Catalytic and Molecular Properties of a Phage-induced Capsular Polysaccharide Depolymerase*

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SUMMARY

Infection of Aerobacter aerogenes with a phage isolated from raw sewage induces the de novo synthesis of a polysaccharide depolymerase active against the capsular polysaccharide of the host organism. The enzyme exists in two forms, soluble and phage-bound. The soluble form has been purified to apparent homogeneity, as judged by disc gel electrophoresis and molecular sieve chromatography, and possesses the following molecular properties: (a) sedimentation coefficient, 10.7 S; (b) Stokes radius, 86.2 Å; (c) molecular weight, 379,000; and (d) frictional ratio, 1.77. Treatment of polysaccharide with sodium dodecyl sulfate at 42–62°C results in dissociation to two apparently nonidentical subunits with molecular weights of 63,200 and 36,400.

Depolymerase is a highly specific glycanohydrolase which randomly attacks the galactosyl-α-1 → 3-galactose linkages of the capsular polysaccharide; one susceptible bond occurs in each tetrasaccharide repeating unit of the polymer. Depolymerase activity may be conveniently assayed by the release of reducing groups; the pH optimum of the enzyme in each tetrasaccharide repeating unit. Reduced C is digested by depolymerase at less than 1% of the rate of capsular polysaccharide. The smallest oligosaccharide that depolymerase will degrade is a dodecasaccharide, termed C, composed of three tetrasaccharide repeating units. Reduced C is digested by depolymerase at 1% of the rate of capsular polysaccharide and is degraded in a highly specific manner; only the galactosyl galactose linkage immediately adjacent to the terminal, nonreducing tetrasaccharide repeating unit is attacked.

The phage-bound and soluble depolymerases are identical with respect to their catalytic properties. Treatment of phage with either 8 M urea or 4 M guanidine-HCl results in quantitative solubilization of the phage-bound depolymerase activity. The solubilized enzyme is indistinguishable from the soluble enzyme isolated from cell lysates as judged by Sephadex G-200 column chromatography.

A common feature of phage infection of encapsulated bacteria is the induction of enzymes that degrade the capsular (or slime) polysaccharide of the host. Such enzymes, commonly termed polysaccharide depolymerases, have been examined from a number of phage-host cell systems (2–7). There is, however, an almost complete lack of information concerning the molecular properties of any of these enzymes. There is only one report of a polysaccharide depolymerase having been purified to apparent homogeneity (8). Furthermore, although it has long been demonstrated that these enzymes are endohydrolases that are, in general, active against only the exopolysaccharide of the host organism, their mode of action has for the most part remained obscure since studies have been conducted in the absence of specific information concerning the structures of their exopolysaccharide substrates. One exception is the study on phage-induced polysaccharide depolymerases active against Klebsiella aerogenes A3(S1) (type 54) slime polysaccharide, which has been structurally characterized (9, 10). Several of these enzymes were isolated and shown to cleave specifically the fucosylglucose linkages present in the slime polysaccharide (11, 12).

A strain of Aerobacter aerogenes previously has been described which produces a capsule polysaccharide composed of galactose, mannose, and glucuronic acid in a molar ratio of 2:1:1, respectively (13, 14). Structural analysis of this capsule polysaccharide has shown that it is composed of a linear sequence of tetrasaccharide repeating units of the following structure (15):

\[
\begin{align*}
3 \rightarrow & \text{d-Galactose} \xrightarrow{1α3} \text{d-mannose} \xrightarrow{1α3} \text{d-galactose} \xrightarrow{1α} \\
1 \rightarrow & \text{d-glucuronic acid}
\end{align*}
\]

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A bacteriophage, virulent for this strain of *A. aerogenes*, has been isolated and shown to induce the formation of a polysaccharide depolymerase active against this capsular polysaccharide (13). This enzyme has been shown to be a glycanohydrolase which specifically cleaves the galactosylgalactose linkages in the capsular polysaccharide (15). This report describes the purification and molecular properties of this phage-induced enzyme. In addition, further studies on the catalytic properties of this enzyme are presented.

**EXPERIMENTAL PROCEDURE**

**Organism and Growth Conditions**—The strain of *A. aerogenes* used in these studies was kindly supplied by the Department of Microbiology of this institution. Growth of the organism on solid media yields colonies which are highly mucoid due to the production of large amounts of capsular polysaccharide. When grown in synthetic medium containing [U-14C]glucose as the sole source of carbon, nearly 30% of the total radioactivity present in stationary phase cultures was accounted for as purified capsular polysaccharide (15). Detailed structural characterization of this polysaccharide is described in the accompanying paper (15).

For production of polysaccharide depolymerase, *A. aerogenes* routinely was grown in a chemically defined medium of the following composition (grams per liter): (NH₄)₂SO₄, 10; Na₂HPO₄, 10; KH₂PO₄, 3; K₂SO₄, 1; NaCl, 1; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.001; FeSO₄·7H₂O, 0.001; casamino acids, 0.5; and glucose, 10. Glucose was prepared as a 50% solution, sterilized separately, and aseptically added to the sterilized salts medium. Organisms were grown at 37° in New Brunswick fermentors with mechanical agitation and forced aeration.

