Pectic Acid Lyases of *Bacillus polymyxa*¹

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Four enzymes were separated from an extracellular preparation of *Bacillus polymyxa* by carboxymethylcellulose column chromatography. The pH optima were 8.3 to 8.5, 8.7 to 8.9, 9.2 to 9.4, and 9.5 to 9.6. All of the enzymes required calcium ion for maximum activity, whereas strontium ion was only partially effective in stimulating activity. Cobalt was the only other cation tested which was effective in two of the enzymes. The lyases seem to attack a calcium salt-bridged substrate. $K_m$ and $V_m$ data of the four enzymes on various oligomers are presented as well as paper chromatographic evidence of preferred sites of attack. All of the enzymes are endo-enzymes which, based upon their characteristics, were classed into two types.

Some confusion exists as to the number of different pectic acid lyases excreted by *Bacillus polymyxa*. Nagel and Vaughn (12, 13) found two to four enzymes depending upon the procedure used for isolation. Although minor differences were observed between the different preparations, it was concluded that they represented a single enzyme. Nagel and Anderson (11) isolated a single enzyme and further characterized it as to pH optimum on different substrates. The $K_m$ and $V_m$ values on acid-soluble pectic acid, tetra- and tri-galacturonic acids, and unsaturated tetra-galacturonic acid were determined. It was demonstrated that $K_m$ was markedly influenced by chain length with an inverse correlation being observed. This is consistent with the observation that the enzyme(s) attacked pectic acid in a random manner.

Many organisms can elaborate several enzymes that attack pectic acid in different ways (16, 18, 23). In addition, Hasegawa and Nagel (5) showed that an endo-lyase which was more random in character than those apparently produced by *B. polymyxa* was produced by another *Bacillus* species. Because of these observations, it was deemed to be of value to isolate the different lyases of *B. polymyxa* and to characterize them. Evidence for the presence of four enzymes, which can be classed into two groups, is presented.

**MATERIALS AND METHODS**

**Separation of the lyases.** The methods of culturing *B. polymyxa* and isolating the culture medium containing all of the lyases have been described (12). The culture medium was dialyzed against cold, running tap water (ca. 15°C) for about 36 hr. All subsequent operations were done in a cold room (4°C). The crude enzyme was precipitated with three volumes of cold acetone (4°C) and centrifuged for 7 min at 4,600 × g. The precipitate was dissolved in a minimum amount of 0.001 M sodium phosphate (pH 5.2) and dialyzed against four changes of distilled water for a total of 24 hr. The dialyzed enzyme was loaded onto a carboxymethylcellulose (CMC) column (2 by 30 cm; Carl Schleicher and Schuell Co., Keene, N. H.) previously equilibrated with 0.001 M sodium phosphate (pH 5.2). After loading and washing with 5 to 10 ml of cold distilled water, the column was eluted with a linear gradient (1,200 ml total) of sodium phosphate (pH 6.5) from 0.001 to 0.1 M. Fractions of approximately 5 ml were collected. The fractions of individual peaks were pooled and crystalline bovine plasma albumin (Armour Pharmaceutical Co.) was added to give a final concentration of 0.1%.

**Repurification of the individual lyases.** Further purification of each enzyme, after desalting on Sephadex G-25 (Pharmacia Fine Chemicals), was attempted by chromatographing on CMC by using a linear gradient of sodium chloride in 0.01 M imidazole (pH 7.0). Enzymes P₂ and P₃ were purified from the respective pooled fractions obtained from the separation shown in Fig. 1, and enzymes P₁ and P₄ were purified from the pooled peaks obtained in a duplicate run (data shown in parentheses in Table 1).

