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Mode of Action of Pectic Enzymes

II. Further Purification of Exopolygalacturonate Lyase and Pectinesterase from *Clostridium multifermentans*[1]

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Exopolygalacturonate lyase and pectinesterase from *Clostridium multifermentans* were purified 156-fold and 178-fold, respectively, by gel filtration chromatography on Sephadex G-200. The activities of both enzymes coincided in a single protein peak. Profiles of the two activities also coincided in diethylaminoethyl-cellulose chromatography and zonal centrifugation. These studies indicated that the esterase and the lyase were either complexed or similar molecular species. The former seems more probable because of the relatively high molecular weight. Both activities were most stable at *p*H 6.0. The esterase was inactivated rapidly at *p*H 5 or 7. Lyase preparations were freed of pectinesterase activity by heating for 30 min at 38 C and *p*H 7.0.

Clostridium multifermentans ferments polygalacturonic acid and produces exopolygalacturonate lyase (EC 4.2.99.3) and pectinesterase (EC 3.1.1.11). Previous studies (5) showed that the lyase degrades polygalacturonate by removing units of an unsaturated digalacturonic acid from the reducing ends of substrate molecules. Highly esterified pectin cannot be degraded by this enzyme unless pectinesterase is also present to hydrolyze the methoxyl groups. These studies indicated that the terminally acting lyase might be useful as a tool in investigating the pattern of the action of tomato pectinesterase on molecules of highly esterified pectin. Reexamination of the methods previously developed for purification of the lyase (4, 6) showed, however, that clostridial pectinesterase was not consistently removed as reported. For example, a typical preparation contained 1.4 units of lyase and 0.25 units of esterase. Although the amount of residual esterase activity was small, it was sufficient to interfere in the studies planned with tomato pectinesterase.

This prompted a reinvestigation of methods for the purification of clostridial exopolygalacturonate lyase. Purification was directed toward two goals. First, it was necessary to produce lyase preparations that were completely free of pectinesterase. The second objective was to develop methods for purification of the clostridial esterase, so that a comparison could eventually be made between the mode of action of this enzyme and the one from tomatoes. In addition, this paper describes several properties of clostridial pectinesterase and exopolygalacturonate lyase.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick.

MATERIALS AND METHODS

Organism and cultural conditions. The strain of *C. multifermentans* was generously supplied by R. H. Vaughn, Department of Food Science and Technology, University of California, Davis. General methods and media for production of exopolygalacturonate lyase and pectinesterase were as previously described (4, 6), except that cultures were incubated for 5 days at 21 C.

Substrates and enzyme assays. Pectin N.F. (no. 3442) and polygalacturonic acid (no. 3491) were obtained from Sunkist Growers Inc., Corona, Calif. Exopolygalacturonate lyase acting on 0.5% polygalacturonic acid was measured spectrophotometrically at 235 nm as previously described (4). One unit of activity is that amount of enzyme which produces 1 μmole of unsaturated digalacturonate per min at *p*H 8.0.

Pectinesterase activity was measured in a *p*H-stat with a 20-ml reaction mixture containing 0.5% pectin N.F. and 0.05 M NaCl. The assay method was as previously described for tomato pectinesterase by Lee and Macmillan (2), except that the *p*H and temperature were maintained at 7.5 and 26 C, respectively. One unit of pectinesterase is that amount of enzyme releasing 1 μmole of carboxyl groups per min under the above conditions.

Specific activities for both enzymes are expressed in units per milligram of protein. Protein concentration was determined by the method of Lowry et al. (3), with bovine serum albumin as the standard.

Adsorbants and gels. Calcium phosphate gel was

prepared by the method of Kunitz (1). Diethylaminoethyl (DEAE)-cellulose (Mann Research Laboratories, 1475) was washed with acid and base, and then equilibrated in 1 M sodium acetate buffer, *p*H 5.5. Immediately prior to use, the gel was equilibrated in 0.05 M sodium acetate buffer, *p*H 5.5.

