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Polygalacturonic Acid *trans*-Eliminase of *Xanthomonas campestris*

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Polygalacturonic acid *trans*-eliminase from the culture fluid of *Xanthomonas campestris* was purified 66-fold by acetone precipitation, citrate extraction and chromatography on diethylaminoethyl- and carboxymethyl-cellulose. The optimum pH is 9·5 in glycine–sodium hydroxide buffer. Up to 1mM-calcium chloride brings about a remarkable stimulation of the enzyme activity and, at this concentration, no other cations promote or inhibit enzyme action except Ba^{2+} ions, which cause complete inhibition. The enzyme degrades polygalacturonic acid in a random manner; it does not act upon polygalacturonate methyl glycoside, although it can cleave partially (68%) esterified pectin. The end products from polygalacturonic acid at 46% breakdown are unsaturated di- and tri-galacturonic acids, in addition to saturated mono-, di- and tri-galacturonic acids. Pentagalacturonic acid is split preferentially into saturated dimer plus unsaturated trimer, or into saturated trimer plus unsaturated dimer; at a lower rate, it is also split into monomer and unsaturated tetramer. Unsaturated pentamer is split into unsaturated dimer plus unsaturated trimer. Tetragalacturonic acid is split somewhat preferentially at the central bond to form dimer and unsaturated dimer, but it is also split into monomer and unsaturated trimer. Unsaturated tetramer is split only at the central bond to yield only unsaturated dimer. Trigalacturonic acid is split into monomer and unsaturated dimer. Unsaturated trimer is cleaved into saturated dimer and probably 4-deoxy-L-5-*threo*-hexoseulose uronic acid, which has not yet been directly identified. Neither saturated nor unsaturated digalacturonic acid is attacked. The unsaturated digalacturonic acid was isolated and proved to be *O*-(4-deoxy-β-L-5-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid.

Relatively little has been reported on the pectic enzymes of the phytopathogenic bacteria of the genus *Xanthomonas*. The deficiency probably stems from the primarily necrotic nature of the plant diseases caused by xanthomonads; certainly the predominant symptoms are not suggestive of much pectolytic activity in the sense of soft rots. Nevertheless, there is evidence that pectic substances can be degraded *in vitro* by some xanthomonads. The relevant earlier literature is cited by Starr & Nasuno (1967), who report that 16 out of 27 xanthomonad cultures (representing nine out of 19 *Xanthomonas* spp.) form pectinesterase and polygalacturonic acid *trans*-eliminase, but that none forms the hydrolytic polygalacturonase.

The detailed enzymology of the breakdown of pectic substances which has been reported for *Xanthomonas* (Smith, 1958*a*,*b*) has not progressed to the degree of clarification now available for the pectinolytic enzymes from other bacterial genera [*Erwinia carotovora* (Starr & Moran, 1962; Nasuno & Starr, 1966; F. Moran, S. Nasuno & M. P. Starr, unpublished work), *Bacillus polymyxa* (Nagel & Vaughn, 1961*a*,*b*, 1962), *Pseudomonas fluorescens* (Fuchs, 1965) and *Clostridium multifermentans* (Macmillan & Vaughn, 1964)] in which eliminative splits have been elucidated, sometimes in addition to the classical hydrolytic action.

The present paper deals with the purification and characterization of the polygalacturonic acid *trans*-eliminase found extracellularly in culture fluids of *Xanthomonas campestris*. In addition, it presents the action patterns of the purified enzyme on oligogalacturonides.

METHODS

Preparation of adsorbents. DEAE-cellulose was obtained from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). The cellulose was converted into the chloride form with 1·0M-tris–HCl buffer, pH 7·2, after pretreatment with NaOH and decantation of fine particles. Then the column of the adsorbent was washed thoroughly with 0·05M-tris–HCl buffer, pH 7·2. CM-cellulose of Bio-Rad Laboratories was first equilibrated with 0·1M-sodium citrate buffer, pH 5·5. After decantation of fine particles, the column was washed with 10 vol. of 0·05M-sodium citrate buffer, pH 5·5.