The entire sample of P₄ was desalted on a large Sephadex G-25 column (4.3 by 36.5 cm, about 520 ml), whereas only portions of the pooled peaks of the other three enzymes, sufficient enzyme for one column separation, were desalted on a smaller Sephadex G-25 column (2 by 20 cm, about 90 ml). All samples were concentrated before G-25 chromatography by placing them in a dialysis bag and covering with powdered sugar. The following units of activity were used for the purification of P₁, P₂, P₃, and P₄; 38.2, 50.5, 42.3, and 25.5, respectively.

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Elution on the second CMC column (1.1 by 40 cm) was accomplished with a linear gradient of sodium chloride (0.0 to 2.0 m) in 500 ml (total volume) of 0.01 m imidazole (pH 7.0). The effluent was collected in 5-ml fractions. After locating the activity, 0.01 ml of 5% bovine plasma albumin was added to the most active fractions. The fractions (0.01 ml each) were assayed for activity at both pH 8.5 and 9.4, and an activity ratio was calculated therefrom. Only those fractions whose ratios agreed within 5 to 7% were pooled and used for further study.

Substrates. Acid-soluble pectic acid (ASPA) was prepared by the procedure of McCready and Seegmiller (8). The saturated and unsaturated oligogalacturonides (the terminal galacturonide on the nonreducing end contains an unsaturated bond between carbons 4 and 5) used were those isolated and characterized by Nagel and Wilson (15).

Analyses. Enzyme activity was determined with a solution containing 0.001 m calcium, 0.05% ASPA, and 0.05% each of glycine-NaOH (pH 9.4) and glycine (pH 9.4) in a total volume of 3 ml. A portion (0.01 to 0.1 ml) of each fraction was used to determine activity. The rate of change in optical density was measured at 232 nm. An enzyme unit is that amount of lysase causing the release of 1 μmole of product per min at 25 C. A molar absorptivity (22) of 4,600 m<sup>−1</sup> cm<sup>−1</sup> was used for this calculation. Protein was estimated by measuring the optical density at 280 nm or more precisely by the method of Lowry et al. (7). Salt concentration of the fractions obtained from the G-25 columns was determined by conductance measurements.

Optimum pH. It was found that storage of the combined reagents before addition of enzyme resulted in a change in some component of the assay medium which affected the pH optimum of the enzymes. Because of this observation, separate stock solutions of buffer, calcium chloride, and ASPA were prepared. The proper amounts of the solutions were then mixed just before assay. The reaction mixtures contained 0.05 m each of Tris and glycine, 0.001 m calcium, and 0.05% ASPA. The total volume was 3.0 ml.

Viscosity reduction and bond breakage. The viscosity reduction which results from bond breakage was determined in a capillary viscometer with solutions which contained 0.5% (w/v) pectic acid (no. 3491, Sunkist Growers, Corona, Calif.), 0.1 m buffer at the optimum pH, and 0.001 m calcium. The buffers and pH values used for assay of enzymes P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, and P<sub>4</sub> were 0.05 m Tris and 0.05 m glycine at pH 8.95, 0.1 m Tris at pH 8.35, 0.05 m Tris and 0.05 m glycine at pH 9.3, and 0.1 m Tris at pH 8.5, respectively. The amount of bond cleavage was estimated by measuring the optical density changes at 232 nm in a DU spectrophotometer by using a silica cuvette with a 1-mm light path. All reaction rates were measured at 25 C.

Cation requirements. The cation requirement of the four enzymes was determined in a freshly mixed assay mixture containing 0.05% ASPA, 0.01 m cation, and 0.1 m buffer at the pH optimum of the enzymes. The cations tested were the chloride salts of calcium, strontium, magnesium, barium, cadmium, nickel, manganese, and cobalt. The stimulatory effect of potassium (0.01 m) was tested in an assay mixture containing 0.001 m calcium.

The effect of calcium concentration on the degradation of trimer was determined with enzyme P<sub>2</sub>. The reaction mixture contained 0.004 m trimer, 0.1 m Tris-hydrochloride (pH 8.35), various calcium ion concentrations (0.001 to 0.1 m) and 0.05% ASPA in a total volume of 3 ml. The data are plotted as activity versus the ratio of normality of calcium to the normality of carboxyl groups.