DEAE-, QAE-, CM- and SE-Sephadex; Sephadex G-75, G-100, G-150, G-200; and Sepharose 4B were obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J. All were prepared for use according to the directions supplied by the manufacturer. Specific conditions and other details of the methods used for column chromatography are reported in the section on results.

RESULTS

Carbon source for enzyme production. It was previously shown (6) that the lyase was produced when *C. multifermentans* was grown in media containing polygalacturonate or pectin N.F. Although pectinesterase was formed with polygalacturonate as the substrate, no information was available on the production of this enzyme with pectin N.F. The relative amounts of the two enzymes are similar no matter which pectic material was employed as the carbon source, and neither enzyme is produced in the presence of glucose alone (Table 1).

Stability of pectinesterase and exopolygalacturonate lyase at various pH values. From preliminary studies with these two enzymes, it was suspected that the esterase was less stable than the lyase. In the following experiment, enzyme stability was measured at different *p*H values.

Samples of crude enzyme, previously dialyzed overnight against 0.001 M $CaCl_2$, were adjusted to various *p*H values with dilute HCl or NaOH and incubated in a water bath at 38 C. This elevated temperature was arbitrarily chosen to accelerate inactivation and shorten the incubation period. Samples of each preparation were removed periodically and assayed for esterase and lyase activities. The results in Fig. 1 show that the lyase was

TABLE 1. *Production of pectinesterase and polygalacturonate (PGA) lyase by Clostridium multifermentans*

Substrate[a]	PGA lyase (units/ml)	Pectinesterase (units/ml)
Polygalacturonic acid..	0.34	0.43
Pectin N.F.....	0.15	0.18
Glucose.....	0	0

[a] Medium contained 0.5% yeast extract, 0.1% K_2HPO_4, 0.05% sodium thioglycolate, and 0.5% substrate. The *p*H was 7.3. After incubation for 6 days at 21 C, the cells were removed, and the broth was analyzed for enzyme activities.

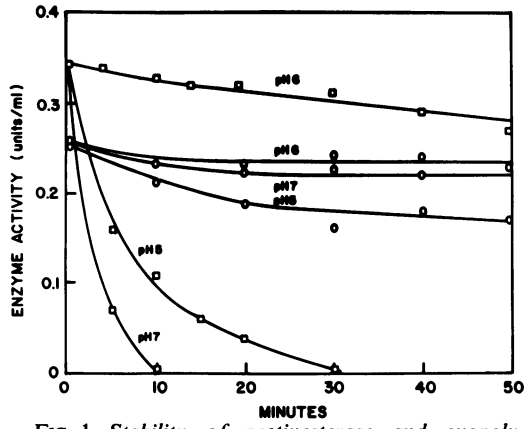

FIG. 1. *Stability of pectinesterase and exopolygalacturonate lyase at various pH values. The temperature was 38 C. Symbols:* □, *pectinesterase;* ○, *exopolygalacturonate lyase.*

relatively stable between *p*H values of 5 to 7. In contrast, pectinesterase activity decreased rapidly with time at *p*H values of 5 or 7. The esterase was most stable at *p*H 6.0. The instability at other *p*H values explains the discrepancy found in repeating the original partial purification procedure. In the earlier work, crude enzyme solutions were routinely dialyzed at *p*H 8.0, and presumably this treatment rapidly inactivated the esterase. In repeating the purification, dialysis was conducted at *p*H 6.0, and considerably more esterase remained active.

Effect of $CaCl_2$ on lyase stability. It was generally observed that lyase preparations containing $CaCl_2$ remained active longer than those without this salt. This stability effect was studied at several *p*H values by heating samples of crude enzyme, containing various amounts of added $CaCl_2$, for 30 min at 38 C (Fig. 2). At all *p*H values tested, it was found that the lyase is most stable in 0.005 M $CaCl_2$. The activity of the original enzyme preparation was 0.21 unit/ml. Thus, many of the samples actually increased in activity after incubation in $CaCl_2$. Although divalent cations are required for lyase activity, these observed increases cannot be attributed to a lack of calcium in the original reaction mixture. It was previously shown (6) that 0.0005 M $CaCl_2$ was sufficient for maximal activity, and this amount was incorporated in all reaction mixtures.

