Mode of Action of Pectic Enzymes. III. Site of Initial Action of Tomato Pectinesterase on Highly Esterified Pectin*

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ABSTRACT: The mode of action of tomato pectinesterase was studied with polymethylpolygalacturonic acid methyl glycoside as substrate. Exopolygalacturonate lyase produced by *Clostridium multifermentans* was used to determine the amount of enzymatic deesterification occurring at the "reducing" ends of polymethylpolygalacturonic acid methyl glycoside chains. Both the lyase and the tomato esterase were included in one reaction mixture and the respective activities were monitored simultaneously. Fifty per cent of the tomato pectinesterase activity was initiated near the reducing ends of highly esterified pectin molecules. The rest of the esterase activity occurred at some secondary locus or loci, perhaps next to free carboxyl groups as has been suggested for orange pectinesterase. Although both tomato pectinesterase and exopolygalacturonate lyase activities decreased as the reaction proceeded, the molar ratio of the lyase activity to the esterase activity increased gradually to approximately 0.5. Since the lyase can only act at the reducing end and since each unsaturated digalacturonate molecule contains two carboxyl groups, this suggested that eventually all of the pectinesterase activity occurred at the reducing ends of the molecules. These results could be caused by inhibition of pectinesterase by carboxyl groups produced in the interior portion of the chain.

While it is known that pectinesterase (EC 3.1.1.11) acting on pectins produces blocks of free carboxyl groups (Deuel and Stutz, 1958; Kohn *et al.*, 1968), some question remains as to the site or sites of initiation of enzymatic attack. Although Solms and Deuel (1955) correlated the activity of orange pectinesterase with the presence of free carboxyl groups in the pectin molecules, these authors have not excluded the possibility that enzymatic deesterification is initiated from either end of the pectin molecule. An approach to determining the location of the initial attack was suggested by the studies of Macmillan *et al.* (1964) on exopolygalacturonate lyase (EC 4.2.99.3) from *Clostridium multifermentans*. This enzyme degrades polygalacturonic acid from the reducing end of the molecule, removing units of *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid (or α,β-unsaturated digalacturonic acid). Exopolygalacturonate lyase will not degrade glycosidic linkages in pectins which are highly esterified with methanol. In the present study this clostridial enzyme was used analytically to determine the amount of pectinesterase activity acting at the "reducing" ends of highly esterified pectin molecules.

Materials and Methods

Substrates. Pectin, N.F. (No 3442), and polygalacturonic acid (No 3491) were obtained from Sunkist Growers, Inc., Corona, Calif. A highly esterified pectin (polymethylpolygalacturonic acid methyl glycoside) was prepared by the method of Morell and Link (1933) and Morell *et al.* (1934) by refluxing polygalacturonic acid in HCl and methanol. The product has a degree of esterification of 95.8%. With an average chain length of 33 as reported by Jansen *et al.* (1948) this would correspond to approximately one free acid group per chain.

Measurement of Enzymatic Activities. Pectinesterase activity was measured in a pH-Stat by the method described earlier (Lee and Macmillan, 1968). One unit of pectinesterase is the amount of enzyme that releases 1 μmole of carboxyl groups/min from 0.5% pectin N.F. at 30° and pH 7.0. Exopolygalacturonate lyase was assayed spectrophotometrically at 235 nm. Routine assays were conducted at pH 8.0 as previously described (Macmillan and Phaff, 1966) with a reaction mixture containing 0.5% polygalacturonic acid, 0.0005 M $CaCl_2$, and 0.033 M Tris-HCl buffer (pH 8.0). At this pH a molar extinction coefficient, ϵ_{235}, of 4600 was used to calculate the amount of unsaturated digalacturonic acid produced by the enzyme. When lyase activity was measured at pH 7.0, the Tris buffer was replaced with 0.033 M phosphate buffer (pH 7.0) and unsaturated digalacturonic acid was calculated on the basis of an ϵ_{235} value of 4700. One unit of exopolygalacturonate lyase is that amount of enzyme that produces 1 μmole of unsaturated digalacturonic acid/min under the specified conditions.

Preparations of Pectinesterase and Exopolygalacturonate Lyase. Highly purified tomato pectinesterase was prepared as described earlier (Lee and Macmillan, 1968). The final preparation had a specific activity of 1150 units/mg of protein and was essentially free of polygalacturonase activity.

