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Partial Purification and Characterization of a Polysaccharide Depolymerase Associated with Phage-Infected *Erwinia amylovora*

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***Erwinia amylovora* infected with bacteriophage ERA103 produced an enzyme which degraded the extracellular polysaccharide of noninfected cells. The depolymerase enzyme was purified 15-fold by a procedure which included ammonium sulfate precipitation, ultracentrifugation, CM-Sephadex batchwise separation, Sephadex G-50 column chromatography, and Sephacryl S-200 column chromatography. The enzyme had a molecular weight of approximately 21,000 and a pH optimum of 6.0. Activity was enhanced by supplements of 2-mercaptoethanol or dithiothreitol.**

Polysaccharide depolymerases have been demonstrated in *Pseudomonas aeruginosa* (3), *Klebsiella* sp. (11, 12), *Alcaligenes faecalis* (13), *Enterobacter aerogenes* (18), and *Rhizobium trifolii* (7) upon infection with bacteriophages. All of these are encapsulated bacteria. Phage infection results in the induction of enzymes that degrade the capsular polysaccharide of the host.

There is a preliminary report that *Erwinia amylovora*, the fireblight pathogen of rosaceous plants, also produces a phage-associated enzyme that degrades the extracellular polysaccharide of noninfected cells (6).

Because little is known about the ecological role of this phage-encoded enzyme, the purpose of this study was to isolate the bacteriophage and purify and characterize a polysaccharide depolymerase from phage-infected *E. amylovora* NCPPB 595.

The bacteriophage was isolated from fire blight-infected apple and pear tissue obtained from Michigan, Montana, Colorado, Illinois, and Washington. Enrichment for bacteriophage was done as previously described (16). Although several bacteriophages were isolated, those purified produced a turbid halo around the plaque. The phages were further purified by three successive plaque pickings, and high-titer lysates were obtained by adding phages at a ratio of 1:10 phage/CFU. After incubation for 3 to 4 h at 25°C, the preparations were chloroform treated and stored at 4°C.

The phage isolate that was purified further was designated phage ERA103, according to the guidelines of Ackermann et al. (1). Phage ERA103 was concentrated by the polyethylene glycol procedure of Yamamoto and Alberts (17).

Many phages were isolated from the different *E. amylovora* strains. These results were similar to those of Richie and Klos (10). Host-range studies showed no differences among the four plaques with turbid halos. Phage ERA103 produced a clear plaque of 1 to 3 mm, surrounded by a turbid halo of 5 to 7 mm. *Erwinia herbicola* M232A was insensitive to all the phages. The purification of ERA103 by plaque picking, lysate production, and polyethylene glycol precipitation yielded 10 ml of high-titer lysate (10^{11} to 10^{12} PFU/ml) per 100 ml of culture. Pellets from the polyethylene glycol treatment contained more than 99% of the total PFU.

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Depolymerase activity in phage-infected bacteria was produced in the following manner. Bacterial cells were grown in 100 ml of N broth (15) supplemented with 0.25% yeast extract for 18 h at 25°C on a New Brunswick shaker at 250 rpm. This culture was used to inoculate 2,000 ml of N broth supplemented with 0.25% yeast extract. This was propagated at 25°C and 250 rpm. Early-log-phase cells (4.0×10^8 CFU/ml) of this culture were infected with phage at a multiplicity of infection of 2. Incubation was continued for 10 h. The lysates were stored at 4°C for 11 h and then processed for purification.