Preparation of substrates. Pectin N.F. (68% esterified) and polygalacturonic acid (no. 491) were obtained from

Sunkist Growers Inc. (Corona, Calif., U.S.A.). They were used without additional treatment for most experiments. A highly purified sodium polygalacturonate, prepared according to Macmillan & Vaughn (1964), was used in experiments dealing with the effect of various cations on the enzyme activity. Polymethyl polygalacturonate methyl glycoside (fully methoxylated 'Link pectin') was prepared by the method of Morell, Baur & Link (1934). *O*-(4-Deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid (unsaturated digalacturonic acid) was kindly supplied by Dr C. W. Nagel, and was also prepared by a modification of the method of Nagel & Vaughn (1961*b*). The analogous unsaturated tri-, tetra- and penta-galacturonic acids were prepared by means of paper chromatography with polygalacturonic acid partially degraded by the action of polygalacturonic acid *trans*-eliminase of *X. campestris*. Pure D-galacturonic acid was obtained from the commercial product (Eastman Organic Chemicals) by recrystallization from acetone. Di- and tri-galacturonic acids were prepared by the procedure of Luh & Phaff (1954*b*). Tetragalacturonic acid was isolated by the method of Demain & Phaff (1954). Pentagalacturonic acid was isolated by paper chromatography from the degradation products of polygalacturonic acid acted upon by yeast polygalacturonase; the purified yeast enzyme was prepared by the method of Luh & Phaff (1954*a*) by using *Saccharomyces fragilis* (which was supplied through the courtesy of Dr H. J. Phaff).

Culture methods. The particular strain of *Xanthomonas campestris* (ICPB-XC135) used in this study was one of the more actively pectolytic of the xanthomonads surveyed by Starr & Nasuno (1967). It forms pectinesterase and polygalacturonic acid *trans*-eliminase, but no hydrolytic polygalacturonase. The basal culture medium contained: pectin N.F., 5g.; yeast extract (Difco), 4g.; $(NH_4)_2SO_4$, 5g.; KH_2PO_4, 1g.; $MgSO_4,7H_2O$, 0·5g.; $CaCl_2,2H_2O$, 0·1g.; $MnSO_4,H_2O$, 2μg.; $ZnCl_2$, 10μg.; H_3BO_3, 1μg.; $FeCl_3,6H_2O$, 20μg.; 1000ml. of water, adjusted to pH6·8 with NaOH. A solution containing the pectin was autoclaved apart from the other components and the two portions were mixed before use; final pH was 6·8.

Assay methods. One unit of polygalacturonic acid *trans*-eliminase is the amount of enzyme that releases 1μmole of aldehyde groups from polygalacturonic acid/min. at pH9·5 at 30°. The enzyme activity was always assayed by the measurement of changes in extinction at 235mμ in reaction mixtures. The reaction mixture consisted of enzyme, 0·25% sodium polygalacturonate, 1mM-$CaCl_2$, and 0·05M-glycine–NaOH buffer, pH9·5, in a total volume of 2ml. The release of 0·1μmole of aldehyde groups in a 2ml. reaction mixture is equivalent to an increase of 0·26 in the extinction at 235mμ under the above conditions. The cleavage of glycosidic bonds of the substrate by polygalacturonic acid *trans*-eliminase was also measured by the hypoiodite method (Jansen & Macdonnell, 1945).

In the experiments to examine the effect of temperature on enzyme activity, the reaction mixture contained enzyme, 0·25% substrate, 1mM-$CaCl_2$ and 0·05M buffer, final pH8·5 (tris–HCl) or 9·5 (glycine–NaOH), in a total volume of 6ml. Triplicate 1ml. samples were removed from the reaction flasks at 5min. intervals, transferred to 2ml. of 3mM-EDTA solution to halt further reaction, and the extinction was measured at 235mμ.

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) by using crystalline bovine serum albumin as a standard. In fractions eluted from columns, protein was estimated by comparison at 280 and 260mμ with the method of Warburg & Christian (1942).