The procedures used for preparing exopolygalacturonate lyase were based on those previously used (Macmillan and Phaff, 1966; Miller and Macmillan, 1970). One liter of crude culture broth from *C. multifermentans*

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was dialyzed overnight against 0.001 M $CaCl_2$. The dialyzed solution was heated at 38° for 15 min at pH 7.0 to inactivate the clostridial pectinesterase. Exopolygalacturonate lyase activity, essentially unaffected by this heat treatment, was then adsorbed on 5% (v/v) calcium phosphate gel. The lyase was eluted with 0.1 M phosphate buffer (pH 8.0) and dialyzed overnight at 5° against distilled water. The dialyzed enzyme was treated with 50% (v/v) calcium phosphate gel and then dialyzed again overnight against distilled water. The final preparation contained 3 units of exopolygalacturonate per mg of protein and was free of pectinesterase activity.

Results

Effect of pH on Highly Esterified Pectin. Preliminary experiments indicated that polymethylpolygalacturonic acid methyl glycoside undergoes some chemical saponification at pH 8.0. Ten milliliters of a 0.5% solution of this highly esterified pectin produced 0.447 μmole of carboxyl groups/min at a temperature of 30°. This represents almost 1% deesterification per hour. Chemical deesterification also occurs at pH 7.0, but at a greatly reduced rate. Reaction mixtures containing 0.5% highly esterified pectin and 0.0005 M calcium chloride produced 0.078 μmole of carboxyl groups/min at 30° and pH 7.0. This is approximately one-sixth the rate at pH 8.0. The following studies on the mode of action of tomato pectinesterase, therefore, were generally conducted at pH 7.0 to minimize the error due to nonenzymatic deesterification.

Preliminary Studies with Pectinesterase and Exopolygalacturonate Lyase. Initial experiments were designed to test whether clostridial exopolygalacturonate lyase could be used to measure the amount of pectinesterase activity occurring at reducing ends of pectin molecules. Although several alternative approaches to this problem were possible, the most direct method was to include both enzymes in a single reaction mixture containing highly esterified pectin and compare the resulting esterase and lyase activities. A predetermined amount of pectinesterase (0.031 unit) was added to reaction mixtures containing 0.5% polymethylpolygalacturonic acid methyl glycoside, 0.0005 M $CaCl_2$, 0.033 M phosphate buffer (pH 7.0), and 0.25 unit of exopolygalacturonate lyase in a final volume of 3.3 ml. Changes in absorbance were measured at 235 nm and the results are shown in Figure 1. The upper and lower lines are controls. The upper curve shows the activity of polygalacturonate lyase when assayed on 0.5% polygalacturonic acid at pH 7.0. It can be seen from the lower curve, however, that this same amount of lyase had almost no activity when assayed on highly esterified pectin in the absence of pectinesterase. When both enzymes were present with highly esterified pectin as substrate (middle curve), the lyase produced 0.0047 μmole of unsaturated digalacturonic acid/min. Pectinesterase, assayed under similar conditions in a separate reaction mixture, produced 0.031 μmole of carboxyl groups/min. Since each unsaturated dimer contains two free carboxyl groups, esterase activity at the reducing ends of molecules, as measured by production of deesterified substrate for the lyase, accounts for approximately 30% of the total esterase activity. From this it was concluded that a significant amount of tomato pectinesterase activity acts at the reducing ends of pectin molecules.

The 30% value above was obtained by comparison of the activities of the two enzymes which had been assayed in separate reaction mixtures under somewhat different conditions. While the lyase was assayed in phosphate buffer and in a 3.3-ml reaction volume containing 0.3 ml of each enzyme, the esterase was assayed in a pH-Stat without any buffer in a 20-ml reaction volume containing 1.0 ml of each enzyme. The following procedure for monitoring both enzyme activities simultaneously in a single reaction vessel was developed so that more significance could be attached to the exact quantitative correlation of two enzyme activities.