K<sub>m</sub> and V<sub>m</sub> determinations. The K<sub>m</sub> and V<sub>m</sub> constants were determined for the four enzymes with seven saturated and two unsaturated oligogalacturonides. The constants were obtained by the method of Line-weaver and Burk (6). No determination was made with unsaturated trimer because no reliable assay was available. The assay solutions contained 0.1 m buffer at the optimum pH of the enzyme with the exception of enzyme preparation P<sub>1</sub> which was assayed at pH 8.3. Tris-hydrochloride buffer was used for enzymes P<sub>1</sub> and P<sub>2</sub>, whereas 0.05 m NaOH buffer was used for enzymes P<sub>3</sub> and P<sub>4</sub>. The assay solutions also contained 0.01 m calcium, except in the case of ASPA for which a concentration of 0.001 m was used. Sufficient enzyme to give a measurable rate was used. The constants for enzymes P<sub>1</sub> and P<sub>2</sub> were determined at 0.001 m calcium. The activity measurements were recorded at 25 C and 232 nm on a Beckman DU spectrophotometer equipped with a Gilford automatic recording apparatus and thermostapapers.

Paper chromatography of products. The susceptibility of different bonds in a substrate to enzyme attack was examined by paper chromatography. The reaction mixtures contained 0.001 m calcium, 0.1 m Tris-hydrochloride (pH 8.0), 0.01 m substrate, and the following units of enzyme: P<sub>1</sub>, 0.068; P<sub>2</sub>, 0.184; P<sub>3</sub>, 0.088; and P<sub>4</sub>, 0.041 in a total volume of 1 ml. The reactions were run at pH 8.0 to minimize denaturation of the enzyme. The chromatograms were developed in pyridine-ethyl acetate-acetic acid-water (5:5:1:3, solvent system 1) for various time periods. The chromatograms showing octamer degradation by the four enzymes were also developed in a new solvent system containing pyridine-ethyl acetate-acetic acid-water (3:5:2:3, solvent system 2). Saturated and unsaturated uronides were well separated in 16 hr with solvent system 2. All of the uronides of DP (degree of polymerization) lower than four were clearly separated. The saturated and unsaturated tetramers were not separated, although longer elution times would probably result in their separation. The spots were visualized by the O-aminobiphenyl reagent (1).

RESULTS

Separation of the lysases. The separation of the crude enzyme preparation on CMC into four lysases was achieved (Fig. 1), and the results of the first column separation are shown in Table 1. The reproducibility of the technique is indicated by the data (shown in parentheses) from a duplicate experiment. The relative distribution of the four lysases was reasonably constant between the two separations (one major component,
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![Graph](image)

**FIG. 1.** Elution pattern of the lyases of *B. polymyxa* on carboxymethyl cellulose. Symbols: O, activity; •, absorbancy (A) at 280 nm.

| Sample               | Volume (ml) | Activity (units/ml) | Total units | Protein (mg/ml) | Specific activity (units/mg) | Yield (%) | Purification |
|----------------------|-------------|---------------------|-------------|----------------|-----------------------------|-----------|--------------|
| Crude enzyme...      | 200 (180)*  | 9.8 (8.6)           | 1960 (1548) | 0.713 (0.810)  | 13.6 (10.6)                 | 100 (100) | 1.0 (1.0)    |
| Acetone precipitate  | 96 (60.5)   | 17.2 (25.4)         | 1651 (1536) | 0.900 (1.138)  | 19.1 (22.3)                 | 84 (99)   | 1.4 (2.1)    |
| CMC-load...          | 24.5 (27)   | 2.8 (1.8)           | 69 (49)     | 0.212 (0.116)  | 13.2 (15.4)                 | 4 (3)     | 0.9 (1.5)    |
| P1                   | 87.5 (95.5) | 7.4 (5.5)           | 648 (523)   | 0.038 (0.034)  | 195 (162)                  | 41 (35)   | 14 (15)      |
| P2                   | 141.5 (118.5)| 2.8 (2.8)          | 396 (332)   | 0.019 (0.006)  | 311 (467)                  | 25 (22)   | 23 (44)      |
| P3                   | 145 (123)   | 2.2 (2.5)           | 319 (308)   | 0.009 (0.013)  | 244 (192)                  | 20 (21)   | 18 (18)      |

