Purification and Properties of Polysaccharide Depolymerase Associated with Phage-infected *Pseudomonas aeruginosa* 

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**SUMMARY**

A polysaccharide depolymerase was purified 1,688-fold from crude lysates produced by the propagation of a specific bacteriophage in *Pseudomonas aeruginosa*. Conventional methods of protein fractionation were employed, namely, salting out with ammonium sulfate, Sephadex gel filtration, and high speed centrifugation. The purified depolymerase behaves as a single entity when tested by electrophoresis in acrylamide gel. On the basis of gel filtration data the molecular weight was estimated to be 180,000. Maximal enzymatic activity was found at pH 7.5. Polysaccharide depolymerases have been reported in various phage-infected bacteria (1–3), and recently it was observed that *Pseudomonas putida* also produces a depolymerase when infected by phage (4). A system consisting of phage-infected *Pseudomonas aeruginosa* was reported to produce a polysaccharide depolymerase (5), and reaction of this enzyme with polysaccharide resulted in decreased viscosity, as well as in measurable increases in the levels of hexosamines, hexoses, and reducing substances, thereby distinguishing it from other known phage-associated depolymerases. Although a partial purification of the enzyme has been described (5), a substantial purification of the enzyme is necessary in order to clearly characterize the enzyme and thus permit further study of its properties, mechanism of action, and possible role in the virus life cycle.

The present article describes the purification of a polysaccharide depolymerase obtained from phage-infected cultures of *P. aeruginosa*, as well as some of the properties of this enzyme.

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**EXPERIMENTAL PROCEDURE AND RESULTS**

**Bacterial Materials**

*P. aeruginosa* strain BI and *Pseudomonas* phage 2, which have been previously described (5), were used in this study. Trypticase soy broth and agar were employed for bacterial cultivation and phage propagation. Bacteriophage titrations were performed by the soft agar layer method and the drop method as described by Adams (6).

**Chemicals**

Trypticase soy agar and broth were purchased from Baltimore Biological Laboratory, Baltimore, Maryland. A standard of n-glucosamine hydrochloride, obtained from Eastman, was included in each hexosamine determination. Crystalline bovine albumin, obtained from Nutritional Biochemicals, was used in the construction of a standard curve in protein determinations based on the method of Lowry et al. (7). Bovine serum albumin, Fraction V, used in experiments dealing with molecular weight determinations, was purchased from Pentex Inc., Kankakee, Illinois. Alcohol dehydrogenase and bovine y-globulin were purchased from Sigma. Sephardex G-200 was obtained from Pharmacia; polyethylene glycol, from Union Carbide Chemicals Company, New York; collodion sac, from Schleicher and Schuell Company, Keene, New Hampshire; acrylamide and vertical gel electrophoresis cell, from E. C. Apparatus Company, Philadelphia, Pennsylvania.

**Substrate**

Polysaccharide was prepared from uninfected cultures of *P. aeruginosa* strain BI cultivated on sheets of cellophane overlaying Trypticase soy agar as previously described (5). Briefly, polysaccharide was extracted from the slime layer in 0.15 M sodium chloride, precipitated by the addition of an ethyl alcohol-acetone mixture (equal volumes), dialyzed, and lyophilized.
Phage-associated Polysaccharide Depolymerase

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2078 Phage-associated Polysaccharide Depolymerase units.

Purification of polysaccharide depolymerase from phage-infected P. aeruginosa. Details are presented in text. PFU, plaque-forming units.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
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<td>5,382</td>
<td>10,168</td>
<td>1.9</td>
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<td>100</td>
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<tr>
<td>2. Ammonium sulfate</td>
<td>10</td>
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<td>95.8</td>
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<tr>
<td>3. Sephadex G-200</td>
<td>52</td>
<td>1.66</td>
<td>2,207</td>
<td>1,330</td>
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<td>22</td>
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<tr>
<td>4. Sephadex G-200</td>
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<td>0.66</td>
<td>2,112</td>
<td>1,200</td>
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</tbody>
</table>

Fig. 1. Elution profile from Sephadex G-200 column chromatography. Details are presented in text. PFU, plaque-forming units.

Estimation of Enzyme Activity

This procedure was previously described (5) and only a brief description is presented here. Depolymerase activity was measured by the release of hexosamine from polysaccharide substrate. The amount of hexosamine was determined at various time intervals, after stopping the reaction by chilling, by the method of Rondle and Morgan (8) as modified by Boas (9). The unit of enzyme activity was expressed in terms of nanomoles of hexosamine released per min per mg of protein.

Source of Depolymerase

The appearance of the polysaccharide depolymerase in cultures of P. aeruginosa strain BI after infection with phage 2 has been described (5). Large quantities of crude enzyme were obtained from soft agar layer cultures of phage-infected cells which were harvested in 0.02 M sodium phosphate buffer, pH 7.5, and centrifuged at 16,300 x g for 30 min. The supernatant fluids containing crude enzyme and phage were usually frozen at -20°C.