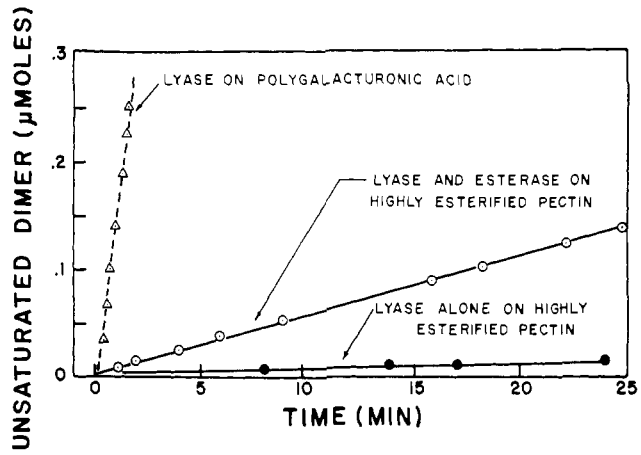

FIGURE 1: Effect of tomato pectinesterase on the activity of polygalacturonate lyase acting on highly esterified pectin. Reaction mixtures contained 0.5% polygalacturonic acid (broken line) or polymethylpolygalacturonic acid methyl glycoside (solid lines), 0.005 M $CaCl_2$, 0.033 M phosphate buffer (pH 7.0), and 0.25 unit of lyase in a final volume of 3.0 ml. In addition, the reaction mixture represented by the middle curve contained 0.031 unit of tomato pectinesterase in a final volume of 3.3 ml.

Procedure for the Simultaneous Measurement of Both Lyase and Esterase Activity. Reaction mixtures for the simultaneous measurement of pectinesterase and exopolygalacturonate lyase activity contained 0.125% polymethylpolygalacturonic acid methyl glycoside, 0.0005 M $CaCl_2$, and appropriate amounts of both enzymes in a final volume of 10 ml. Reactions were conducted under nitrogen at 30° in the thermostatically controlled vessel of a Radiometer pH-Stat. Pectinesterase activity was calculated from the amount of 0.02 N NaOH required to maintain a pH of 7.0. Lyase activity was determined by circulating the solution from the reaction vessel through a 2-mm flow cell in a Gilford Model 2000 recording spectrophotometer. The concentration of unsaturated digalacturonic acid was calculated from the increase in absorbance at 235 nm as discussed previously. This system could detect a maximum of 15 optical density units or 32 μmoles of unsaturated digalacturonic acid. This amount would correspond to 100% degradation of 0.125% polymethylpolygalacturonic acid methyl glycoside.

Site of Action of Tomato Pectinesterase. Figure 2 shows the results of an assay of 2.8 units of tomato pectinesterase and 1.5 units of exopolygalacturonate lyase acting simultaneously on highly esterified pectin. In the first few minutes of the reaction, unsaturated digalacturonic acid produced by the lyase accounted for about 50% of the deesterification produced by the esterase. Therefore, since the lyase can only act at the reducing ends of chains, this indicated that a

TABLE I: Comparison of Initial Rates of Exopolygalacturonate Lyase and Tomato Pectinesterase Acting Simultaneously on Highly Esterified Pectin.

Amount of Enzyme Added[a]			Initial Enzyme Rates[b]		
Esterase (units)	Lyase (units)	2 × Lyase/Esterase	Esterase (μmoles/min)	Lyase (μmoles/min)	2 × Lyase/Esterase
0.12	0.90	15.0	0.11	0.024	0.44
0.57	1.6	5.6	0.47	0.11	0.47
0.62	1.6	5.2	0.62	0.14	0.45
0.90	0.75	1.7	0.75	0.21	0.56
2.8	1.5	1.1	2.8	0.67	0.48
2.1	0.75	0.71	2.1	0.58	0.55
3.0	1.1	0.67	3.0	0.82	0.55
2.8	0.75	0.54	2.6	0.62	0.48
3.0	0.75	0.50	1.7	0.47	0.55
					Av 0.50

[a] As determined in separate assays of esterase on highly esterified pectin and lyase on polygalacturonic acid. [b] As determined by simultaneously monitoring both enzymes acting together on highly esterified pectin.

considerable amount of the initial pectinesterase activity must also be occurring at the reducing ends. If the esterase acted entirely randomly or even entirely linearly beginning from some locus other than the reducing end, much less than 50% correlation between the two initial rates would be expected.

Effect of Enzyme Concentration on Correlation of Initial Esterase and Lyase Activities. In interpreting the above experiment, it was imperative to determine whether pectinesterase was indeed limiting the activity of polygalacturonate lyase on highly esterified pectin. Table I shows the results of a series of experiments employing various amounts of esterase and lyase in the presence of 0.0005 M $CaCl_2$ and 0.125% highly esterified pectin. The amount of enzyme added in each case was determined on the following basis. Pectinesterase was assayed on 0.125% highly esterified pectin at pH 7.0 in the absence of lyase. Exopolygalacturonate lyase was assayed on 0.125% polygalacturonic acid at pH 7.0 in the absence of pectinesterase. The activities of both enzymes acting simultaneously on 0.125% polymethylpolygalacturonic acid methyl glycoside were continuously monitored by the method described above. The lyase unit, based on the concentration of unsaturated digalacturonic acid, corresponds to two esterase units. For comparison, therefore, the ratio of these enzymes is expressed as twice the polygalacturonate lyase activity divided by the pectinesterase activity. On this basis, the ratio of the initial activities, as determined simultaneously, varied between 0.44 and 0.56. These variations fit no apparent pattern and appeared to be independent of the amounts of the two enzymes added. It is clear, however, that in each case pectinesterase limited the initial activity of polygalacturonate lyase. For example, even when the ratio of the enzymes added (2 × lyase/esterase) is 15, the ratio of the initial rates was only 0.44.