Stock cultures of *A. aerogenes* were maintained at −15° in Trypticase soy broth containing 15% glycerol; vials containing 1 ml of bacterial suspension were grown in Trypticase soy broth overnight and plated on the same medium containing 2% agar. Single mucoid colonies were isolated and used to inoculate media for further studies.

**Bacteriophage**—A virulent bacteriophage for *A. aerogenes* was isolated as follows: 25 ml of raw sewage was clarified by passage through a bacterial filter and then was added to a flask containing 5 ml of 5-fold concentrated Trypticase soy broth (15 g/100 ml) and inoculated with 1 ml of a log phase culture of *A. aerogenes*. After 5 hours of incubation of 37° on a rotary shaker, cells were removed by centrifugation. A 10-ml aliquot of the supernatant fluid was diluted to 20 ml with 0.5% sterile Trypticase soy broth and inoculated with 1 ml of a log phase culture of *A. aerogenes*. Complete lysis occurred after incubation for 2 hours at 37°. Appropriate dilutions of the crude lysate were plated with *A. aerogenes* with the soft agar layering technique according to Adams (16). Several types of plaques were observed although the predominant morphology characteristically exhibited a translucent, depressed halo surrounding the clear zone of lysis (Fig. 1). This type of plaque morphology is formed characteristicallly by bacteriophage that are capable of inducing the synthesis of a capsular polysaccharide depolymerase in infected host cells (2, 4, 6, 17). A bacteriophage, designated as K-2 in the remainder of this paper, was purified by repeated single plaque isolation as described by Adams (16).

Cell lysates used in the preparation of phage K-2 or polysaccharide depolymerase were prepared routinely as follows: 9000 ml of synthetic medium were inoculated with 1000 ml of an overnight culture of *A. aerogenes*. After incubation for 75 to 85 min at 37°, phage K-2 was added at a multiplicity of infection of 1/25 and incubation was continued; lysis was usually complete within 2 hours (Fig. 2). The lysate was then chilled immediately to 4° and cell debris was removed by centrifugation.

Purified phage suspensions were prepared as follows: solid ammonium sulfate (50 g/100 ml) was added to a cell lysate; the resultant precipitate was dissolved in T2 buffer (see below) and centrifuged at 9000 × g for 30 min to remove insoluble material. The supernatant fluid from the previous step was centrifuged at 78,800 × g, the supernatant was discarded, and the pellet was resuspended in T2 buffer. To 4.5 ml of crude phage suspension were added 2.4 g of CaCl₂ and the suspension was centrifuged to equilibrium (36 hours) at 39,000 rpm in a Beckman SW 39 rotor. The phage were concentrated in a narrow band near the bottom of the tube and were collected by puncturing the bottom of the tube and allowing the contents to flow dropwise from the tube. Phage suspensions were stored at 4° in T2 buffer containing the following components (grams per liter): Na₂HPO₄, 3.0; KH₂PO₄, 1.5; NaCl, 4.0; K₂SO₄, 5.0; CaCl₂·6H₂O, 0.02; and MgSO₄·7H₂O, 0.2. The phage titer was stable for at least 1 year if the suspension contained more than 10⁹ phage per ml.

![Fig. 1. Plaque morphology of bacteriophage K-2 on Aerobacter aerogenes.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

1 Baltimore Biological Laboratories, Baltimore, Maryland.
Density Shift Experiments—Cultures of A. aerogenes were grown in synthetic medium lacking casamino acids; in heavy medium, D₂O replaced 97% of the H₂O. Cultures (20 ml) were grown in 125-ml flasks on a rotary-action shaker at 37° to a density of approximately 70 Klett units. The cells were then centrifuged at 78,800 × g for 2 hours. Supernatants were mixed with ammonium sulfate (4.75 g/10 ml), stirred for 1 hour, and then centrifuged at 78,800 × g for 30 min. Pellets were dissolved in 0.25 ml of 10 mM Tris buffer, pH 7.5, and dialyzed against 1,000 volumes of the same buffer for 100 min.

Prior to isopycnic equilibration centrifugation, 10 ml of each dialyzed sample was mixed with 3.35 ml of 10 mM Tris buffer, pH 7.1, containing 36 μg of catalase (internal marker). The resultant solutions were then mixed with 1.8 ml of saturated (at 4°) cesium chloride solution and centrifuged to equilibrium at 46,000 rpm for 63 hours in a Beckman SW 50 rotor. Fractions were collected by puncturing the bottoms of the tubes.

Depolymerase Assay—For routine assay of depolymerase, incubation mixtures contained the following components in a total volume of 0.25 ml: purified capsular polysaccharide (15), 0.12 mg (equivalent to 150 μmoles of uranic acid); sodium acetate buffer, pH 5.2, 25 μmoles; and appropriate dilutions of the enzyme. Incubation was conducted at 4° and the reaction was followed by removing 50-μl aliquots at appropriate intervals.