* Data in parentheses obtained from a duplicate experiment.

35 to 44%; two lesser components, 21 to 25% each; and one minor component, 3 to 4%.

The percentage recovery from the CMC was fairly high and consistent (90 and 81%, respectively). The concentration of protein found in the crude enzyme is consistent with that reported for other exo-cellular enzymes (3, 5, 8, 21). In the case of two of the enzymes obtained in the first column separation, the protein concentration was too dilute to estimate accurately by the method of Lowry et al. (7).

**Rechromatography of the individual enzymes.** Figure 2 shows the results obtained when enzyme P4 was passed through the Sephadex G-25 column. Complete separation from the bovine plasma albumin was obtained. The only peculiar result was the emergence of the phosphate before the sucrose. This was contrary to the expected separation based on molecular size. An indication of the size of enzyme P4 was shown by its clear separation from the albumin, indicating a small protein. However, charge effects may partially account for the separation. Of the other three enzymes desalted on Sephadex G-25, P2 and P3 were completely separated from the albumin but P1 was not.

The enzymes were eluted from the second CMC column as single peaks (Fig. 3 to 6) without any indication of other activity except for enzyme P1 (Fig. 6). Since enzymes P3 and P4 were not completely separated on the first CMC column (Fig. 1), the pooled fractions of P4 probably included some P2. This activity appeared as a small peak immediately before the main enzyme peak P4. This separation of activities was reproducible. The protein concentrations were too dilute for accurate assay. Therefore, the specific activities were not determined.
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P_{2} was 8.2 to 8.4. Thus, aging of the medium caused a drop of greater than 1 pH unit in the observed pH optimum.

Comparison of viscosity reduction and bond cleavage. Figure 7 shows the relationship between

**Optimum pH.** The pH optima for enzymes P_{1}, P_{2}, P_{3}, and P_{4} are 8.7 to 8.9, 9.5 to 9.6, 9.2 to 9.4, and 8.3 to 8.5, respectively. With the exception of enzyme P_{2}, the usual bell-shaped curves were obtained. When "aged" assay media was used, the observed pH optimum for enzyme

![Fig. 2. Separation of enzyme P_{1} (●) from bovine plasma albumin (○), phosphate buffer (△), and sucrose (■) on a Sephadex G-25 column.](image)

![Fig. 3. Chromatography of enzyme P_{1} on the second carboxymethyl cellulose column. Symbols: ○, activity; □, absorbancy (A) at 280 nm.](image)

![Fig. 4. Chromatography of enzyme P_{2} on the second carboxymethyl cellulose column.](image)

![Fig. 5. Chromatography of enzyme P_{4} on the second carboxymethyl cellulose column.](image)
crude lyase activity were cadmium, barium, magnesium, nickel, and manganese. Maximum activation was shown by calcium with lesser activation by strontium and cobalt. Based on the relative percentage of activity with strontium, enzymes P₁ and P₂ and enzymes P₃ and P₄ form the same set of pairs observed in the viscosity experiment.