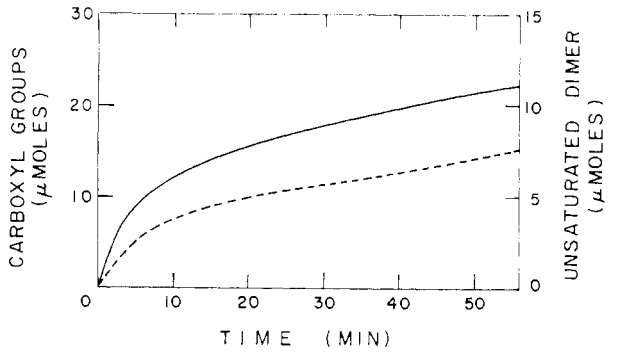

FIGURE 2: Comparison of exopolygalacturonate lyase and tomato pectinesterase activity on highly esterified pectin. The reaction mixture contained 0.125% polymethylpolygalacturonic acid methyl glycoside, 0.0005 M $CaCl_2$, 2.8 units of tomato pectinesterase, and 1.5 units of polygalacturonate lyase in a total volume of 10 ml. The broken line represents unsaturated digalacturonic acid and the solid line represents carboxyl groups as determined by the method described in the text.

Effect of Enzyme Concentration on Correlation of Esterase and Lyase Activities Throughout the Time Course of the Reaction. The activities of various amounts of the two enzymes were monitored simultaneously over prolonged periods of time and the results are shown in Figure 3. The experiment shown by curves labeled 1 was a simultaneous assay of 2.8 units of tomato pectinesterase and 0.75 unit of exopolygalacturonate lyase. Initially, lyase activity accounted for 48% of the enzymatic deesterification (Table I). When the amount of lyase added was doubled (curves 2), the initial rates of pectinesterase and polygalacturonate lyase activity on highly esterified pectin were unaffected. The rates for the esterase and the lyase, however, decreased less rapidly with time than in the previous experiment. After 30 min both enzymes were acting 20 to 30% faster in the reaction mixture shown by curves 2 than in those shown by curves 1. These higher final enzyme rates in the presence of excess lyase (curves 2) may be due to the more rapid degradation of blocks of galacturonic acid residues

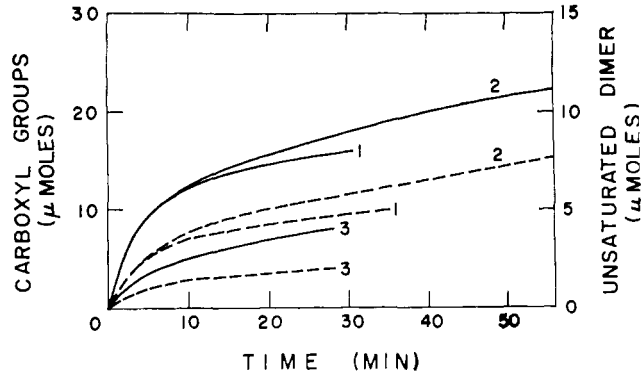

FIGURE 3: Effect of enzyme concentration on correlation of tomato pectinesterase and exopolygalacturonate lyase activity throughout the time course of the reaction. The solid lines represent pectinesterase activity, while the broken lines represent lyase activity. Reaction mixtures contained 0.125% polymethylpolygalacturonic acid methyl glycoside, 0.0005 M $CaCl_2$, and the following amounts of enzyme in a final volume of 10 ml: 2.8 units of pectinesterase and 0.75 unit of lyase in curves 1, 2.8 units of pectinesterase and 1.5 units of lyase in curves 2, and 0.90 unit of pectinesterase and 0.75 unit of lyase in curves 3. Both enzymes were monitored simultaneously by the method described in the text.