Each aliquot was assayed for reducing sugar by the method of Park and Johnson (18) with galactose as a standard. One unit of enzyme is defined as that amount which catalyzes the formation of 1 pmole of reducing sugar (galactose equivalents) per min under the conditions specified above and specific activity is defined as units per mg of protein.

Amino Acid Analyses—Amino acid analyses were performed with the Beckman model MS automatic amino acid analyzer. Two aliquots (240 μg each) of purified depolymerase were dialyzed exhaustively against distilled water and then evaporated to dryness. One sample was oxidized with performic acid for 1 hour at 4° (19). Both samples were then suspended in 1 ml of 6 N HCl which had been thoroughly flushed with Nz. Hydrolysis was carried out for 24 hours at 110° in evacuated, sealed Pyrex tubes; HCl was then removed under reduced pressure and the residues were dissolved in 0.01 N HCl prior to analysis. Tryptophan was determined spectrophotometrically (20) with 160 μg of purified depolymerase.

Spectrophotometric Determinations—All spectrophotometric analyses were carried out on a Gilford spectrophotometer. Ultraviolet absorption spectra were obtained with a model 15 Cary spectrophotometer.

Protein was estimated by the ultraviolet spectrophotometric method of Waddell (21).

Disc Gel Electrophoresis—Disc gel electrophoresis was conducted at pH 8.3 (22) and at pH 4 (23) on gels containing 7.5% polyacrylamide. The gels at pH 4 were polymerized with riboflavin, and malachite green was used as the tracking dye. Disc gel electrophoresis on gels containing 0.1% SDS and 10% polyacrylamide was performed as described by Weber and Osborn (24).

MATERIALS—Oligosaccharides A, B, and C were isolated from A. aerogenes produced by other strains of A. aerogenes were kindly supplied by Dr. J. F. Wilkinson, Department of Bacteriology, Edinburgh University, Scotland. All other materials were obtained from commercial sources.

RESULTS

Purification of Depolymerase

Three 10-liter cultures of log phase A. aerogenes were infected with phage K-2 and lysis was allowed to proceed to completion. The lysate was chilled to 4° and all subsequent purification procedures were conducted at 0-4° unless otherwise indicated. Following removal of cell debris by centrifugation, ammonium sulfate (43.6 g/100 ml) was added to the lysate and the resultant precipitate was allowed to settle overnight; the major portion of the supernatant was siphoned off and discarded. The remaining suspension was centrifuged at 15,000 × g for 20 min and the pellet was retained.

The pellet was suspended in 148 ml of 10 mM Tris buffer, pH 7.5, stirred for 1 hour, and then centrifuged at 78,800 × g for 1 hour; the resultant supernatant was discarded since sufficient residual ammonium sulfate had been present in the pellet to prevent extraction of depolymerase by the indicated amount of buffer. The majority of the depolymerase activity was then extracted from the pellet by the following procedure: the pellet was resuspended in sufficient 10 mM Tris buffer, pH 7, containing 1% ammonium sulfate, to give a final volume of 310 ml and the resultant suspension was dialyzed against three changes (12 liters each) of the same buffer over a period of 22 hours. The suspension was then centrifuged at 12,000 × g for 10 min and the pellet was discarded. Phage were removed from the supernatant by centrifugation at 78,800 × g for 90 min. The supernatant fluid was diluted to 1400 ml with 10 mM Tris buffer, pH 7, and quantitatively adsorbed to DEAE-cellulose by batchwise addition of adsorbent. The depolymerase containing resin was packed into a column (4 × 26 cm), washed with buffer, and then eluted with a linear gradient of KCl in the same buffer. Depolymerase activity eluted as a single peak at approximately 0.18 M KCl (Fig. 3). Prior to the final step of purification, the majority of the KCl was removed from the depolymerase solution by concentrating the solution to 52 ml by pressure dialysis and then diluting to 500 ml with 10 mM Tris buffer, pH 7.

The depolymerase solution was passed through a column of...
FIG. 3. DEAE-cellulose chromatography of depolymerase. Partially purified enzyme was quantitatively adsorbed to a column of DEAE-cellulose (4 × 26 cm) equilibrated with 10 mM Tris buffer, pH 7.0. Elution was performed with a 2000-ml linear gradient of KCl (10 to 250 mM) in the above buffer at 22° and 15 ml-fractions were collected. Fractions 70 to 110 were pooled.

![Graph](image1)

FIG. 4. CM-cellulose chromatography of depolymerase. The enzyme (2760 units) was adsorbed to a column of CM-cellulose (4 × 40 cm) equilibrated with 10 mM Tris buffer, pH 7.0. The column was eluted with a 2000-ml linear gradient of KCl (10 to 300 mM) in the same buffer at 22° and 16-ml fractions were collected. Fractions 34 to 42 were pooled.

