Partial Purification and Characterization of a Polymannuronic Acid Depolymerase Produced by a Mucoid Strain of *Pseudomonas aeruginosa* Isolated from a Patient with Cystic Fibrosis

W. MICHAEL DUNNE, JR.,* AND FRANCIS L. A. BUCKMIRE

Department of Microbiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Received 28 January 1985/Accepted 21 June 1985

An exopolysaccharide depolymerase was isolated from a mucoid strain of *Pseudomonas aeruginosa* of cystic fibrosis origin. Purified preparations of the depolymerase showed maximum activity against the unacetylated polymannuronic acid exopolysaccharide from the same strain and little activity against commercially prepared alginate. The evidence suggests that the enzyme is either periplasmic in location or associated with the outer cell membrane and is released extracellularly, in the absence of cell lysis, after a reduction of the culture magnesium (Mg2+) concentration below 3.0 mM. The depolymerase is also released after the addition of sublethal concentrations of EDTA to cultures containing 3.0 mM Mg2+. A survey of additional mucoid *P. aeruginosa* isolates recovered from patients with cystic fibrosis showed that nearly 60% demonstrated similar depolymerase activity while none of the nonmucoid revertants of the parent strains produced detectable depolymerase activity.

Mucoid strains of *Pseudomonas aeruginosa* (PaM) commonly colonize the respiratory tract of children and adolescents with cystic fibrosis (6, 12, 15, 27). The factors which promote chronic colonization are uncertain, but unsuccessful elimination of this condition can lead to increased morbidity and mortality (11, 13).

Most PaM produce an alginic acid-like exopolysaccharide (EPS) composed predominantly of D-mannuronic acid with lesser amounts of its 5′ epimer, L-guluronic acid (5, 16, 30, 31). In nature, alginic acid is also produced by several species of brown algae (3, 18, 21, 22, 24) and by the soil bacterium *Azobacter vinelandii* (17). Unlike the polymer produced by the brown algae, bacterial alginates may contain acetyl-substituted mannuronic acid (9, 16, 31). The alginic acid-like EPS of PaM is considered a virulence factor by virtue of its anti-phagocytic properties (1, 35). In addition, the EPS may promote bacterial adherence and microcolony formation in the lungs (26).

Recently, Dunne and Buckmire (W. M. Dunne, Jr., and F. L. A. Buckmire, Microbios, in press) have characterized two PaM (PM1 and PM9) isolated from patients with cystic fibrosis. Both strains produced an EPS composed of mannuronic acid only with no detectable guluronic acid. The EPS from strain PM9 but not PM1 contained O-acyl-substituted mannuronic acid. When the concentration of the only divalent cation present in the minimal growth medium (Mg2+) was 3.0 mM or greater, the EPS isolated from either strain was of uniform size (as determined by polyacrylamide gel electrophoresis [PAGE] and molecular sieving chromatography) and of high molecular weight (ca. 20 × 106). If the Mg2+ concentration was reduced to 1.5 mM or below during growth, the molecular size of EPS from strain PM1 but not PM9 was reduced. The addition of sublethal concentrations of EDTA to cultures of PM1 with 3.0 mM Mg2+ caused the appearance of low-molecular-weight EPS (<104) with simultaneous disappearance of existing high-molecular-weight polymers. In purified form, however, the molecular size of either high- or low-molecular-weight EPS could not be changed with added Mg2+ or EDTA. The results indicate that the EPS from strain PM1 is degraded by an enzyme with polymannuronic acid depolymerase activity.

In this report, we describe the partial purification and characterization of a polymannuronic acid depolymerase produced by a mucoid strain of *Pseudomonas aeruginosa* of cystic fibrosis origin. The evidence indicates that the enzyme (i) is released into the extracellular medium when the Mg2+ concentration is reduced below a threshold level (independent of cell lysis) (ii) is contained within the periplasmic space or is associated with the cell wall when sufficient Mg2+ is present, and (iii) shows substrate specificity for different alginic acid-like polysaccharides. In addition, we show that similar depolymerase activity is expressed by a number of PaM isolated from patients with cystic fibrosis.

**MATERIALS AND METHODS**

Bacterial strains and culture media. All PaM used in this study were recovered from patients with cystic fibrosis on primary cultures of sputum. Nonmucoid variants occurred spontaneously during subculture of parent mucoid strains. The mucoid strains used as a source of EPS substrates (PM1 and PM9) and depolymerase (PM1) have been characterized previously as having methods of identification, storage, and propagation (Dunne and Buckmire, in press). Bacteria were cultured in a chemically defined liquid medium base referred to as V-B medium containing 0.5% sodium gluconate as the sole carbon source. The concentrations of Mg2+ (MgCl2 · 6H2O) and Ca2+ (CaCl2) in the growth medium were varied as described in the text. Cultures were grown at 37°C with constant shaking, and cell growth was monitored turbidimetrically at 600 nm.