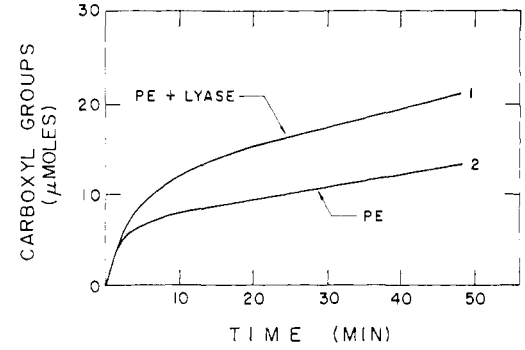

FIGURE 4: Activity of tomato pectinesterase in the presence (1) and absence (2) of exopolygalacturonate lyase. Reaction mixtures contained 0.125% polymethylpolygalacturonic acid methyl glycoside, 0.0005 M $CaCl_2$, 2.8 units of pectinesterase, and, in the reaction mixture represented by curve 1, 1.5 units of lyase.

formed at the reducing ends of highly esterified pectin chains. This could be considered a reduction in the steady-state concentration of anhydrogalacturonic acid on the pectin chain resulting in a decrease in product inhibition of pectinesterase (Lee and Macmillan, 1968). In the reaction mixture represented by curves 3, pectinesterase activity was reduced to 0.90 unit, while polygalacturonate lyase activity was returned to its original level of 0.75 unit. Although the initial enzyme rates were lower than in curves 1 and 2 above because of the decreased pectinesterase activity, exopolygalacturonate lyase still accounted for approximately 50% of the initial pectinesterase activity. In all three experiments as the reaction proceeded the lyase accounted for increasing amounts of the pectinesterase activity. In curves 2 of Figure 3, for example, exopolygalacturonate lyase initially accounted for 50% of the esterase activity. After 50 min, however, exopolygalacturonate lyase accounted for 95% of the pectinesterase activity occurring at that time. This suggested that in the latter stages of the reaction most of the tomato pectinesterase activity was occurring at the reducing ends of pectin chains.

Effect of Polygalacturonate Lyase on Pectinesterase Activity. Pectinesterase activity was measured on polymethylpolygalacturonic acid methyl glycoside both in the presence and in the absence of polygalacturonate lyase (1.5 units). The results (Figure 4) show that the initial esterase activities in both assays were the same. The rate of saponification decreased less rapidly, however, in the presence of lyase than in its absence. Polygalacturonic acid has been shown to be a competitive inhibitor of tomato pectinesterase (Lee and Macmillan, 1968). This experiment indicated that enzymatically deesterified blocks on the pectin molecule were inhibiting tomato pectinesterase. Since exopolygalacturonate lyase can only degrade pectin substances from the reducing ends, it follows that many of these blocks must be located near the reducing ends of the pectin chains. In the presence of polygalacturonate lyase, the terminal acid groups were removed from the substrate molecule and less inhibition occurred.

Discussion

The present studies rule out several possible patterns of action for enzymatic deesterification of pectins. If tomato pectinesterase saponified pectins in a random manner, only a very small percentage of the initial deesterification would occur near the reducing ends of the chains. With an average chain length of 33, the statistical probability of initial deesterification occurring at reducing ends is $1/33$. As deesterification proceeded, and the number of ester groups decreased, the statistical probability of deesterification occurring at the reducing ends would increase. Exopolygalacturonate lyase, acting terminally from reducing ends, would initially account for very little of the pectinesterase activity. With time, the rate of lyase activity would increase slowly until enzymatic saponification was nearly complete. The highest rate of exopolygalacturonate lyase activity would occur near the end of the reaction, after most of the methyl ester groups were removed.

The present study also eliminates the possibility that pectinesterase saponifies pectins linearly beginning only from loci within the chain. Pectinesterase acting from within pectin chains could possibly continue working in either or both of two directions. If deesterification proceeded only toward the nonreducing ends, no exopolygalacturonate lyase activity would be possible. Even if pectinesterase worked toward the reducing ends, exopolygalacturonate lyase activity would be initially quite small. Lyase activity would be expected to increase with time as pectinesterase reached the reducing ends of more pectin molecules.

Kohn *et al.* (1968) showed that tomato pectinesterase saponifies pectins in a linear manner. Their results, however, provide no information on the initiation points, or sites of enzymatic attack. Solms and Deuel (1955) suggested that orange pectinesterase saponifies ester linkages only when they are next to acid residues. Presumably, saponification could begin at any free acid group, but it proceeded linearly along the chain producing blocks of completely deesterified residues.