![Graph](image2)

**TABLE I**

| Fraction | Units | Protein mg | Specific activity | Purification |
|----------|-------|------------|------------------|-------------|
| I. Ammonium sulfate pellet... | 5870 | 3410 | 1.7 | 1 |
| II. Second extract of I....... | 4070 | 750 | 6.6 | 4 |
| III. DEAE-cellulose........ | 4160 | 48.5 | 86 | 51 |
| IV. CM-cellulose*.............. | 675 | 2.88 | 235 | 140 |

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

* Only 60% of the enzyme from the previous step was used.

**Catalytic Properties of Depolymerase**

**Endohydrolase**—The action of depolymerase on capsular polysaccharide is characterized by: (a) a rapid reduction in the viscosity of the substrate and (b) a relatively slow release of reducing groups (Fig. 5). These results strongly suggest an endohydrolytic mode of action. Under conditions of limited hydrolysis of the capsular polysaccharide, the release of reducing groups is linear with respect to both time and enzyme concentration (Fig. 6) and is the basis of the enzyme assay.

**Effect of pH, Buffer Concentration, and Temperature**—Maximum depolymerase activity was observed at pH 5.2 (Fig. 7) in 100 mM acetate buffer. The buffer concentration was important; lowering of the acetate concentration below 40 mM resulted in significant loss of depolymerase activity. Depolymerase activity increased with increasing temperature up to 55°, indicating a relatively heat-stable protein. Heating of the enzyme at 65° for 10 min resulted in 52% inactivation.

**Inhibitors**—Depolymerase activity did not require the addition of metal ions and was not inhibited by a variety of chelating agents, including: EDTA, potassium cyanide, o-phenanthroline, and S-hydroxyquinoline. Of the various metal ions tested for inhibition, only Fe³⁺ markedly affected depolymerase activity; the presence of 5 × 10⁻³ mM FeCl₃ resulted in 50% inhibition.
**FIG. 6** (left). Depolymerase assay. Assays were performed as described under “Experimental Procedure” with the following quantities of purified depolymerase (ng): ●—●, 1.6; ○—○, 3.2; and ×—×, 8.0.

**FIG. 7** (right). The effect of pH on depolymerase activity. Assays were conducted in either 100 mM acetate buffer (●—●) or 100 mM Tris buffer (○—○) at the indicated values of pH. Activity is expressed relative to that observed at pH 5.2 in 100 mM acetate buffer.

**TABLE II**

| Substrate                        | Relative activity |
|----------------------------------|-------------------|
| Capsular polysaccharide          | 1.00              |
| Capsular polysaccharide (NaIO₄, NaBH₄) | <0.01             |
| Reduced oligosaccharide C        | 0.01              |
| Oligosaccharide B                | 0.00              |

- a See accompanying paper for structures (15).
- b The specific activity of depolymerase obtained when capsular polysaccharide was used as substrate is arbitrarily set at 1.00.

**Dithiotreitol** and **p-chloromercuribenzoate** had no effect on either depolymerase activity or stability of the enzyme.

**Substrate Specificity**—Previous studies on the oligosaccharide products formed by depolymerase digestion of capsular polysaccharide have clearly demonstrated that depolymerase specifically cleaves the α-galactosylgalactose linkages present in the capsular polysaccharide (15). Further aspects of substrate specificity of depolymerase were investigated by measuring its relative activity against a number of compounds containing α-galactosylgalactose linkages (Table II).

Treatment of capsular polysaccharide with NaIO₄ selectively oxidizes the glucuronate branches leaving the polysaccharide backbone unaltered; the resultant product is therefore a high molecular weight polysaccharide in which the mannose residues are now substituted at C-2 by negatively charged acetals (15). Following reduction with NaBH₄, this product was tested as a substrate for depolymerase. As indicated in Table II, although this product did serve as a substrate for depolymerase, its rate of degradation was less than 1% of the rate obtained with intact capsular polysaccharide as substrate.

Oligosaccharide C is a dodecasaccharide composed of three tetrasaccharide repeating units (15). Treatment of oligosaccharide C with depolymerase has been shown to result in its degrada-

**Fig. 8**. Scheme illustrating depolymerase digestion of [H-]NaBH₄-reduced oligosaccharide C. Gal-OH*, radioactively labeled [H]galactitol.