In similar experiments, 0.001 M $MgCl_2$, $MnCl_2$, and $ZnCl_2$ were compared for ability to stabilize the lyase. $MnCl_2$ proved to be as effective as $CaCl_2$, whereas the other two salts had no effect.

Preliminary purification steps. The procedures employed were based on those reported earlier.

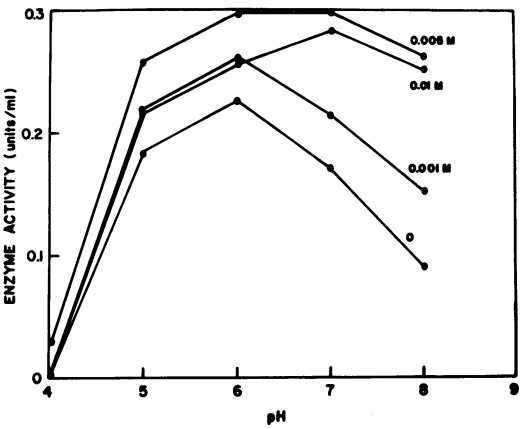

FIG. 2. *Effect of various amounts of $CaCl_2$ on stability of exopolygalacturonate lyase. Samples of crude enzyme which had been dialyzed overnight against distilled water were adjusted to various pH values and heated at 38 C for 30 min.*

Precautions were taken to keep the *p*H near 6.0 to minimize inactivation of either of the enzymes. Unless otherwise stated, all purification steps were conducted at 5 C.

Crude cell-free culture broth (5 liters) was filtered through celite, dialyzed for 24 hr against 0.001 M $CaCl_2$, and then 250 ml of calcium phosphate gel was added to adsorb the lyase and the esterase. The mixture was stirred for 10 min and centrifuged. Five hundred milliliters of 0.1 M potassium phosphate buffer (*p*H 6.0) was added to the packed gel, and the resulting slurry was mixed for 10 min to elute both enzymes. After centrifugation, the gel was suspended in 200 ml of the same buffer and centrifuged again. The two eluates were combined, dialyzed against distilled water, and lyophilized. The dry powder was taken up in 25 ml of distilled water and centrifuged to remove an insoluble residue. This preparation contained 43 units of exopolygalacturonate lyase per ml with a specific activity of 2.8, and 70 units of pectinesterase per ml with a specific activity of 4.5. At this stage in the purification reported earlier, there was no pectinesterase activity. Enzyme prepared as above was used in the following purification studies.

Gel filtration chromatography. Both enzymes were excluded from columns of Sephadex G-75, G-100, and G-150 but were retained slightly on Sephadex G-200. The elution pattern in Fig. 3 shows that the lyase and the esterase appeared together in a single peak. Fractions 70 to 88 were combined and lyophilized. The powder was taken up in 19 ml of distilled water and rechromatographed on Sephadex G-200 (Fig. 4). Once again the enzymes were in the same peak. The specific activities of the lyase and esterase in the combined fractions (70 to 85) increased to 50 and 48, respectively. A summary of this purification is shown in Table 2.

Since the active peak on Sephadex G-200 came off the column quite near the void volume, it appeared possible that a separation of the two enzymes might be obtained on Sepharose 4-B. This was not the case (Fig. 5). Since the specific activities of the eluate were not as high as with