Polymethylpolygalacturonic acid methyl glycoside is

predominantly a single substance of molecular weight between 5000 and 7000 corresponding to a chain of about 33 anhydrogalacturonic acid methyl ester residues (Jansen *et al.*, 1948). As this material is 96% esterified, an average chain contains 32 methyl ester groups and one free acid group. This acid group can be located anywhere along the chain (Kohn and Furda, 1967a). Tomato pectinesterase may act, as does the orange enzyme, at loci next to free carboxyl groups, as well as at the reducing ends of pectin chains. If this is so, it would explain why exopolygalacturonate lyase was initially only able to degrade about half of the deesterified product produced by tomato pectinesterase. Assuming that tomato pectinesterase has the same affinity for ester groups next to free carboxyl groups as for ester groups at the reducing ends of polymethylpolygalacturonic acid methyl glycoside molecules, approximately 50% of the enzymatic deesterification would occur at the reducing ends of the chains, and the rest would occur next to the free carboxyl group somewhere within the chain. Thus, exopolygalacturonate lyase, acting linearly from the reducing ends, would only be able to initially degrade half of the total product produced by the action of pectinesterase.

As the reaction proceeds, blocks of anhydrogalacturonic acid residues begin to accumulate. Those acid groups produced at the reducing ends are removed by the lyase. Those produced in the interior portions of the chains, however, cannot be removed by the lyase and eventually competitively inhibit the activity of pectinesterase. Although both tomato pectinesterase and exopolygalacturonate lyase activities decreased with time, the molar ratio of the lyase activity to the esterase activity increased gradually and approached the value 0.5. (This ratio would be 1.0 on the basis of 2 × lyase/esterase as expressed in Table I.) Since exopolygalacturonate lyase can only act at the reducing end this ratio suggested that eventually all of the pectinesterase activity was occurring at the reducing end of molecules.

At this time it is possible to conclude with certainty only that tomato pectinesterase activity acts at both the reducing ends and some second locus or loci on highly esterified pectin chains. Reexamination of the pH optimum reported previously (Figure 7; Lee and Macmillan, 1968), however, lends support to the carboxyl group–reducing group hypothesis. There is a broad pH optimum between pH 6.0 and 9.0 with an apparent peak at pH 8.5. Saponification rates at above pH 7.0 were obtained by bringing the pH of the reaction mixture to the appropriate value, and adding enzyme. As a result of the alkaline conditions, some free carboxyl groups were generated both before and during enzymatic hydrolysis. Since alkaline saponification is a random process (Kohn and Furda, 1967a,b), these new carboxyl groups could act as initiation points for enzymatic attack. The number of sites for enzymatic attack would be, therefore, increased, and higher esterase activities would result.

The work presented here was not intended to imply that orange pectinesterase and tomato pectinesterase act differently on pectins. No direct experimental comparison was made between the two enzymes. The conclusion that orange pectinesterase hydrolyzes only methyl ester groups next to free carboxyl groups was based partly on the fact that the orange enzyme had a higher activity on pectin presaponified with alkali than on pectin presaponified to the same degree of esterification with the orange pectinesterase (Solms and Deuel, 1955). This same evidence might also be interpreted to indicate that large blocks of free carboxyl groups, produced enzymatically with orange pectinesterase, were capable of inhibiting the further action of orange pectinesterase. Alkaline deesterified pectin would not inhibit as much as enzymatically deesterified pectin since the carboxyl groups would be randomly distributed rather than in large blocks. Furthermore, in addition to hydrolyzing the ester bond, treatment of pectin with alkali is known to produce reducing groups by cleavage of the glycosidic bonds by a *trans*-elimination reaction (Neukom and Deuel, 1958). The orange enzyme may act faster on alkali deesterified pectin not only because of the random distribution of carboxyl groups, but also because of the larger number of free reducing terminals. Thus, there is no experimental evidence proving either similarities or differences between the tomato and the orange enzyme.

The use of polymethylpolygalacturonic acid methyl glycoside as substrate in these experiments might be questioned since it is possible that the methyl group at position one might interfere with the action of the lyase. This was obviously not the case, however, since the lyase in combination with tomato pectinesterase readily attacks this substrate. After the lyase removes the first unsaturated digalacturonate from the molecule there would be a reducing group present on the chain which remains. Naturally occurring pectin would be less suitable as substrate in these experiments since the distribution of carboxyl groups is not well characterized. Conceivably there could be either a random or a blockwise arrangement of carboxyl groups in pectins depending on the manner of isolation and on whether they had been acted upon by plant enzymes. Use of chemically esterified pectin eliminated this uncertainty.

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