**Fig. 9**. Disc gel electrophoresis of purified depolymerase. In each case, 40 μg of enzyme were applied and migration was from top to bottom for a sufficient period of time to move the tracking dye to within 0.5 cm of the bottom of the gel. Each gel was 6 cm long.
Depolymerase was hydrolyzed in 6 N HCl as described under "Experimental Procedure." Over-all recovery on each analysis was 85%.

| Amino acid      | Composition | Residues/1000 residues |
|-----------------|-------------|------------------------|
| Aspartic acid   | 181.5       | 129                    |
| Glutamic acid   | 137.6       | 84                     |
| Glycine         | 148.5       | 108                    |
| Alanine         | 109.2       | 76                     |
| Valine          | 98.2        | 70                     |
| Leucine         | 92.1        | 68                     |
| Isoleucine      | 77.2        | 55                     |
| Serine          | 115.9       | 82                     |
| Threonine       | 105.1       | 75                     |
| Half-cystine    | 14.0*       | 10                     |
| Methionine      | 10.7*       | 8                      |
| Proline         | 47.0        | 34                     |
| Phenylalanine   | 64.1        | 46                     |
| Tyrosine        | 48.2        | 34                     |
| Histidine       | 25.2        | 18                     |
| Lysine          | 55.0        | 40                     |
| Arginine        | 73.6        | 52                     |
| Tryptophan      | 18.1*       | 13                     |

* Determined as cysteic acid after performic acid oxidation.
† Determined as methionine plus methionine sulfone after performic acid oxidation.
‡ Spectrophotometric analysis indicated a tyrosine to tryptophan ratio of 2.67 (20).

Depolymerase exhibited a preference for either of the two galactosylgalactose linkages present in this substrate. Oligosaccharide C was reduced with [3H]NaBH₄ in order to label the terminal, reduced tetrasaccharide repeating unit. The resultant product was exhaustively digested with depolymerase and then chromatographed on Sephadex G-25. Analysis of the fractions for radioactivity revealed that only the octasaccharide region contained the radioactive label. This clearly demonstrates that depolymerase is unable to cleave the α-galactosylgalactose linkage immediately adjacent to the terminal, reduced tetrasaccharide repeating unit in this substrate (Fig. 8).

Depolymerase was completely inactive against the synthetic substrate p-N0-phenyl-α-D-galactosyanoside and the capsular polysaccharides of A. aerogenes strains 243 (25) and A381 (9, 10).

**Molecular Properties of Depolymerase**

**Criteria of Purity**—Disc gel electrophoresis of purified depolymerase at pH 4 revealed a single protein component which migrated toward the cathode (Fig. 9). Elution of slices of an unstained control gel permitted demonstration of depolymerase activity coincident with the protein band. Electrophoresis of depolymerase at pH 8.3 revealed a protein component which barely entered the gel, migrating toward the anode. Again, enzymatic activity was found to be coincident with the protein band.

Purified depolymerase was subjected to molecular sieve chromatography on a Sephadex G-200 column (Fig. 10). All of the protein emerged as a single symmetrical peak just within the void volume of the column. Depolymerase activity was coincident with the protein peak and exhibited a constant specific activity in all fractions, again suggesting that purified depolymerase was homogeneous.

**Ultraviolet Spectrum**—The purified depolymerase preparation exhibited a typical protein ultraviolet absorption spectrum with an absorption maximum at 278 nm. Assuming a molecular weight of 379,000, the ε₂₈₀ was calculated to be 4.1 × 10⁴ M⁻¹ cm⁻¹. There was no significant absorption by the protein at wave lengths greater than 310 nm.

**Amino Acid Composition**—The amino acid composition of depolymerase is presented in Table III. None of the common amino acids was missing and no unusual amino acids were detected. The partial specific volume (ᵦ) of depolymerase was calculated from its amino acid composition as described by Schachman (26) and found to be 0.727 cc per g.

**Stokes Radius and Diffusion Coefficient**—The Stokes radius of depolymerase was obtained by molecular sieve chromatography with Sephadex G-200 according to the procedure of Ackers (27). The pore radius of the column was determined with catalase, bovine serum albumin, and hemoglobin as standards (Table IV). On the basis of its elution characteristics on the calibrated column, the Stokes radius of depolymerase was calculated to be 98.2 Å. This value for the Stokes radius was then used to calculate the diffusion coefficient of depolymerase by means of the Stokes-Einstein equation (61); a value of 2.49 × 10⁻⁹ cm² sec⁻¹ was obtained.

**Sedimentation Coefficient**—The sedimentation coefficient of depolymerase was determined by centrifugation in sucrose density gradients as described by Martin and Ames (32). Sedimentation coefficients of the two standards, catalase and Escherichia coli alkaline phosphatase, were assumed to be 11.3 S (28) and 6.3 S (33), respectively. The sedimentation behavior of depolymerase relative to the two standards is shown in Fig. 11. The average value of sₑₘ obtained from six separate determinations was 10.7 S.

**Molecular Weight and Frictional Ratio**—The molecular weight of the native enzyme was calculated from the experimentally
Determination of Stokes radius

Depolymerase (92 µg) was chromatographed at 22° on a Sephadex G-200 column (2.5 × 33 cm) equilibrated with 10 mM Tris buffer, pH 7.0, containing 1% ammonium sulfate. Catalase, bovine serum albumin, and hemoglobin were used as standards. Bovine serum albumin and hemoglobin were determined by absorbance at 280 nm and 415 nm, respectively; catalase and depolymerase were monitored by their respective enzymatic activities. The pore radius of the column and the Stokes radius of depolymerase were calculated as described by Ackers (27).

| Compound         | Stokes radius a | Pore radius b |
|------------------|-----------------|---------------|
| Blue dextran     | 47.5            |               |
| Depolymerase     | 58.4 (86.2 ± 3) | (234 ± 8)     |
| Catalase         | 73.7            | 52.2         |
| Bovine serum albumin | 89.9         | 36.1        |
| Hemoglobin       | 101.8           | 24.4         |
| TZO              | 131.7           |              |

a Each value represents the average of at least two separate determinations.