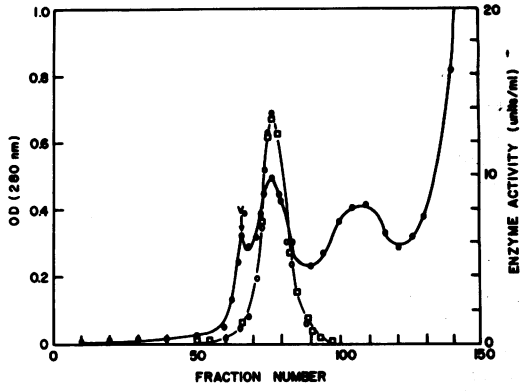

FIG. 3. *Ascending gel filtration of exopolygalacturonate lyase and pectinesterase on Sephadex G-200. Symbols: ●, protein, absorbance at 280 nm; ○, exopolygalacturonate lyase; □, pectinesterase. The sample (25 ml) was applied to the bottom of a column (5 by 86 cm) and eluted with 0.02 M potassium phosphate buffer (pH 6.0). The void volume was 650 ml. Fractions (10 ml) were collected with a flow rate of 40 ml per hr.*

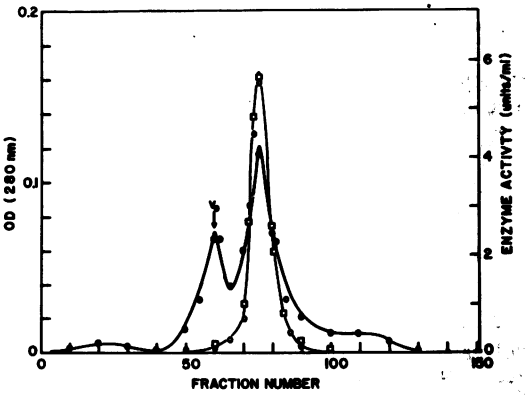

FIG. 4. *Second gel filtration of exopolygalacturonate lyase and pectinesterase on Sephadex G-200. Symbols: ●, protein, absorbance at 280 nm; ○, exopolygalacturonate lyase; □, pectinesterase. Conditions are as stated in the legend to Fig. 3. The sample (10 ml) was a portion of the concentrated material from pooled fractions 70 to 88 in the first gel filtration.*

TABLE 2. *Purification of pectinesterase and exopolygalacturonate lyase*

Fraction	Vol	Total protein	Exopolygalacturonate lyase			Pectinesterase		
			Total units	Specific activity	Recovery	Total units	Specific activity	Recovery
	ml	*mg*		*units/mg*	%		*units/mg*	%
Dialyzed fermentation broth	5,000	6,250	2,000	0.32	100	1,700	0.27	100
Eluate from calcium phosphate gel	700	383	1,250	3.3	63	1,660	4.3	98
Concentrated eluate	25	388	1,080	2.8	53	1,750	4.5	103
Eluate from Sephadex G-200	190	59	1,260	21.3	63	1,350	22.9	79
Concentrated eluate	19[a]	42	1,100	26.2	55	1,375	32.7	81
Eluate from Sephadex G-200	160	8	400	50.0	38	384	48.0	43

[a] Concentrated eluate (10 ml) was applied to the second G-200 column. Values for volume, total protein, and total units reported in the final step are those actually found, but the per cent recovery has been corrected to correspond to 19 ml.

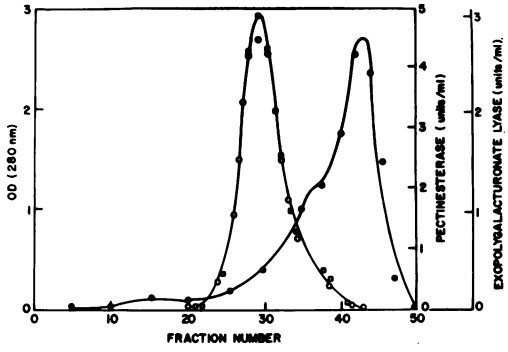

FIG. 5. *Elution profile for ascending gel filtration on Sepharose 4-B. Symbols:* ●, *protein, absorbance at 280 nm;* ○, *exopolygalacturonate lyase;* □, *pectinesterase. The sample (3 ml) containing 33 units of exopolygalacturonate lyase and 40 units of pectinesterase per ml was applied to the bottom of a column (2.5 by 35 cm) and eluted with 0.02 M potassium phosphate buffer, pH 6.0. The void volume was 60 ml. Fractions (4 ml) were collected with a flow rate of 20 ml/hr.*