The effect of calcium concentration on activity of enzyme P₃ is shown in Fig. 8. Maximum activation occurred on an equivalence basis of 1.0, although a slight increase in activity was observed with higher ratios. These data indicate that the interaction of calcium, trimer, and en-

| Enzyme | Per cent relative activity |
|--------|---------------------------|
|       | Calcium | Strontium | Cobalt |
| P₁    | 100     | 12        | 12     |
| P₂    | 100     | 8         | 0      |
| P₃    | 100     | 21        | 4      |
| P₄    | 100     | 28        | 0      |

**Fig. 6.** Chromatography of enzyme P₄ on the second carboxymethyl cellulose column.

**Fig. 7.** Relationship between the percentage of bond breakage and the percentage of specific viscosity.

viscosity changes and bond degradation by the purified enzymes. The percentages of bonds cleaved for a reduction of 50% in specific viscosity for enzymes P₁, P₃, P₃, and P₄ were 2.9, 3.0, 2.5, and 2.3%, respectively. Thus, the four enzymes are classified as endo-enzymes. The data further indicate that the four enzymes form two groups based on their relative “endo” character. Thus, enzymes P₃ and P₄ behave more as endo-enzymes than P₁ and P₂ since less bond breakage is required for the same viscosity change.

**Cation requirements.** Table 2 shows the relative activities of the four enzymes in the presence of 0.001 M calcium, strontium, and cobalt ions. Additional cations which had no effect on the

**Fig. 8.** Effect of the ratio of calcium to carboxyl groups on the activity of enzyme P₃ with trimer as the substrate.
zyme probably satisfies the requirements of an interaction between cation and substrate. The metal combines with the substrate rather than the enzyme.

**Kₘ and Vₘ determinations.** Table 3 lists the experimental values of Kₘ and Vₘ obtained for the four enzymes. There are a number of observations common to all of the enzymes. In the series of saturated uronides, Vₘ was essentially constant for the longer chain uronides for any particular enzyme. The deviation from a constant Vₘ within the series of saturated uronides occurred with enzymes P₁ and P₂ in their attack on trimer and tetramer. In contrast, the Vₘ values of enzymes P₁ and P₃ with these substrates were relatively consistent with the Vₘ values of the longer oligogalacturonides. In one case (enzyme P₂ with tetramer), the value was high. No explanation can be given for this observation.

The Kₘ values showed a pattern expected for endo-enzymes. In each case, an inverse correlation between Kₘ and chain length was observed, indicating that when more sites for enzyme attack are available on the substrate, relatively less substrate is required to saturate the enzyme. This, in turn, supports the contention that the enzymes can attack more than one site on the longer oligogalacturonides. However, were this the only factor, one would expect the apparent Kₘ for tetramer to be one-half that of trimer, since, as will be shown later, there is only one site of attack in the case of the latter, whereas tetramer has two potential sites of attack.

The Kₘ and Vₘ constants of the unsaturated uronides (Table 3, bottom) indicate both differences and similarities. These values showed little differences for saturated and unsaturated uronides of either DP with enzyme P₁. The Vₘ of unsaturated tetramer with enzyme P₂ was unusually high, but that of unsaturated pentamer was similar to that obtained with saturated pentamer. The Kₘ constants of P₂ with unsaturated tetramer and unsaturated pentamer were two and seven times greater than the corresponding saturated substrates. These data indicate a decreased affinity due to the double bond.

In the case of enzyme P₄, the Vₘ values for the tetramer and pentamer were greater by factors of 16 and 23, respectively, than that of the corresponding unsaturated uronides. A similar but less marked trend was observed with enzyme P₄. The Kₘ constants of the unsaturated substrates were less than those of their saturated counterparts of the same DP. Both of these observations indicate that the double bond resulted in an increased affinity of the substrates for enzymes P₃ and P₄ as well as a more stable E-S complex.

