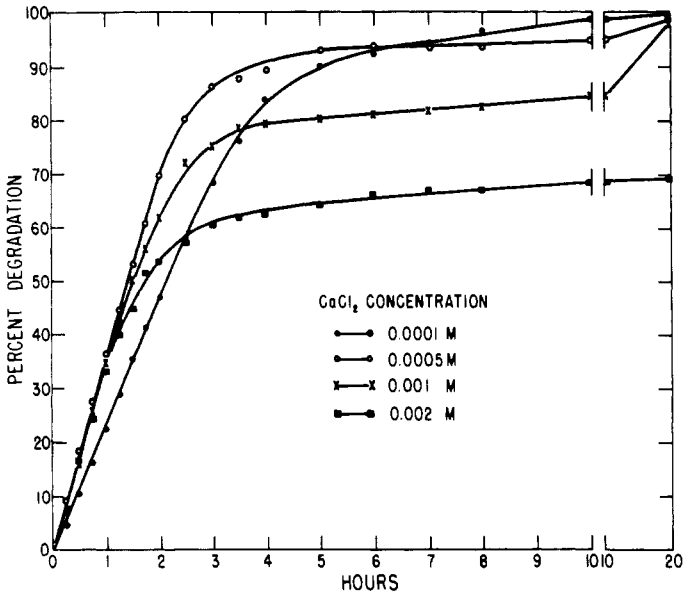

FIG. 8.—The effect of various amounts of calcium chloride on the per cent degradation of polygalacturonic acid. Reaction conditions were the same as reported in Fig. 7 except that 0.075 unit of PATE per ml was employed.

could well be the actual substrate being fermented when *C. multifermentans* is grown on polygalacturonic acid.

PATE was found in cell-free dialyzed broth from all the cultures grown on substrates of pectic origin (other than galacturonic acid). It is significant that with the oligogalacturonides the amount of PATE produced varied with the chain length of the substrate. The longer the chain the more PATE was present in the culture after 3 days.

That PATE is an inducible enzyme was suggested in the preceding experiment since none was detected in cultures grown on glucose or galactose. That glucose might repress PATE production was indicated since no PATE was detected in the culture broth when *C. multifermentans* was grown in the presence of a mixture of glucose and polygalacturonic acid.

Discussion

The results presented above show that *C. multifermentans* produces an enzyme which degraded polygalacturonic acid by a *trans*-eliminative mechanism forming unsaturated digalacturonic acid. Although pectin N.F. is also attacked by this enzyme, it appears that the specificity is for the nonesterified portion of pectin molecules since highly esterified pectin (Link pectin) was not attacked. Therefore the name "polygalacturonic acid-*trans*-eliminase" is justified.

The techniques employed in this work for the identification and isolation of unsaturated digalacturonic acid were the same as reported by Nagel and Vaughn (1961b) and its chromatographic behavior indicates that the compound isolated here is the same as produced by PATE from *Bacillus polymyxa*. However, Hasegawa and Nagel (1962) stated that the molar extinction coefficient for unsaturated digalacturonic acid was 3300. Later, they repeated the determination and obtained a value of 4500 (C. W. Nagel, personal communication).

In contrast to the PATE produced by *Bacillus polymyxa*, the only product detected in the reaction of clostridial PATE with polygalacturonic acid was unsaturated digalacturonic acid. This is a strong implication that polygalacturonic acid is degraded terminally rather than randomly. With random cleavage, higher oligouronides would be detected early in reactions. Further evidence for the terminal nature of the attack and identification of small amounts of additional products is presented in the accompanying publication.

The method used for determining the absence of polygalacturonase in PATE preparations may not necessarily be applicable to systems containing other *trans*-eliminases. This method is dependent on the formation of unsaturated digalacturonic acid throughout an entire reaction. The randomly acting *trans*-eliminases, such as the one formed by *Bacillus polymyxa*, also produce from polygalacturonic acid unsaturated products of a higher degree of polymerization than dimer. If the extinction values for these unsaturated compounds is not the same as that of unsaturated dimer, the change in absorbancy in a reaction mixture might not be related in a uniform manner to the production of reducing groups.

The function of calcium and other divalent cations as an absolute requirement for activity has not been determined. The implication that these cations may be necessary as a link between enzyme and substrate can be postulated but not verified without additional knowledge on the inhibitory effects of excess calcium in reaction mixtures.