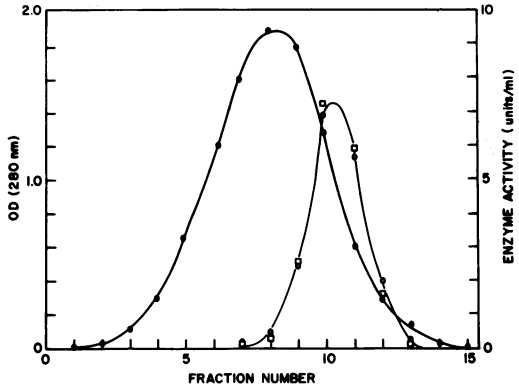

FIG. 6. *Rate-zonal sedimentation profile. Symbols:* ●, *protein, absorbance at 280 nm;* ○, *exopolygalacturonate lyase;* □, *pectinesterase. The sample (20 ml) contained 30 units of pectinesterase and 28 units of exopolygalacturonate lyase per ml. Fractions (30 ml) were collected.*

Sephadex G-200, this procedure was not incorporated into the purification scheme.

Zonal centrifugation. The behavior of pectinesterase and exopolygalacturonate lyase in gel filtration chromatography suggested that they had molecular weights sufficiently high for separation by zonal centrifugation. A partially purified preparation (20 ml) was pumped to an initial starting radius of 4 cm over a 400-ml gradient [10 to 30% (w/w) sucrose] in a B-XIV zonal rotor (International Equipment Co., Needham Heights, Mass.). After 16 hr at 35,000 rev/min, there was some separation from the major protein peak (Fig. 6), but once again the two activities coincided. Although specific activity for both the esterase and lyase increased about fourfold, there appeared to be no particular advantage for incorporating this procedure into the purification scheme.

Ion-exchange chromatography. A number of Sephadex ion exchangers, equilibrated with 0.05 M sodium acetate buffer (*p*H 5.4) or 0.05 M histidine-hydrochloride buffer (*p*H 6.5), were screened for ability to adsorb exopolygalacturonate lyase. Equal volumes of each of the swollen exchangers and a lyase preparation containing 2 units/ml were mixed, and the slurry was allowed to settle for 1 hr. The supernatant liquid was decanted and assayed for lyase activity. All of the activity was adsorbed by DEAE- and QAE-Sephadex A-50 at *p*H 5.4 and 6.5, and none was adsorbed by CM- or SE-Sephadex C-50.

An active fraction from the second column of Sephadex G-200 was chromatographed on col-

umns of DEAE-Sephadex and DEAE-cellulose. It was found that NaCl could be used to elute both the esterase and the lyase. Although numerous experimental conditions were investigated, the enzymes always appeared together in the same peak. An example of a typical elution pattern on DEAE-cellulose is shown in Fig. 7.

None of the attempts with ion-exchange chromatography were successful in increasing specific activities of either enzyme. In fact, pectinesterase was always partially inactivated. This inactivation was not necessarily directly caused by treatment with the ion-exchanger, since it was generally noted that the esterase was much more sensitive to inactivation after the second gel-filtration step. For example, the specific activity for pectinesterase in one preparation dropped from 48 to 14 during lyophilization, and then to 11 after chromatography on DEAE-cellulose. The specific activity of the lyase decreased only slightly during these operations.

Properties of clostridial pectinesterase. The enzyme preparation used in the following studies was partially purified by adsorption on calcium phosphate gel and contained 15.0 units of pectinesterase and 8.0 units of exopolygalacturonate lyase per ml. The activity of pectinesterase at various *p*H values was determined at 26 C in a reaction mixture containing 0.5% pectin N.F. and 0.05 M NaCl. The *p*H was automatically controlled with a *p*H-stat. Pectin N.F. undergoes some chemical deesterification at *p*H values above 7.0 (2). Rates for this chemical saponification were measured at various alkaline *p*H values and subtracted from those obtained enzymatically. The corrected values plotted in Fig. 8 show that the esterase activity increased steadily from *p*H 5 to 9 and then rapidly decreased between *p*H 9 and 9.5. Enzyme activities were difficult to measure at alkaline *p*H values, since the rate of deesterification decreased rapidly with time, probably a result of enzyme inactivation (Fig. 1). The points plotted in Fig. 8 are estimations of the rates during the first 2 min of each reaction. It was impractical to measure activity routinely at the apparent *p*H optimum of 9.0. Thus, a *p*H value of 7.5 was chosen for the standard assay as a compromise between highest amount of activity versus duration of the initial linear rate.