Paper chromatography. The products formed by the action of polygalacturonic acid *trans*-eliminase were separated at room temperature by descending development on Whatman no. 4 paper with pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by vol.) for 16–18hr. (Nagel & Vaughn, 1961*b*). The reaction mixtures contained enzyme, 0·25% substrate and 1mM-$CaCl_2$ in 0·05M-glycine–NaOH buffer, pH9·5; they were incubated at 30° unless otherwise stated. Products of yeast polygalacturonase action (see Preparation of substrates, above) were separated by butan-1-ol–acetic acid–water (4:2:3, by vol.) for 18–20hr. Whatman no. 3MM paper and a development time of 20–48hr. were used for preparative chromatography. Before application to the paper, the solution was treated with Dowex 50 (H^+ form). A solution of 0·04% bromophenol blue in 95% ethanol adjusted to pH7·0 with NaOH and a $AgNO_3$ reagent (Block, Durrum & Zweig, 1955) were used for detection of oligogalacturonides. The αβ-unsaturated compounds were located by spraying with a solution containing 0·01% quinine sulphate and 0·04N-sulphuric acid in 95% ethanol (Edstrom & Phaff, 1964).

RESULTS

Course of enzyme excretion by Xanthomonas campestris. When *X. campestris* was grown on a rotary shaker (3cm. amplitude and 100 rotations/min.) at 28° for 48hr. in 2l. flasks containing 400ml. of the basal pectin medium, there was a progressive increase in polygalacturonic acid *trans*-eliminase activity in the culture fluid after the cells entered the logarithmic phase of growth. Excretion of enzyme almost paralleled growth, and reached a maximum after 36–48hr. There was a slight loss in enzymic activity beyond that point. When polygalacturonic acid replaced pectin as the substrate, cell growth and excretion of the enzyme reached the maximum at 24hr. of incubation, and there was a loss in enzyme activity on incubation of the culture beyond 24hr. No polygalacturonic acid *trans*-eliminase was produced with galacturonic acid or glucose as the substrate.

Pectinesterase activity was found in the culture fluid only with pectin as the substrate, but not with polygalacturonic acid, galacturonic acid or glucose. Not even a trace of the hydrolytic polygalacturonase was detected in the culture fluids with or without pectic substrates (Starr & Nasuno, 1967). Furthermore, no excretion of polygalacturonase by *X. campestris* was detected upon varying the culturing conditions such as incubation temperature, degree of aeration [shaken cultures (3cm. amplitude at 100 rotations/min.) or static cultures (1·5 and 3cm. deep)], initial pH, and nitrogen sources or other constituents of the culture medium.

Purification of enzyme. For the purification of polygalacturonic acid *trans*-eliminase from *X. campestris*, 0·5% polygalacturonic acid, rather than pectin, was used as the substrate in the basal medium, because pectinesterase was not produced with this substrate and thus did not have to be removed. The bacteria were grown for 24hr. under the conditions described in the preceding section.

The enzyme was first precipitated at 4° from the centrifuged culture fluid by the dropwise addition of an equal volume of cold acetone chilled to −20°. The precipitate was centrifuged and extracted with 0·2M-sodium citrate buffer, pH5·5. The buffer extract was then dialysed against 5mM-tris–hydrochloric acid buffer. The dialysed enzyme solution was then placed on a DEAE-cellulose column (15cm. high and 1·2cm. diam.) equilibrated at pH7·2 with 0·05M-tris–hydrochloric acid buffer. Under these conditions, 75% of the non-enzymic protein was retained on the column and the polygalacturonic acid *trans*-eliminase passed through, which resulted in a fourfold increase in specific activity.

Next, the solution which passed through the DEAE-cellulose column was adjusted to pH5·5 with citric acid and applied to a column (15cm. high and 1cm. wide) of CM-cellulose equilibrated at pH5·5 with 0·05M-sodium citrate buffer. Fractions of volume 3ml. were collected. The stepwise elution was carried out with 5mM-sodium citrate buffer, pH5·5, containing, in turn, 0·05M-, 0·5M- and 1·0M-sodium chloride. A typical elution is represented by the curves of Fig. 1. In fractions 45–50, approximately half of the enzyme applied to the column was recovered in citrate buffer containing 0·5M-sodium chloride. The overall purification procedure is summarized in Table 1. The final purification of the enzyme resulted in about 66-fold increase in specific activity of the enzyme.