The polysaccharide depolymerase was partially purified by the five-step process described in Table 1. Initial studies indicated that the depolymerase activity was associated only with phage-infected cells. No enzymatic activity was detected in either the stock phage preparations or in noninfected *E. amylovora* NCPPB 595. The enzyme was precipitated at 45% $(NH_4)_2SO_4$ saturation. Other depolymerases isolated from *Klebsiella* sp. (11, 12) and *E. aerogenes* (18) can also be precipitated with ammonium sulfate. Ethanol precipitation was also attempted but resulted in a diminished yield. The ultracentrifugation procedure demonstrated that the activity was present in the supernatant and that no activity was associated with the pellet. The recovered enzyme was treated with CM-Sephadex C-50 in a batchwise procedure followed by gel filtration with Sephadex G-50 and Sephacryl S-200. Attempts to elute the enzyme from anion-exchange columns were unsuccessful. The depolymerase purified from *P. aeruginosa* is also difficult to purify from anion exchangers (3). Several protein peaks absorbing 280-nm light were observed in the Sephadex G-50 elution profile; depolymerase activity was associated with only one of them. Two protein peaks were observed in the elution profile of the Sephacryl S-200 column (data not shown).

The S-200 fraction exhibited activity from pH 3.0 to 8.0, with optimal activity at pH 6.0 (determined by using citrate and Tris hydrochloride buffers). Enzymatic activity was stable after storage at −20°C for several weeks. The optimal temperature for activity was 30°C (tubes were preincubated for 10 min before addition of the substrate and then further incubated during the reaction period).

One of the products of the enzymatic reaction was galactose, as determined by the paper chromatographic technique

TABLE 1. Summary of partial purification of depolymerase from phage-infected *E. amylovora* NCPPB 595[a]

Step	Vol (ml)	Enzyme units/ml[b]	Total enzyme units	Protein (mg/ml)	Enzyme sp act[c]	% Recovery	Purification (fold)[d]
45% $(NH_4)_2SO_4$ precipitate	30	528	15,840	2.4	220	100	
82,000 × *g* supernatant	30	361	10,830	1.4	258	68	1.2
CM-Sephadex C-50 batchwise	15	250	3,750	0.6	417	23	1.9
Sephadex G-50 column	12	277	3,324	0.12	2,309	21	10.4
Sephacryl S-200 column	3	333	999	0.10	3,330	6	15.0

[a] All fractionation steps were performed at 4°C, with reduction in volume of fractions accomplished with Carbowax 20. The active lysate from 2 liters of phage-infected cells was centrifuged at 8,000 × *g* for 30 min to remove bacterial cells. Ammonium sulfate was added slowly to the supernatant to give a final concentration of 45% saturation and precipitated for 18 h. The ammonium sulfate precipitate was centrifuged at 82,000 × *g* for 2 h. The supernatant from the ultracentrifugation step was then subjected to a batchwise separation with CM-Sephadex C-50 equilibrated in 0.01 M citrate buffer (pH 6.0) containing 0.01 M 2-mercaptoethanol. The slurry was filtered through a Büchner funnel, washed with buffer, dialyzed, and concentrated. The concentrated preparation was applied to an ascending column (2.6 by 65 cm) of Sephadex G-50 equilibrated with 0.01 M citrate buffer (pH 6.0) containing 0.01 M 2-mercaptoethanol. The concentrated post-G-50 fraction was then chromatographed on an ascending column (2.6 by 75 cm) of Sephacryl S-200 equilibrated with 0.01 M citrate buffer (pH 6.0) containing 0.01 M 2-mercaptoethanol.

[b] One unit is the amount of enzyme required to produce 1 μmol of galactose equivalent per min under standard assay conditions. Depolymerase activity was assayed by following the release of galactose from the polysaccharide substrate by the method of Fairbridge et al. (5). Polysaccharide was prepared from uninfected cultures of *E. amylovora* NCPPB 595 cultivated on sheets of cellophane overlaying tryptic soy agar, as described by Liu et al. (9).

[c] Units per milligram of protein, determined by the microbiuret method of Koch and Putman (8).

[d] Because of interfering substances, depolymerase was not assayed in crude lysates; any purification achieved by the initial ammonium sulfate fractionation is therefore not included.

of Trevelyan et al. (14). The capsule of *E. amylovora* has been reported to be composed of 61 to 69% galactose (4).