Purification of Depolymerase

All manipulations were conducted at 0-4°C, except as otherwise noted.

Step 1. Crude Lysate—After thawing, the crude depolymerase preparation was centrifuged at 16,300 x g for 30 min to sediment a fine precipitate which formed. The formation and elimination of this precipitate did not affect enzyme or plaque titers of the lysate. The titer of bacteriophage in the supernatant fluid was 5 x 10⁶ plaque-forming units per ml. Other characteristics of the crude lysate are shown in Table I.

Step 2. Ammonium Sulfate Fractionation—Before ammonium sulfate fractionation took place, the crude lysate was centrifuged twice at 78,480 x g for 90 min. Solid ammonium sulfate was added slowly with stirring to the supernatant fluids to give a final concentration of 30% of saturation, and the solution was allowed to remain at 4°C overnight. The very fine precipitate which developed was sedimented at 78,480 x g, and the supernatant fluid was drawn off, then brought to 45% of saturation with the addition of solid ammonium sulfate. After remaining at 4°C overnight the precipitate was sedimented at 34,800 x g for 90 min, resuspended in 16 ml of 0.005 M sodium phosphate buffer, pH 7.5, and dialyzed against the same buffer. As shown in Table I, ammonium sulfate fractionation resulted in a purification of 50-fold. At this stage the concentration of bacteriophage was considerably decreased to 6.5 x 10⁵ plaque-forming units per ml.

Step 3. Passage through Sephadex G-200—The protein solution (16 ml) was concentrated in a collodion sac by ultrafiltration (10) against 0.005 M sodium phosphate buffer, pH 7.5, to a volume of 5 ml and placed on a Sephadex G-200 column of 2.5 x 83 cm, with a bed volume of approximately 460 ml. The Sephadex column was prepared by equilibration with 0.005 M sodium phosphate buffer, pH 7.5, and elution was accomplished at a flow rate of 7 drops per min in the same buffer. The depolymerase activity was present in 52 ml of the effluent which followed the void volume, and a 702-fold purification was achieved. A major portion of phage activity (7.0 x 10⁵ plaque-forming units per ml) was eluted in those fractions preceding the depolymerase peak; however, because of some overlapping, a depolymerase activity. The elution profile is illustrated in Fig. 1.

Electrophoretic Analysis

After the second passage through Sephadex G-200 in Step 4 of the purification procedure, enzymatically active Fractions 18, 19, and 20 were pooled and concentrated by dialysis against a suspension of polyethylene glycol, then further concentrated in a collodion sac to 0.01 the original volume. The protein sample (approximately 200 µg/0.1 ml) in 5% sucrose was carefully placed into a vertical gel electrophoresis cell. In a separate slot, bovine albumin was run as a control. The procedure followed was essentially that described by Raymond (11). The results are presented in Fig. 2 which shows the electrophoretic pattern obtained in acrylamide gel. Only one band was discernible and it was located approximately 3 cm from the origin, or about halfway between the origin and the albumin front. To determine if all the enzyme activity was present in the position of the single band, corresponding segments of unstained acrylamide gel were cut off, homogenized in 1 ml of 0.016 M sodium phosphate buffer, pH 8.0, and tested for depolymerase activity. All enzyme activity was found to be present in that segment corresponding to the position of the single band.
Properties of Enzyme

The molecular weight of the purified depolymerase was determined by the gel filtration technique described by Andrews (12). This was accomplished by comparing the effluent volume of the enzyme from a Sephadex G-200 column with that of several proteins with established molecular weights. The molecular weights of the proteins used in standardizing the column were taken from the literature as follows: bovine serum albumin, Fraction V, 67,000 (13); alcohol dehydrogenase, 126,000 (12); and bovine γ-globulin, 169,000 (14). The column of Sephadex G-200, 2.5 × 91 cm, was washed with 0.1 M KCl in 0.05 M Tris buffer at pH 7.6. A 1.3-ml sample containing 200 µg of each protein was added to the top of the column and washed through with the KCl-Tris solution at a flow rate of 25 ml per hour at room temperature (20-22°C). Fractions, 1 ml each, were collected and proteins were estimated spectrophotometrically at 215 nm as recommended by Andrews (12). Those fractions containing absorbing material were assayed for depolymerase activity. The molecular weight of the enzyme was determined by comparing its elution volume with those of several proteins of known molecular weight. As shown in Fig. 3, a linear relationship existed between the effluent volumes and the logarithm of the molecular weights of the proteins tested. The curve was extrapolated to include the experimental point for the effluent volume of the polysaccharide depolymerase. From the position of this point, a molecular weight for the enzyme of 180,000 was calculated.

The enzymatic activity of the purified depolymerase as a function of pH indicated a maximal activity at pH 7.5. These results are similar to determinations previously obtained with partially purified enzyme (5).