Optimum pH *and stability.* The effect of pH on the activity of polygalacturonic acid *trans*-eliminase is shown in Fig. 2. It is apparent that the optimum

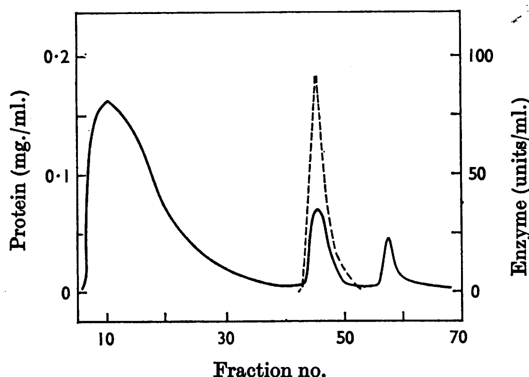

Fig. 1. CM-cellulose chromatography of polygalacturonic acid *trans*-eliminase from *Xanthomonas campestris* XC135. The eluting solution consisted of 5mM-sodium citrate buffer with stepwise increasing concentrations of NaCl: 0·05M, 0·5M and 1·0M. Fractions of volume 3ml. were collected. ——, Protein; ----, enzyme.

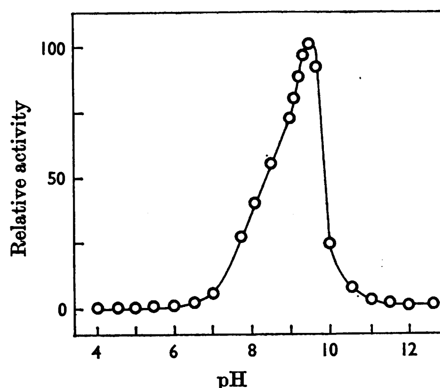

Fig. 2. Effect of pH on the activity of polygalacturonic acid *trans*-eliminase from *Xanthomonas campestris* XC135. Buffer systems (0·05M) used were: sodium acetate (pH4·0–5·5), sodium succinate (pH6·0–7·0), tris–HCl (pH7·5–9·0) and glycine–NaOH (pH9·0–12·5).

Table 1. *Summary of the purification of polygalacturonic acid* trans-*eliminase from* Xanthomonas campestris *XC135*

Purification step	Volume (ml.)	Total enzyme units	Total protein*	Specific activity (units/mg. of protein)	Recovery (%)
Crude culture fluid†	350	1750	110	16	100
Dialysed buffer extract	40	1670	35	48	95·5
After DEAE-cellulose	44	1380	7	197	79
After CM-cellulose	12	693	0·66	1050	40

* Determined by the method of Lowry, *et al.* (1951).

† Dialysed against 0·05M-tris–HCl buffer, pH7·2, for determination of enzyme activity and protein.

pH for activity lies at about 9·5 in glycine–sodium hydroxide buffer. The enzyme solution was most stable between pH 6 and 9·5. No apparent loss of activity of the purified enzyme was detectable after a few weeks in the frozen state at −20°. As is common with enzymes, the thermal behaviour of polygalacturonic acid *trans*-eliminase depends a great deal on the pH of the system. The data of Fig. 3 show that the maximum activity of the enzyme was found between 25° and 30° at pH 9·5 (glycine–sodium hydroxide buffer) and at 45° at pH 8·5 (tris–hydrochloric acid buffer). These are somewhat lower than the values reported for the similar enzymes of *Bacillus polymyxa* (Nagel & Vaughn, 1961*a*) and of *Erwinia carotovora* (Starr & Moran, 1962).