**Paper chromatography of products.** The results obtained from paper chromatographic examination of products formed by the enzymes from the different substrates are summarized in Table 4. Only the glycosidic bonds (numbered from the reducing end) which were initially attacked are listed. Photographs of the chromatograms are also shown (Fig. 9, 10). The four enzymes appear to be of two types with respect to specificities, enzymes P₁ and P₄ being more endo in character than enzymes P₁ and P₂. (Photographs of the chromatograms for enzyme P₁ are not shown but are essentially identical to those of enzyme P₄.) Some further general observations are possible. Bond 1 of all substrates tested was not attacked by any of the enzymes. The only substrates attacked similarly by all four enzymes were the trimers. The differences between the enzymes became apparent only when tetramer and the longer uronides were substrates. Enzymes P₁ and P₂ attacked tetramer at bonds 2 and 3 but showed a strong preference for bond 2. Enzymes P₃ and

| Substrate   | P₁      | P₂      | P₃      | P₄      |
|-------------|---------|---------|---------|---------|
|             | Kₘ (µ)  | Vₘ     | Kₘ (µ)  | Vₘ     | Kₘ (µ)  | Vₘ     | Kₘ (µ)  | Vₘ     |
| ASPA        | 2.42 × 10⁻⁴ | 2.36   | 1.31 × 10⁻⁴ | 5.59   | 1.17 × 10⁻⁴ | 3.36   | 2.8 × 10⁻⁴ | 3.07   |
| Octamer     | 4.57 × 10⁻⁴ | 4.19   | 3.66 × 10⁻⁴ | 6.00   | 4.26 × 10⁻⁴ | 4.35   | 17.7 × 10⁻⁴ | 3.26   |
| Heptamer    | 3.71 × 10⁻⁴ | 3.3    | 3.71 × 10⁻⁴ | 5.53   | 1.44 × 10⁻⁴ | 6.02   | 20 × 10⁻⁴ | 2.82   |
| Hexamer     | 6.67 × 10⁻⁴ | 6.7    | 5.80 × 10⁻⁴ | 6.67   | 1.28 × 10⁻⁴ | 4.00   | 38.7 × 10⁻⁴ | 3.05   |
| Pentamer    | 9.45 × 10⁻⁴ | 5.89   | 4.59 × 10⁻⁴ | 5.89   | 4.59 × 10⁻⁴ | 3.84   | 130 × 10⁻⁴ | 3.22   |
| Tetramer    | 5.88 × 10⁻⁴ | 11.75  | 505 × 10⁻⁴ | 8.28   | 2.430 × 10⁻⁴ | 0.133 | 460 × 10⁻⁴ | 0.3    |
| Trimer      | 3.11 × 10⁻⁴ | 3.1    | 2.070 × 10⁻⁴ | 8.28   | 2.430 × 10⁻⁴ | 0.133 | 460 × 10⁻⁴ | 0.3    |
| u-Pentamer  | 1.075 × 10⁻⁴ | 4.12   | 1.825 × 10⁻⁴ | 12.8   | 22.6 × 10⁻⁴ | 0.17   | 46.4 × 10⁻⁴ | 1.06   |
| u-Tetramer  | 214 × 10⁻⁴ | 6.25   | 2.625 × 10⁻⁴ | 112.5  | 126 × 10⁻⁴ | 0.05   | 67 × 10⁻⁴ | 0.33   |

* Values for Vₘ are in units of ΔA per minute.
**Table 4. Bond susceptibility of oligomers degraded by P₁, P₂, P₃, and P₄.**