The effect of NaCl on the activity of the esterase is shown in Fig. 9. Maximal activity, occurring in 0.05 M NaCl, was twice that observed with no added salt. Thus, routine assays were always made in a reaction mixture with 0.05 M NaCl.

The activity of pectinesterase was measured at various temperatures between 22 and 45 C. Maximal activity occurred over a broad range (25 to 35 C). The rates were linear for a longer time near the lower end of this temperature range than at the upper end, and, on this basis, a temperature of 26 C was selected for routine assays.

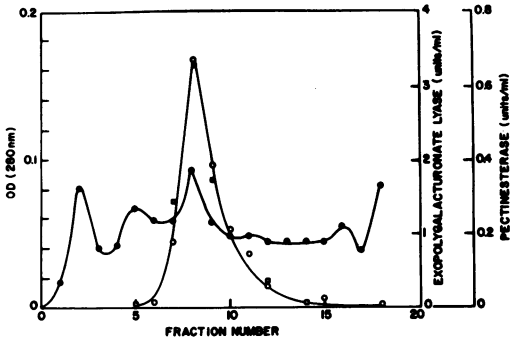

FIG. 7. *Elution profile for descending chromatography on DEAE-cellulose. Symbols:* ●, *protein, absorbance at 280 nm;* ○, *exopolygalacturonate lyase;* □, *pectinesterase. A concentrated sample (1 ml) containing a total of 30 units of exopolygalacturonate lyase and 10 units of pectinesterase was applied to the top of a column (0.9 by 4.5 cm) of DEAE-cellulose. The column was eluted in 6 steps of 12 ml each. The eluting solutions contained 0, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl in 0.5 M sodium acetate buffer (pH 5.5). Fractions (4 ml) were collected.*

DISCUSSION

The purification steps reported here were repeated several times and are quite reproducible. The largest yields of pectinesterase activity were obtained when the purification was conducted at low temperatures and enzyme preparations were kept at *p*H 6.0. The clostridial esterase is much

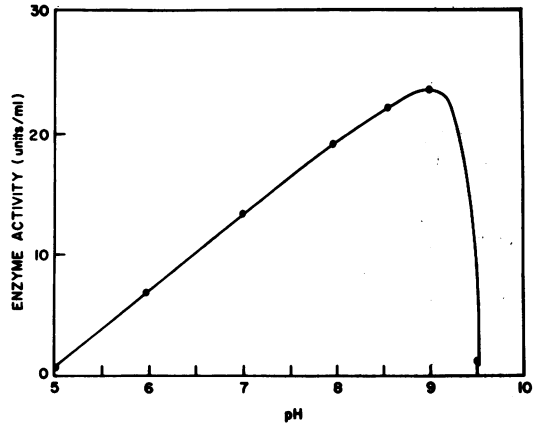

FIG. 8. *Effect of pH on the activity of pectinesterase. Enzyme reactions were continuously monitored in a pH-stat at 26 C. Initial rates were based on the first 2 min of each reaction.*