Subunit Composition—The subunit composition of depolymerase was investigated by disc gel electrophoresis (24) in the presence of 0.1% SDS following preliminary incubation of the enzyme in mercaptoethanol and SDS (0.67% each). As shown in Fig. 12, when preliminary incubation was conducted at 22° a single band was observed upon electrophoresis; its mobility, however, was consistent with that predicted for undissociated enzyme. Prior incubation at 42° or at 62° resulted in the complete disappearance of the slow moving species seen after preliminary incubation at 22°; two faster migrating species, termed Subunit I and Subunit II, were observed instead. Calibration of the gels with standard proteins (Fig. 13) indicated that Subunits I and II corresponded to molecular weights of 63,200 ± 1,200 and 36,400 ± 400, respectively. Prior incubation at either 42° or 62° gave identical results with respect to the relative proportion of Subunit I to Subunit II and thus indicated that Subunit II was not being formed by dissociation of Subunit I. The fact that identical results were also obtained when mercaptoethanol was omitted during the preliminary incubation period would argue against contention that Subunit I is derived from Subunit II by formation of mixed disulfide bonds. These results, therefore, indicated that Subunits I and II represent nonidentical subunits derived from depolymerase.

While it has been established that the intensity of staining with the Coomassie blue stain used on SDS gels is not a completely reliable means of quantitating the amount of protein in each band, scanning of gels stained with this dye indicated that Subunit I and Subunit II occurred in a ratio of approximately 3.2:1.0, respectively. In order to obtain a more reliable estimate of the ratio of protein in the two types of subunits, purified depolymerase was dissociated in mercaptoethanol and SDS as described; the resultant mixture of subunits was fractionated on a Sephadex G-200 column equilibrated with buffer containing 0.1% SDS and 10% polyacrylamide as described by Weber and Osborn (24). Each sample was then subjected to electrophoresis in gels containing 0.1% SDS and 10% polyacrylamide as described by Weber and Osborn (24).Fig. 12. SDS disc gel electrophoresis. Prior to electrophoresis each sample was incubated as follows: 20 µg of purified depolymerase in 0.025 ml of 10 mM Tris buffer, pH 7.5, containing 1% ammonium sulfate was mixed with 0.050 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 1% mercaptoethanol and 1% SDS. Left gel, preliminary incubation was for 3 hours at 22°. Right gel, preliminary incubation was for 3 hours at 62°. Each sample was then subjected to electrophoresis in gels containing 0.1% SDS and 10% polyacrylamide as described by Weber and Osborn (24). I, Subunit I; II, Subunit II.

The molecular properties of depolymerase are summarized in Table V. Assuming the subunit molecular weights shown, the
Arrows Z and ZZ denote the relative mobilities of Subunits I and II obtained by treating depolymerase with mercaptoethanol and SDS (0.1% each) for 3 hours at 62° prior to electrophoresis.

Fig. 14 (right). Separation of depolymerase subunits by gel filtration in 0.1% SDS. Depolymerase (1.07 mg) was dissociated by preliminary incubation with mercaptoethanol and SDS (0.67%) and fractionated in 0.1% SDS. Chromatography was conducted at 22° and 1.7-ml fractions were collected.

**Table V**

| Parameter                  | Method      | Value         |
|----------------------------|-------------|---------------|
| Stokes radius (a)          | Sephadex G-200 | 86.2 A        |
| Diffusion coefficient (D_p,u) | Sephadex G-200 | 2.49 x 10^-2 cm^2 sec^-1 |
| Sedimentation coefficient (s_p,u) | Sucrose gradient | 10.7 x 10^-14 sec |
| Partial specific volume (v) | Amino acid analysis | 0.727 cm^3 per g |
| Molecular weight           | Physical properties | 379,000       |
| Frictional ratio (f/f_o)   | Physical properties | 1.77          |
| Subunit molecular weight   | SDS disc gel electrophoresis | 1: 63,200; II: 36,400 |