Inhibition during the later stages of degradation of polygalacturonic acid in calcium concentrations in excess of 0.0005 M (cf. Fig. 8) may be due to the linkage of two substrate molecules by a calcium bridge across two carboxyl groups. The enzyme, acting terminally, may encounter steric hindrance when approaching these bridges leaving portions of the substrate molecules inaccessible to rapid attack by the enzyme. Thus, in high concentration of calcium, the time necessary to completely degrade the substrate is prolonged. Even calcium concentrations at 0.0005 M may produce some inhibition in the later stages of reactions, since it was found that 0.0001 M calcium allowed complete degradation before that with 0.0005 M calcium even though the initial rate in the former case was considerably lower.

The availability of *trans*-elimination, as compared to hydrolysis, as a mechanism for bacterial pectinolysis

can be evaluated only on the basis of limited data. The known bacterial *trans*-eliminases have all been shown to attack polygalacturonic acid. *Clostridium multifermentans* evidently does not produce a hydrolytic enzyme. Other bacterial species may produce polygalacturonase but a positive identification of this hydrolytic enzyme in bacteria has not yet been made. Prior to the discovery of *trans*-eliminases it was generally observed that crude bacterial pectic enzymes attack polygalacturonic acid at *p*H values above neutrality and that they are stimulated by the addition of calcium. Since these observations were on the basis of either reducing-group production or decreases in viscosity, conceivably either polygalacturonase or *trans*-eliminase could be involved. Proven fungal polygalacturonases, however, have low *p*H optima and no calcium dependence. Thus bacterial polygalacturonases, if they exist, either differ considerably from the fungal type or are not widespread. All of the known bacterial *trans*-eliminases agree with the earlier observations by having both high *p*H optima and calcium dependence. Therefore it is suspected that polygalacturonic acid-*trans*-eliminase, in combination with pectinesterase, constitutes the major enzymic system for pectinolysis by bacteria.

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The Pattern of Action of an Exopolygalacturonic Acid-*trans*-eliminase from *Clostridium multifermentans**

JAMES D. MACMILLAN, HERMAN J. PHAFF, AND R. H. VAUGHN

From the Department of Food Science and Technology, University of California, Davis

Received November 18, 1963

A bacterial polygalacturonic acid-*trans*-eliminase (PATE) of *Clostridium multifermentans* has been shown to remove units of *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid (or α,β-unsaturated digalacturonic acid) from the reducing ends of polygalacturonate chains. The reaction goes to completion. This terminally splitting enzyme has been named "exopolygalacturonic acid-*trans*-eliminase" (exo-PATE) in contrast to the already known randomly splitting endo-PATE produced by other bacteria. Tetra- and trigalacturonic acids are degraded at the same initial rate as polygalacturonate. Each produces 1 mole of unsaturated digalacturonic acid. The balance of the reactions consists of 1 mole of normal digalacturonic acid in case of the tetramer and 1 mole of D-galacturonic acid in case of the trimer. Digalacturonic acid is not attacked by the enzyme. Viscosity decrease of polygalacturonate in relation to per cent cleavage is typical of an end-group attack. Fully esterified pectin is not attacked by exo-PATE unless a source of pectinesterase is also added. By determining the extent of cleavage of partially esterified pectin N.F. some information has been obtained on the distribution of the free carboxyl groups in this substrate.

Within the last decade it has become increasingly apparent that the glycosidic bonds of pectic substances are subject to degradation by a variety of enzymes which differ markedly in both substrate specificity and mode of attack. In 1957, prior to the discovery of enzymes which split pectic substances by a *trans*-elimination mechanism, Demain and Phaff (1957) proposed a classification of the pectic glycosidases based on whether the substrate molecule was split in a terminal or a random manner. Random degradation, the most common type of breakdown encountered, is typified by the endopolygalacturonase produced by *Saccharomyces fragilis*. Demain and Phaff (1954b) found that this enzyme hydrolyzed polygalacturonic acid initially to higher oligouronides, which ultimately became degraded to a mixture of D-galacturonic and digalacturonic acids. Saito (1955) showed that *Asper-*

* These results were presented at the Symposium on Recent Developments in Pectin Chemistry and Pectin Degradation at the 114th American Chemical Society meeting in Los Angeles, California, March 31 to April 5, 1963.