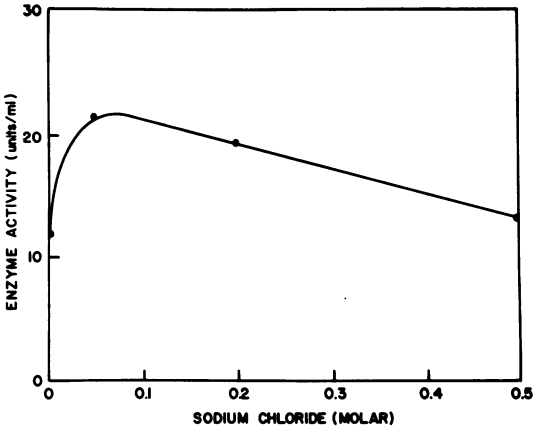

FIG. 9. *Effect of NaCl on pectinesterase activity. Various amounts of NaCl were added to reaction mixtures containing 0.5% pectin N.F. Enzyme activity was measured at pH 7.5 and 26 C.*

less stable than those produced by either *Fusarium oxysporum* f. sp. *vasinfectum* or tomatoes (Miller, Lee, and Macmillan, *unpublished data*). For example, the fungal enzyme remained completely active in the *p*H range of 5.0 to 6.5 when heated for 1 hr at 50 C.

Pectinesterase and exopolygalacturonate lyase activities were measured at various stages during the purification, and both were consistently found in the same fractions. The ratio of the two activities remained constant through many of the purification steps; inconstancies can generally be explained by differential inactivation. Furthermore, the shapes of all of the various chromatographic elution patterns and the rate-zonal sedimentation profile indicate that the two activities were not separated at all from one another by these methods. The coincidence of the two enzyme activities in rate-zonal sedimentations means that the two enzymes must have identical sedimentation coefficients. The increase in specific activity after each purification step was about the same for both enzymes, and the final overall purification was 156-fold for the lyase and 178-fold for the esterase.

There are several possibilities which would explain the inability to separate the two activities by any of the methods employed here. Conceivably the enzymes might exist as two distinct but very similar molecular species, or they could be two or more different species complexed with one another. A third possibility is that of a single molecular species with two separate active sites.

Both clostridial activities were excluded from Sephadex G-150 and were retained on Sephadex G-200. Sephadex G-150 excludes globular proteins with molecular weights of about 400,000 or higher. The pectinesterase from tomatoes (2) and the pectinesterase and polygalacturonate lyase from *F. oxysporum* (Miller and Macmillan, *unpublished data*) are all retained on Sephadex G-75 which retains globular proteins with molecular weights of about 70,000. The larger size of the clostridial enzymes supports the possibility that they are complexed.

In every culture of *C. multifermentans* tested, the amount of pectinesterase present was about equal to the amount of exopolygalacturonate lyase on the basis of total enzyme units. When pectin N.F. was used as the carbon source, considerably less of both enzymes was produced than when polygalacturonic acid was the growth substrate. These results might be expected for the lyase but not for the esterase. For example, *Xanthomonas campestris* produced esterase and lyase on pectin but only lyase on polygalacturonate (7). Although no direct evidence is presented here, it is possible that induction of the two clostridial enzymes is dependent on carboxyl groups rather than on methoxyl groups. If true, this is another indication of the similarity between these two enzymes which might be related to their being complexed.

The results presented here indicate that the clostridial pectinesterase is different from other pectinesterases, and that part of this difference may result from its being complexed with the lyase. The active sites of the two enzymes, however, must be different, since the two activities do not have similar properties in heat and *p*H stability. Furthermore, $CaCl_2$ helps stabilize lyase activity but not esterase activity.

The initial goal of producing lyase preparations completely free of clostridial pectinesterase was not realized through these purification studies. We have, however, taken advantage of stability differences and produced lyase preparations in which the esterase was inactivated by heating for 30 min at 38 C and *p*H 7.0. Lyase prepared this way has been used successfully for investigating the action patterns of tomato pectinesterase (Lee and Macmillan, *in preparation*) and clostridial pectinesterase (Lee, Miller, and Macmillan, *in preparation*). In contrast to the tomato enzyme, the clostridial esterase exhibits an action pattern very similar to exopolygalacturonate lyase; i.e., it hydrolyzes ester linkages in linear sequence beginning from the reducing end of pectin chains. Thus, a physical association or complexing of the two activities might accelerate pectin degradation by facilitating an alternation of deesterification with cleavage of glycosidic bonds.

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