The molecular weight of depolymerase was estimated by ascending gel filtration chromatography with Sephacryl S-200. A molecular weight of 21,000 was obtained (Fig. 1). The molecular weights of other depolymerases are as follows: *R. trifolii*, 440,000 (7); *E. aerogenes*, 379,000 (18); *P. aeruginosa*, 180,000 (3); and *A. faecalis*, 48,000 (13).

The effect of various inhibitors on enzyme activity is shown in Table 2. The enzyme was insensitive to a variety of agents such as sodium azide, potassium cyanide, EDTA, ethylene-di-(*o*-hydroxyphenylacetate), and 8-hydroxyquinoline. Inhibitors such as *p*-chloromercuribenzoate, 2-mercaptoethanol, and dithiothreitol that affect sulfhydryl groups had different effects on the enzymatic activity. However, other sulfhydryl group alkylators such as *N*-ethylmaleimide and iodoacetate had no effect on enzymatic activity. Many of the reported depolymerase enzymes appear to be insensitive to a variety of chemical agents; however, the enzyme from *R. trifolii* was inhibited by EDTA (7).

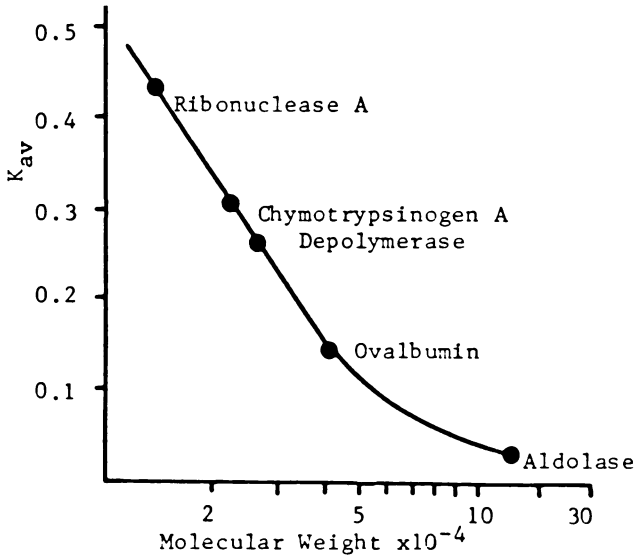

FIG. 1. Molecular weight determination of depolymerase from phage-infected *E. amylovora*. The enzyme was eluted by ascending gel filtration on a column (2.6 by 75 cm) of Sephacryl S-200 in 0.01 M citrate buffer (pH 6.0) containing 0.01 M 2-mercaptoethanol. The elution volume of the enzymatic activity was compared with the elution of volumes of standard proteins (RNase A, chymotrypsinogen A, ovalbumin, and aldolase). $K_{av} = (V_e - V_o)/V_t - V_o)$, where V_e is the elution volume for the protein, V_o is the elution volume, for Blue Dextran 2000, and V_t is the total bed volume.

Enzymes that degrade capsular polysaccharide have been previously described (2, 3). Many of the earlier reports described two polysaccharide depolymerases, one diffusable and the other phage associated (2). In our preparations, we were unable to detect depolymerase in the phage particle itself. The functional role of these phage-encoded depolymerase enzymes remains hypothetical. However, Sutherland and Wilkinson (12) suggest that the enzyme assists in the release of phage particles from infected cells and thus enables the phage to infect a greater number of cells.

TABLE 2. Effect of various chemical agents on depolymerase activity

Agent	Concn (mmol/liter)	Activity (% control)[a]
p-Chloromercuribenzoate	10.0	0
	1.0	0
	0.1	0
Iodoacetate	10.0	100
	1.0	100
	0.1	100
N-Ethylmaleimide	10.0	100
	1.0	100
	0.1	100
2-Mercaptoethanol	10.0	168
	1.0	155
	0.1	135
Dithiothreitol	10.0	258
	1.0	138
	0.1	122

[a] Percent activity is based on a control assay without inhibitors.

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