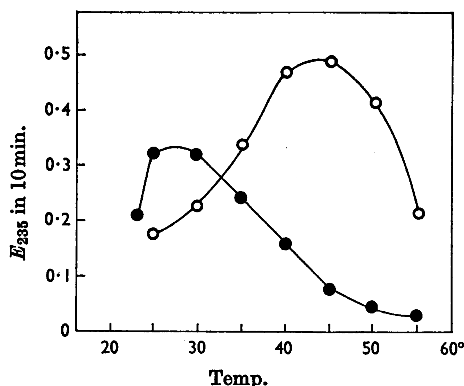

Fig. 3. Relationship, at different pH values, between temperature and the activity of polygalacturonic acid *trans*-eliminase from *Xanthomonas campestris* XC135. ○, Tris–HCl, pH 8·5; ●, glycine–NaOH, pH 9·5.

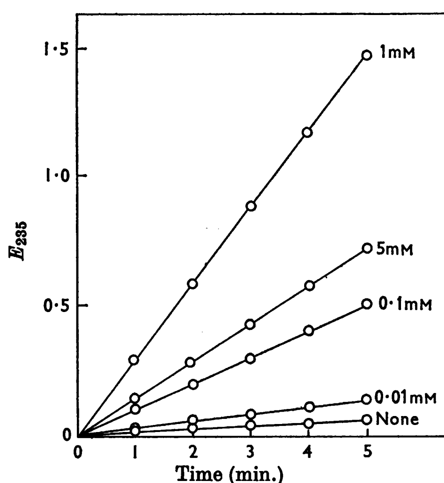

Fig. 4. Effect of $CaCl_2$ on reaction rate of polygalacturonic acid *trans*-eliminase from *Xanthomonas campestris* XC135.

Effects of salts. The addition of calcium chloride, up to 1 mM, caused a marked increase in activity over that of the enzyme alone (Fig. 4). A higher concentration (5 mM) of calcium chloride decreased the reaction rate because of precipitation of the substrate. The addition of 0·03 μM-EDTA, instead of calcium chloride, to the reaction mixture resulted in complete loss of enzyme activity. This activity was completely restored by subsequent addition of 1 mM-calcium chloride.

As is shown in Table 2, stimulation by calcium chloride and inhibition by barium chloride of polygalacturonic acid *trans*-eliminase activity were specific for these two cations; Na^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Sr^{2+} ions were ineffective at 1 mM concentration in affecting activity of the enzyme.

Substrate specificity. Fully esterified polymethyl polygalacturonate methyl glycoside ('Link pectin') was virtually not split, although polygalacturonic acid was rapidly degraded by the concentrated enzyme solution (Fig. 5). Partially esterified (68%) citrus pectin N.F. was attacked at relatively low velocity by the same enzyme preparation, probably by attack on the non-esterified units of the polymer. These results indicate that the polygalacturonic acid *trans*-eliminase of *X. campestris* is specific for polygalacturonic acid rather than for pectin.

Reaction products from polygalacturonic acid treated with trans-*eliminase.* The random mechanism of attack on polygalacturonic acid by *X. campestris* polygalacturonic acid *trans*-eliminase was first shown indirectly by the 50% loss of viscosity at approximately 3% cleavage of the glycosidic bonds. This was further supported by

Table 2. *Effect of salts* (1 mM) *on activity of polygalacturonic acid* trans-*eliminase from* Xanthomonas campestris *XC*135

The reaction mixture contained enzyme, 0·25% polygalacturonic acid, 1 mM-salt, 0·05 M-glycine–NaOH buffer, pH 9·5. The enzyme activity in the absence of additional salt was taken as the control (1·00 = activity).

Salt	Activity relative to control
None	1·00
NaCl	1·05
KCl	1·52
$CaCl_2$	22·00
$MgCl_2$	1·23
$MnCl_2$	1·13
$ZnCl_2$	1·10
$BaCl_2$	0
$Sr(NO_3)_2$	1·03

paper-chromatographic analysis of the reaction products. The results (Table 3) show that higher oligogalacturonides predominated in the initial stages of the reaction. There was a progressive increase in the concentration of unsaturated di- and tri-galacturonic acids, with a concomitant decrease in the higher oligouronides as the reaction proceeded. At 46% cleavage of the glycosidic bonds of the substrate, unsaturated di- and tri-galacturonic acids accumulated as predominant end products; saturated mono-, di- and tri-galacturonic acids remained in rather small quantities.