| Enzyme | Reaction | Trimer | Tetramer | Pentamer | Hexamer | Heptamer | Octamer | Octamer |
|--------|----------|--------|----------|----------|---------|----------|---------|---------|
| P₁     | NR       | 1      | 1        | 1        | 1, 4    | 1, 5     | 1, 6    | 1, 6, 7 |
|        | R        | 2      | 2 > 3    | 2 = 3    | 2 > 3 > 4 | 2 = 3 = 4 | 5       | 2 > 3, 4, 5 |
| P₂     | NR       | 1      | 1        | 1, 4     | 1, 4, 5  | 2 > 3 > 4 | 1, 5, 6  | 1, 6, 7  |
|        | R        | 2      | 2 > 3    | 2 > 3    | 2 > 3 > 4 | 2 > 3 = 4 | 5 > 4 = 3 | 2 > 4 ≥ 3, 5 |
| P₃     | NR       | 1      | 1, 2     | 1, 2     | 1, 2, 6  | 1, 2, 6, 7 | 1, 2, 6, 7 | 1, 2, 6, 7 |
|        | R        | 2      | 3        | 3 > 4    | 3, 4, 5  | 4 > 3 = 5 | 3 ≤ 4 > 5 | 3 ≥ 4 = 5 |
| P₄     | NR       | 1      | 1, 2     | 1, 2     | 1, 2, 6  | 1, 2, 6, 7 | 1, 2, 6, 7 | 1, 2, 6, 7 |
|        | R        | 2      | 3        | 3 > 4    | 3 > 4    | 3 ≤ 4 > 5 | 3 ≥ 4 = 5 | 4 > 3 = 5 |

* Trimer through octamer, solvent system 1; last column, solvent system 2; R = reaction; NR = no reaction; numbers indicate bond.

**Fig. 9. Paper chromatograms of products produced from tetramer, pentamer, hexamer, and heptamer by enzymes P₂, P₃, and P₄.** Solvent system 1. A, monomer; B, unsaturated dimer; C, dimer; D, unsaturated trimer; E, trimer; F, unsaturated tetramer; and G, tetramer; sampling indicated in hours.

P₄, conversely, attacked tetramer at bond 3 only. The principal distinguishing characteristic for all substrates besides the trimers was the preference of enzymes P₁ and P₂ for attack at bond 2, as opposed to the preference of enzymes P₃ and P₄ for the interior bonds.

The action patterns of the four enzymes on the unsaturated uronides are not shown. All of the enzymes attacked unsaturated trimer only at bond 2. The degradation of unsaturated pentamer yielded two products, unsaturated dimer and unsaturated trimer. The same products could arise from attack at either bond 2 or 3 and no preference could be assigned. The degradation of unsaturated tetramer by enzymes P₁ and P₂ occurred at bond 2. Enzymes P₃ and P₄ also degraded unsaturated tetramer principally at bond 2, with lesser attack occurring at bond 3. Therefore, the presence of the double bond inhibits attack by the latter enzymes at the ad-
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Fig. 10. Comparison of the effect of two solvent systems on the migration of products obtained with octamer as the substrate. Top, solvent system 1; bottom, solvent system 2. Symbols used are identical to those in Fig. 9.

adjacent glycosidic bond since these enzymes preferentially attacked bond 3 of the saturated tetramer.

DISCUSSION

That four enzyme activities are separable by CMC column chromatography has been amply demonstrated. This, in turn, confirms the observation of Nagel and Vaughn (13), who also obtained four enzymes with the use of column chromatographic techniques. Since similar products appeared to be produced by the different enzymes and all of the enzymes seemed to require calcium ion, it was concluded by Nagel and Vaughn that they represent different forms of a single enzyme. No investigation was made of the cation requirements nor of the pH optima of the purified preparations.

The demonstration of four different pH optima ranging from 8.3 to 9.6 falls exactly in the range of pH optima reported for other lyases (4, 5, 10, 17). The observation that the pH optimum is markedly affected by storing the buffer-substrate-calcium chloride mixture for a period of time is difficult to explain. The changes apparently are correlated with the pH of the medium, since the effect seems to be greater at the higher pH values. The absorption of CO2 could result in the removal of calcium which, in turn, is necessary for enzyme activity. Other degradative changes such as migration of the carbonyl group at the reducing end of the molecule can occur under alkaline conditions. However, since the substrate has a chain length of 12 to 14 units, it is difficult to see how this type of change could have such a profound effect on the activity of
the enzymes except in the case of exo-lyases which attack from the reducing end of the molecule (9, 18). In any case, to avoid this problem the individual components were not mixed together until just before assay. In addition, the alkaline buffer solutions were stored in such a manner as to preclude absorption of CO₂.