**Summary of molecular properties of depolymerase**

Phage Induction of Capsular Polysaccharide Depolymerase Synthesis

Depolymerase activity was not detectable in extracts of uninfected *A. aerogenes*. This enzymatic activity is formed only in response to infection with phage K-2. Under conditions in which the latent period is 40 min, the earliest detection of depolymerase activity in cell-free extracts was approximately 30 min after infection; the enzymatic activity present in infected cells continues to increase until cell lysis occurs. Although these data indicate that the appearance of depolymerase activity is a result of phage infection, it does not prove that the depolymerase molecule is synthesized de novo during this period. In view of the high degree of specificity of the depolymerase for the capsular polysaccharide of the host, it seemed plausible that the appearance of depolymerase activity in infected cells could be the result of phage-directed modification of a pre-existing host cell protein. Density shift experiments were therefore performed with the *A. aerogenes*-phage K-2 system essentially as described by Chrisepeels et al. (36). These experiments involved shifting the density of the growth medium immediately prior to phage infection, and the isolation of soluble depolymerase from cell lysates and determination of its density by isopycnic equilibrium centrifugation in cesium chloride gradients. Heavy and light media were prepared by dissolving the salts plus glucose in either 97% D_2O or H_2O, respectively. As shown in Fig. 15, the density of soluble depolymerase was found to be a function of the density of the medium at the time of phage infection. It was concluded therefore that depolymerase is a phage-induced enzyme synthesized de novo following phage K-2 infection of the *A. aerogenes*.

**Phage-bound Depolymerase**

Approximately 10 to 25% of the total depolymerase activity present in lysates of *A. aerogenes* may be removed from solution by centrifugation at 100,000 x g for 90 min. This procedure also removes greater than 95% of the phage and suggests that depolymerase may exist in a phage-bound form as well as the soluble form described above. This was indeed the case since the ratio of enzyme to phage remained essentially constant through repeated centrifugations and isolation of the phage in CsCl gradients. Phage-bound depolymerase exhibited catalytic properties identical with those of the soluble enzyme: (a) a pH optimum
of 5.2 for the rate of formation of reducing termini with capsular polysaccharide as substrate; and (b) extensive digestion of capsular polysaccharide with phage-bound depolymerase yielded oligosaccharides with galactose residues at their reducing termini and which were indistinguishable from the oligosaccharides produced by the soluble enzyme.

Phage-bound depolymerase tightly adheres to the phage; treatment with various concentrations of salts, drastic changes in pH, detergents, chelating agents, or proteolytic enzymes did not achieve detectable solubilization of the activity or loss of phage-bound activity. It was observed, however, that treatment of the phage (30 min, 4°C) with either 8 M urea or 4 M guanidine-HCl resulted in the quantitative solubilization of the enzyme. After removal of the solubilizing reagent by dialysis, solubilized depolymerase exhibited catalytic properties identical with those of the enzyme normally occurring in lysates in soluble form. In addition, the solubilized enzyme was indistinguishable from purified depolymerase in its elution characteristics from Sephadex G-200, suggesting further that the molecular properties of the phage-bound enzyme are similar, if not identical, to those of the soluble enzyme.

**DISCUSSION**

Infection of *A. aerogenes* with phage K-2 is associated with the *de novo* synthesis of a capsular polysaccharide depolymerase, part of which is bound to the phage and appears to be an integral part of phage structure, the major portion is, however, in a soluble form in cell lysates. The soluble form has been purified to apparent homogeneity as judged by disc gel electrophoresis and molecular sieve chromatography. The electrophoretic properties of the enzyme suggest that it is a basic protein; this was further indicated by the observation that depolymerase may be adsorbed to resins of CM-cellulose at relatively high pH values. The basic nature of depolymerase may facilitate interaction of the enzyme with its polyanionic substrate.

No cofactor requirements were detected for depolymerase. The apparent lack of a requirement for divalent cations is of interest in view of the fact that depolymerase may be considered to be an extracellular enzyme. It may differ therefore from the extracellular enzymes of bacterial origin which are generally found to contain firmly bound cations that are usually essential for activity (37).

As demonstrated in the accompanying paper (15), depolymerase is a glycanohydrolase which specifically cleaves only the galactosyl-α-1→3-galactose linkages in the *A. aerogenes* capsular polysaccharide. It is apparent from these studies that this attack occurs at random along the capsular polysaccharide molecule as judged by the extremely rapid loss of viscosity of the capsular polysaccharide solution relative to the release of reducing groups. With regard to substrate specificity of depolymerase it should be noted that the rate of release of reducing groups declined steadily after approximately 20% of the total reducing groups had been released. This observation suggests that the lower molecular weight oligosaccharides produced by random attack of depolymerase on capsular polysaccharide are less effective as substrates for depolymerase than is the intact, high molecular weight capsular polysaccharide.

The dodecasaccharide, oligosaccharide C, is the smallest oligosaccharide produced by depolymerase digestion of capsular polysaccharide that retains the structural characteristics essential for depolymerase activity. Although quantitative measurements of its rate of degradation by depolymerase have not been obtained, the fact that it is the only depolymerase-susceptible oligosaccharide found in "exhaustive" digests of capsular polysaccharide suggests that it is a particularly poor substrate. Therefore, its actual rate of degradation by depolymerase may be comparable to the rate of degradation of reduced oligosaccharide C, which is cleaved at only 1% of the rate of capsular polysaccharide. These results are consistent with the above conclusion that low molecular weight oligosaccharides are relatively poor substrates for depolymerase.