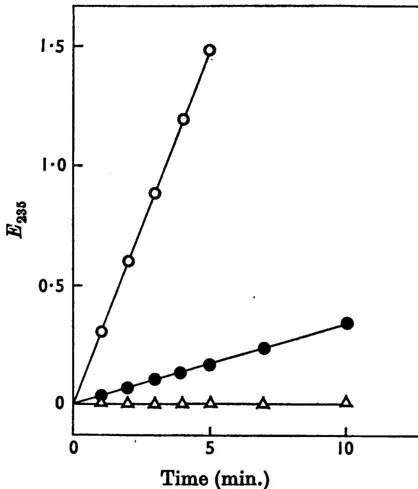

Fig. 5. Time-course of action of polygalacturonic acid *trans*-eliminase from *Xanthomonas campestris* XC135 on polygalacturonic acid (○), pectin N.F. (●) and polymethyl polygalacturonate methyl glycoside (fully methoxylated 'Link pectin', △).

Action of trans-*eliminase on saturated oligogalacturonides.* Table 4 shows the paper-chromatographic analysis of the breakdown products of saturated penta-, tetra- and tri-galacturonic acids by purified polygalacturonic acid *trans*-eliminase from *X. campestris*. Saturated mono-, di- and tri-galacturonic acids, in addition to unsaturated di- and tri-galacturonic acids, were found as the reaction products from pentagalacturonic acid. The appearance of both saturated and unsaturated dimer indicated that enzymic attack occurred at the second and third glycosidic bonds of the pentamer. A simultaneous split of the fourth bond also was suggested by the appearance of trace amounts of monomer early in the reaction. Tetragalacturonic acid was split into saturated mono- and di-galacturonic acids and unsaturated dimer and trimer. Although tetramer was attacked at the second and third bonds, it seemed to be cleaved preferentially at the central bond. Trigalacturonic acid gave clear spots of monomer and unsaturated dimer (but not saturated dimer), indicating that only the second glycosidic bond of the trimer was split by the enzyme. Digalacturonic acid was not attacked, even after 8 days of incubation. These results show clearly that the glycosidic bond next to the reducing end is protected in some way from the action of *Xanthomonas* polygalacturonic acid *trans*-eliminase.

Cleavage of unsaturated oligogalacturonides. Table 5 summarizes the results of cleavage of unsaturated penta-, tetra- and tri-galacturonic acids by *X. campestris* polygalacturonic acid *trans*-eliminase. Unsaturated pentamer was rapidly split into unsaturated dimer and trimer; no unsaturated

Table 3. *Products from polygalacturonic acid acted upon by polygalacturonic acid* trans-*eliminase from* Xanthomonas campestris *XC*135

The reaction mixture contained 0·25% polygalacturonic acid, 1mM-$CaCl_2$ in 0·05M-glycine–NaOH buffer, pH 9·5, and 0·35 unit of enzyme/ml. Spots were detected by the quinine sulphate and $AgNO_3$ reagents. (+), Weak spots; ++++, intense spots; –, no spot detectable.

Time	Galacturonides							Degradation
	Penta-*	Tetra-*	Tri-	Tri-*	Di-	Di-*	Mono-	(%)
0 min.	–	–	–	–	–	–	–	0
5	++	++	(+)	(+)	(+)	–	–	9·1
10	++	++	+	+	(+)	+	–	19·4
30	++	++	+	+	(+)	++	–	25·7
1 hr.	++	++	+	++	+	+++	–	31·2
2	(+)	+	+	++	+	+++	–	39·1
4	–	+	+	++	+	+++	–	40·0
7	–	(+)	+	+++	++	++++	(+)	42·2
12	–	–	+	+++	++	++++	+	44·3
24	–	–	+	+++	++	++++	+	46·0

* Unsaturated compounds.

Table 4. *Degradation products from saturated oligogalacturonides acted upon by polygalacturonic acid* trans-*eliminase of* Xanthomonas campestris *XC*135

The reaction mixture contained 0·5% substrate, 1mM-$CaCl_2$ and 7·5 units of enzyme/ml. in 0·05M-glycine–NaOH buffer, pH 9·5. Spots were detected by the quinine sulphate and $AgNO_3$ reagents. Digalacturonic acid was not attacked. (+), Weak spot; +, a moderate to intense spot; −, no spot detectable.