Although all of the lyases which attack pectic acid have been shown to require calcium, the observations concerning the effect of other cations on activity have differed markedly depending upon the source and type of lyase. For instance, Nasuno and Starr (17) showed that the lyase of a Xanthomonas had a specific requirement for calcium. Strontium ion had no effect, whereas the same ion was at least partially effective in restoring the activity of an exo-lyase of Clostridium (9). The same effect was observed with the purified enzymes of B. polymyxa. Based upon the relative activity with strontium ion, the enzymes can be divided into two groups.

Calcium ion, which is required for activity of the enzymes, only affects the observed Kₘ values of the shorter chain uronides where equivalent concentrations of calcium (0.001 M) approached values less than unity when compared to the available carboxyl groups. These results are consistent with the results obtained with trimer as the substrate with enzyme P₂. The observed activity was proportional to the calcium ion concentration when the ratio of calcium ions to carboxyl groups remained below 0.5. Above this ratio, the effect of calcium was diminished. Thus, one can conclude that the enzymes actually attack the calcium salt of the oligogalacturonides. That the break in activity occurs at a molar ratio (calcium to carboxyl) of 0.5 rather than 1 suggests that salt-bridged substrates may be degraded.

Why an organism would produce pairs of enzymes (which seem to duplicate each other) raises the question of whether a protease is present which could produce fragments from the parent enzymes. Earlier research (14) has demonstrated that lysis of the cells of B. polymyxa occurs after the stationary phase of growth in an inorganic salts medium with pectin as the carbon source. However, extracellular enzyme activity did not decrease during lysis of the cells. To minimize the danger of release of proteolytic enzymes, growth was followed and the cells were removed by centrifugation at the end of the logarithmic growth phase.

Based upon (i) kinetic data, (ii) evidence obtained by paper chromatography of the products, and (iii) viscosity data, there is little doubt that enzymes P₁ and P₂ are endo-enzymes. These enzymes are similar to the endo-pectic acid lyase of Bacillus (5) with some minor exceptions, the most notable being that the latter enzyme appeared to be inactive toward the unsaturated trimer and attacked the saturated trimer only very slowly.

The results with enzymes P₁ and P₂ are not so clear. The paper chromatographic evidence would indicate an exo-type enzyme. Yet the Kₘ data and the viscosity data indicate that the enzymes are endo-enzymes. (In the case of the Kₘ data, it is assumed that chain length would have no influence on the binding of an exo-enzyme and thus the Kₘ would be constant.) Two conclusions are possible: (i) enzymes P₁ and P₂ consist of a mixture of an exo- and an endo-enzyme or (ii) they are endo-enzymes showing a preference for attack at bond 2.

An enzyme similar in action was described by Avigad and Bauer (2). Their levann-6-fructan-hydrolase (levan hydrolase) gave an essentially constant Vₘ and a decreasing Kₘ with an increasing substrate DP. No extensive data on either the Kₘ or Vₘ values were presented. Neither were the cleavage patterns indicating susceptible bonds presented. The action patterns from amylotriose and its tetramer, pentamer, and hexamer homologs degraded by salivary amylase indicated a marked preference for bond 2 from the reducing end of the molecule and the enzyme is an endo-enzyme (19, 20). This enzyme presented an action pattern similar to enzymes P₁ and P₂ in its preference for bond 2.

Perhaps the most convincing evidence for a homogeneous enzyme preparation is obtained from paper chromatography of the products formed from different substrates. From these results, it is apparent that enzyme P₂ would have to consist almost entirely of an exo-enzyme to account for the products observed. Yet the viscosity data and the Kₘ data indicate that this is not true. Thus, it is our conclusion that enzymes P₁ and P₂ are endo-enzymes which show a preference for attack at bond 2.

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