The effect of heat treatment on the purified depolymerase in 0.1 M sodium phosphate buffer, pH 7.5, showed that approximately 50% of the enzyme activity was lost after exposure at 70°C for 5 min.

Discussion

A number of hydrolytic enzymes produced by phage-infected bacteria are now known. These have proven to be valuable tools for investigating anatomical relationships, and, as an adjunct to chemical methods, they provide a means of studying composition and structure of the bacterial cell. In addition, the phage-associated depolymerases may be extremely useful in studying bacterial and other polysaccharides, since purely chemical attack often results in excessive hydrolysis, which does not permit isolation of structurally informative fragments. Although several polysaccharide depolymerases have been isolated from phage-infected systems, their usefulness has been restricted because the only detectable feature of their activity has been a decrease in viscosity of polysaccharide substrates. The mode of action of these depolymerases is unknown. The lack of diffusible products following enzyme action, together with the decrease in viscosity, would suggest that some internal linkage is broken, although the products of the cleavage must still be relatively large (3). More promising, when reacted with polysaccharide obtained from the slime layer of P. aeruginosa, the depolymerase associated with phage-infected P. aeruginosa has been reported to release hexosamines, hexoses, and reducing substances (5). Based on this, a method of assay was described (5) which proved to be useful in studying the kinetics of enzyme formation following phage infection of a sensitive host cell.

The Pseudomonas phage depolymerase described in this article was purified 1,688-fold from crude lysates produced by the propagation of specific bacteriophage in cultures of P. aeruginosa. Conventional methods of protein fractionation were satisfactorily employed, namely, salting out with ammonium sulfate, Sephadex filtration, and high speed centrifugation. When tested by electrophoresis in acrylamide gel, the purified enzyme appeared as a single band in which all enzymatic activity was localized. The determination of depolymerase activity as a function of pH indicated a maximum at pH 7.5. On the basis of gel filtration data, this enzyme was found to have a molecular weight of 180,000. The technique of employing gel filtration for molecular weight determinations was reported by Whitaker (15) and Andrews (12) to be highly accurate for most proteins.

Like the Pseudomonas enzyme, the depolymerases isolated from the Escherichia coli and Klebsiella phage systems were

![Fig. 2. Electrophoresis of purified polysaccharide depolymerase in acrylamide gel.](http://www.jbc.org/)

![Fig. 3. Determination of the molecular weight of the polysaccharide depolymerase by gel filtration on a Sephadex G-200 column (2.5 × 91 cm) calibrated with proteins of known molecular weights.](http://www.jbc.org/)
found to be precipitable between 40 to 50% of saturation of ammonium sulfate. However, attempts to elute the *Pseudomonas* depolymerase from columns of DEAE-cellulose with increasing concentrations of sodium chloride proved unsatisfactory, a method used successfully by Sutherland and Wilkinson (3) in the partial purification of the *Escherichia coli* phage depolymerase. With regard to pH, a rather broad maximum was reported for the *Azotobacter* depolymerase, pH 7.5 to 8.5, with a sharp drop as the pH decreased below 7. The depolymerase isolated by Adams and Park (1) from a *Klebsiella* phage system was found to retain 10% of the original enzyme activity after exposure at 70° for 30 min. In the *Escherichia coli* phage system studied by Sutherland and Wilkinson (3), all measurable activity was destroyed after 5 min at 70°, and in the *Pseudomonas* system described in this paper 50% of the purified depolymerase activity was destroyed after treatment at 70° for 5 min. Although the heat inactivation experiments of the latter two systems were performed under comparable conditions, the data are difficult to equate because of differences in the state of purity of the enzymes. This was quite evident within the *Pseudomonas* system itself in which a partially purified depolymerase preparation retained approximately 90% of its initial activity after treatment at 70° for 5 min, whereas more highly purified preparations were significantly less heat-stable as indicated above.

At the present time, it is reasonable to assume that the *Pseudomonas* depolymerase was synthesized in the bacterial cell at the direction of a specific phage genome. The enzyme cannot be detected in uninfected bacteria or in lysates of the same host cell when infected by other phage (3). Preliminary data, based on fractionation by differential centrifugation and column chromatography, suggest that the enzyme may exist in two forms: (a) a freely diffusible protein, and (b) firmly attached to the phage particle. The reports by Adams and Park (1) and Ekland and Wyss (2) suggest similar possibilities in the systems studied by them.

A functional role of polysaccharide depolymerases associated with phage-infected bacteria remains to be ascertained. In addition, to supplying information for replication of DNA and virion proteins, other genetic information supplied by phage may provide for the synthesis of substances which influence the processes of adsorption, penetration, and release of phage from the infected cell. One may speculate, for example, that such enzymes confer selective advantages to the phage by facilitating adsorption onto encapsulated or slime-producing bacteria. Although it is generally agreed that the processes of adsorption, penetration, and release of phage from the infected cell are enzymatic, little is known to satisfactorily explain the mechanisms involved, and it is quite likely that more than one enzyme may be required to complete a single process.

REFERENCES

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