	Galacturonides formed				
	Saturated			Unsaturated	
Substrate	Tri-	Di-	Mono-	Tri-	Di-
Pentagalacturonic acid (in 1hr.)	+	+	(+)	+	+
Tetragalacturonic acid (in 1hr.)	−	+	(+)	(+)	+
Trigalacturonic acid (in 24hr.)	−	−	+	−	+

Table 5. *Degradation products from unsaturated oligogalacturonides acted upon by polygalacturonic acid* trans-*eliminase of* Xanthomonas campestris *XC*135

See legend of Table 4 for the experimental conditions. Unsaturated digalacturonic acid was not attacked. The unsaturated monomer is assumed to be 4-deoxy-L-*threo*-5-hexoseulose uronic acid.

	Unsaturated galacturonides formed		
Substrate	Tri-	Di-	Mono-
Unsaturated pentagalacturonic acid (in 1hr.)	+	+	−
Unsaturated tetragalacturonic acid (in 1hr.)	−	+	−
Unsaturated trigalacturonic acid (in 24hr.)	−	+	+

tetramer was detected. Unsaturated dimer was the sole reaction product of unsaturated tetramer breakdown, and no unsaturated trimer was detected. These results indicate that both unsaturated pentamer and tetramer are split at central bonds rather than at terminal ones. Unsaturated trimer was also subjected to enzymic degradation, and gave spots of unsaturated dimer and of an unknown compound on the paper chromatogram. The material in the unknown spot was not identified, but was assumed from its position to be 4-deoxy-L-*threo*-5-hexoseulose uronic acid. Thus, in this case, it cannot be decided whether bond 1 or 2 of the trimer is cleaved. Unsaturated dimer was not degraded after 8 days of incubation by polygalacturonic acid *trans*-eliminase from *X. campestris*.

Isolation and identification of unsaturated digalacturonic acid. The isolation and identification of unsaturated digalacturonic acid was carried out to determine whether the polygalacturonic acid *trans*-eliminase of *X. campestris* is a true lyase. A reaction mixture was prepared containing 1% polygalacturonic acid, 0·05M-glycine–sodium hydroxide buffer, pH 9·5, 1mM-calcium chloride and 4·2 units of the crude enzyme (dialysed citrate extract) per ml. of reaction mixture; it was incubated at 28°. After 2 weeks of incubation, the major end product was shown by paper chromatography of a sample of the reaction mixture to be unsaturated digalacturonic acid with some unsaturated trigalacturonic acid. The crude strontium salt of unsaturated digalacturonic acid was isolated from the reaction mixture by the method described by Nagel & Vaughn (1961*a*). The crude salt was dissolved in a small volume of water, and ethanol was added drop by drop until a slight turbidity resulted. The solution was allowed to precipitate a crystalline-like material for a few days in a refrigerator. Pure strontium salt was obtained by repeating this procedure two more times. About 2g. of the strontium salt of unsaturated digalacturonic acid was obtained from 10g. of polygalacturonic acid. After strontium was removed by a Dowex (H^+ form) column, the free acid was obtained in a solid form by freeze-drying. The solid material was then dried overnight over calcium chloride under vacuum at 70°.

The identity of the compound as *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid was based on comparisons with an authentic sample of this unsaturated dimer by means of: (a) paper chromatography; (b) the absorption spectrum between 220 and 270mμ; (c) the absorption spectrum after thiobarbituric acid treatment in the range 450–770mμ; (d) the infrared absorption spectrum.

The molar extinction coefficient at 235mμ of the

free acid in distilled water (pH 3·7 at 0·1 mM) was 5200 M^{-1} cm.$^{-1}$. This is somewhat higher than the value reported by Macmillan & Vaughn (1964) under similar conditions.