A tentative model of the substrate binding site of depolymerase is therefore proposed (Fig. 16) on the basis of the following observations: (a) depolymerase is unable to cleave the galactosylgalactose linkage in oligosaccharide B, and (b) depolymerase specifically cleaves only the galactosylgalactose linkage adjacent to the terminal, nonreducing tetrasaccharide repeating unit in reduced oligosaccharide C. According to this model, depolymerase contains binding sites for three sequential tetrasaccharide repeating units; each site must be occupied in order to align properly a galactosylgalactose linkage with respect to the catalytic groups at the active site. Oligosaccharide B, which contains only two tetrasaccharide repeating units, would thus be unable to form a productive enzyme-substrate complex. The relative location of a catalytic site of the enzyme with respect to the binding sites for the three tetrasaccharide repeating units would account for the specific manner in which reduced oligosaccharide C is cleaved.

At present, little information is available concerning which residues of the tetrasaccharide repeating units are involved in the enzyme-substrate interaction. Periodate degradation of the capsular polysaccharide is known to modify the glucuronate branches selectively, leaving the linear polysaccharide backbone unaltered. The procedure does not affect the charge density of the capsular polysaccharide. Nonetheless, the resultant product is an extremely poor substrate for depolymerase (cleaved at less than 1% the rate of the native polymer); these results suggest a rather specific interaction of depolymerase with the glucuronate branches of the capsular polysaccharide. Other residues, however, are undoubtedly involved. Thus depolymerase is inactive against the capsular polysaccharide produced by *A. aerogenes*, serotype 2. This polysaccharide is grossly similar to the one used in these studies in that it contains glucuronate branches attached to mannose; glucose, however, substitutes for galactose and the linkages and anomeric configurations differ (25). It is therefore concluded that the interaction between depolymerase and capsular polysaccharide is very specific. Studies by Sutherland, Jann, and Jann (38) on the phage-induced depolymerases from *A. aerogenes* A8(S1) (type 54) have led to a similar conclusion. In addition to degrading the exopolysaccharide of the host organism, these polysaccharide depolymerases were found to hydrolyze a structurally related polysaccharide produced by *E. coli* K27. Exam-
ination of the structures of the two exopolysaccharides and analysis of the limit oligosaccharides produced by enzymatic digestion of each led to the conclusion that these polysaccharide depolymerases require the sequence α-glucuronosyl-1 → 3-fucosyl-glucose for substrate activity.

The molecular properties of depolymerase are of interest. The native enzyme has an apparent molecular weight of 379,000; treatment with sodium dodecyl sulfate at elevated temperatures results in dissociation to two apparently nonidentical subunits, termed Subunits I and II. The rather harsh conditions (42–62°C, 3 hours) required for complete dissociation indicate that the forces holding the subunits together are strong. This stability apparently accounts for the ability to isolate intact depolymerase from phage by treatment with either 8 M urea or 4 M quanidine-HCl.

The subunit composition of depolymerase is, at present, ambiguous with respect to the number of each type of subunit per molecule of native enzyme. Thus, the experimental results may be interpreted in terms of a model for the native enzyme composed either of 4 moles of each of Subunits I and II or 3 moles of Subunit I and 3 moles of Subunit II. Since most oligomeric enzymes that have been studied appear to be composed of even numbers of subunits, one would be inclined to interpret our results in terms of the native enzyme possessing 4 moles of each of Subunits I and II. However, the occurrence of an uneven distribution of subunits (4 moles of Subunit I and 3 moles of Subunit II) may be rationalized in terms of the possibility that one type of subunit in the enzyme is not involved in its catalytic activity. Since the reaction catalyzed by depolymerase is a simple hydrolytic reaction, and it is not likely that this enzyme is subject to allosteric regulation, it is interesting to speculate that possibly Subunit II is a phase structural protein involved in binding depolymerase subunits (Subunit I) to appropriate sites on the bacteriophage itself. In order to determine the subunit composition of depolymerase in an unambiguous manner, it will be necessary to obtain more accurate determinations of the molecular weights of the individual subunits and of the native enzyme, as well as the mass ratios of Subunit I to Subunit II in the intact enzyme.

Because of the rather high molecular weight of the native depolymerase and the apparent complex subunit structure that it possesses, attempts were made to visualize some aspects of molecular substructure of the enzyme by electron microscopy. Examination of preparations of the homogeneous enzyme stained with ammonium molybdate (at a magnification of approximately ×100,000) revealed multiple subunit structure in particles the dimensions of which were consistent with those estimated for the native depolymerase. It was not possible, however, from these preliminary observations to establish unequivocally the subunit structure of the native protein. Further studies are underway in this laboratory to resolve the subunit structure of the phase-induced depolymerase, to determine the role of the two subunit types in the catalytic activity of the enzyme, and to attempt further to resolve the actual molecular architecture of depolymerase by electron microscopy.

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