DISCUSSION

With the experience gained recently with purification of pectic enzymes from *Erwinia carotovora* (Nasuno & Starr, 1966; F. Moran, S. Nasuno & M. P. Starr, unpublished work), the polygalacturonic acid *trans*-eliminase which occurs in culture fluids of *X. campestris* ICPB-XC135 was purified free from other pectic enzymes.

The presence of 1 mM-Ca^{2+} ions stimulates the activity of *Xanthomonas* polygalacturonic acid *trans*-eliminase, whereas 1 mM-Ba^{2+} ions are inhibitory. No other univalent or bivalent cations have either stimulatory or inhibitory effect at the same concentration. This is in direct contrast with the polygalacturonic acid *trans*-eliminase of *Clostridium multifermentans*, which effects a terminal split on the same substrate and is stimulated to varying extents by 1 mM-Sr^{2+}, -Mg^{2+} and -Ba^{2+} ions (Macmillan & Vaughn, 1964). However, the exclusive stimulatory action of Ca^{2+} ions on *Xanthomonas* polygalacturonic acid *trans*-eliminase activity is consistent with the behaviour of this enzyme from *Erwinia carotovora* (Starr & Moran, 1962), *Bacillus polymyxa* (Nagel & Vaughn, 1961*a*) and *Pseudomonas fluorescens* (Fuchs, 1965).

The optimum pH (9·5) of *Xanthomonas* polygalacturonic acid *trans*-eliminase is at the extreme of those values reported for the enzymes from other bacteria (pH 8·5–9·3). The high optimum pH can be regarded as a defining characteristic of the bacterial polygalacturonic acid *trans*-eliminases, and is one of the properties which differentiates it from fungal pectin *trans*-eliminase. The large difference in the pH optima of bacterial hydrolytic polygalacturonases (optimum pH 5·2) and bacterial polygalacturonic acid *trans*-eliminases enables differentiation of these two enzymes which have similar substrate specificity. The substrate specificity is one of the features of polygalacturonic acid *trans*-eliminase which distinguishes the various bacterial enzymes from the fungal pectin *trans*-eliminase. Although partially esterified (68%) pectin is split at a significant rate, polygalacturonic acid *trans*-eliminase of *X. campestris*, like that of other bacteria, is specifically active on polygalacturonic acid or on de-esterified regions of pectinic acid, but not on completely methoxylated 'Link pectin' (polymethyl polygalacturonate methyl glycoside).

The random mechanism of breakdown of the substrate is clearly demonstrated by the fact that a rapid increase in viscosity is accompanied by a relatively small increase in aldehyde groups, and that saturated and unsaturated higher oligouronides appear early and disappear later as intermediate breakdown products. Thus the *Xanthomonas* enzyme can properly be named endopolygalacturonic acid *trans*-eliminase. The major end products are unsaturated di- and tri-galacturonic acids, although unsaturated trimer is further degraded but at a lower rate.

The survey of the action of *Xanthomonas* polygalacturonic acid *trans*-eliminase on saturated and unsaturated oligogalacturonides gives a clear picture of the final stages of the polymer degradation (Tables 4 and 5). The conclusions from these experiments are that *Xanthomonas* polygalacturonic acid *trans*-eliminase is unable to split the glycosidic bond nearest the reducing end of the chain, and that the enzyme preferentially splits central bonds of either saturated or unsaturated penta- and tetra-galacturonic acids rather than terminal ones, although it also attacks the bond nearest to the non-reducing ends of these substrates, and the second bond of saturated trigalacturonic acid and probably of unsaturated trimer. Similar results were observed for the breakdown of tetramethyl tetragalacturonide by the pectin *trans*-eliminase from *Aspergillus fonsecaeus* (Edstrom & Phaff, 1964), whereas a somewhat preferential split at the third bond was reported for tetramer cleavage by the polygalacturonic acid *trans*-eliminase of *Bacillus polymyxa* (Nagel & Anderson, 1965).

Nothing is yet known with regard to the possible occurrence in *X. campestris* of a cellular (i.e. internal, non-excreted) oligogalacturonide *trans*-eliminase such as that found in *Erwinia carotovora* by F. Moran, S. Nasuno & M. P. Starr (unpublished work).

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