Measurement of enzyme activity. Depolymerase activity was assayed quantitatively by the thiobarbituric acid method previously described for analysis of alginic acid depolymerases (33, 38). Qualitatively, depolymerase activity could be demonstrated by observing a reduction in the size of the EPS substrate with time by PAGE as previously reported by Bucke (4) and modified by Dunne and Buckmire (in press). The reaction mixture for each method consisted

---

* Corresponding author.
of 25 to 100 μl of the depolymerase preparation, 100 μg of substrate, and 0.03 M Tris-hydrochloride buffer (pH 7.5) to a volume of 1.0 ml. Unless otherwise indicated, the concentration of Mgpretreatment in the reaction mixture was 3.0 mM. All reactions were performed at 37°C and initiated by the addition of enzyme. Samples were removed at 5, 10, and 15 min for analysis. All sample analyses were performed in triplicate. A unit of activity was defined as the amount of enzyme required to produce 1 nmol of beta-formyl-pyruvate min⁻¹ ml⁻¹.

Substrates consisted of purified preparations of commercial alginic acid (Aldrich Chemical Co., Milwaukee, Wis.) and EPS extracted from cultures of PM1 and PM9. Two EPS preparations were obtained from each strain; one was from cultures grown in V-B medium with 3.0 mM Mg²⁺ (Mg-EPS) and a second was from cultures supplemented with 0.5 mM Mg²⁺ and 3.0 mM Ca²⁺ (Ca-EPS). Purification methods and chemical characterization of each substrate have been described by Brunne and Buckmire (in press). Briefly, both bacterial EPSs were polyan mannuronic acids with estimated molecular weights exceeding 20 × 10⁶. EPS from strain PM9 contained O-acyl-substituted mannuronic acids at a ratio of 0.11 to 0.14 mol of acetate per mol of mannuronic acid. No differences in chemical composition were detected when Mg-EPS and Ca-EPS from individual strains were compared. Only commercial alginic acid contained detectable guluronan acid.

For determinations of depolymerase activity as a function of salt concentration, MgCl₂·6H₂O, CaCl₂, MgSO₄·7H₂O, NaCl, and KCl were added to the reaction buffer to the final concentrations indicated in the text. For studies of depolymerase activity as a function of pH, 0.01 M potassium phosphate buffer was used for pH values less than or equal to 7.5, and 0.03 M Tris-hydrochloride buffer was used for pH values of 7.5 or greater.

Measurement of glucose-6-phosphate dehydrogenase was based on the formation of NADPH from NADP with a resulting increase in absorbance of 340 nm (25). The duration of the reaction time (5 min) and the pH of the reaction mixture were adjusted to minimize the activity of 6-phosphogluconic acid dehydrogenase. One unit of activity was defined as the amount of enzyme required to form 1 nmol of NADPH min⁻¹ ml⁻¹.

Release of depolymerase with EDTA. Strain M1 was grown in V-B medium containing 3.0 mM Mg²⁺ to an absorbance of 1.0 (early stationary phase, ca. 4 × 10⁹ CFU/ml). Cells were harvested by centrifugation and suspended in 0.1 volume of treatment buffer (0.03 M Tris-hydrochloride, pH 7.5) containing 0, 1.5, 3.0, and 10.0 mM EDTA. Samples were removed after 1, 15, and 30 min of incubation at 37°C, and Mg²⁺ was added to a final concentration equaling that of the EDTA. Cells were again removed by centrifugation, and the depolymerase activity of each supernatant sample was determined. Total cellular depolymerase activity was defined as the amount of enzyme released after total sonic disruption of untreated cells.

For studies involving simultaneous determinations of depolymerase and glucose-6-phosphate dehydrogenase activities, cells were harvested from exponential phase cultures of 0.8, approximately 2 × 10⁹ CFU/ml) and processed in the same manner.

Purification of the polymannuronic acid depolymerase. Strain PM1 was grown in V-B medium with 3.0 mM Mg²⁺ to an absorbance of 1.0. Four liters of culture medium was processed at one time. The cultures were diluted with 1 volume of the same basal medium, and the cells were harvested by centrifugation at 25,000 × g for 60 min. The resulting cell pellets were suspended in 400 ml of treatment buffer containing 3.0 mM EDTA. After 5 min of incubation, the cells were removed from this solution by centrifugation at 15,000 × g for 60 min. Mg²⁺ was added to the supernatant to a final concentration of 3.0 mM. This material constituted fraction I depolymerase. All ensuing operations were conducted at 4°C.

To fraction I depolymerase, solid ammonium sulfate was slowly added with constant stirring to a final saturation of 60%. After each addition of ammonium sulfate, the pH was adjusted to 7.5 with 50% ammonium hydroxide. After 20 min, the precipitate was removed by centrifugation at 23,000 × g for 30 min and discarded. The supernatant was brought to 95% saturation with solid ammonium sulfate and allowed to stand for 30 min before centrifugation. The recovered precipitate was dissolved in 25 ml of treatment buffer and dialyzed overnight against 500 ml of the same buffer. Insoluble material was removed from the nondialyzable extract by centrifugation, and the resulting material was termed fraction II depolymerase.

From 70 to 100 mg of protein from fraction II was applied to a column (1.2 by 20 cm) containing DEAE-Sephacel 6B previously equilibrated with treatment buffer. The column was eluted with the same buffer, and fractions (2 ml) containing greater than 20 U of activity per ml were pooled and stored at −80°C. The resulting preparations were composed of fraction III depolymerase.

Fraction III depolymerase (ca. 2 mg of protein) was then applied to a column (1.2 by 14 cm) containing CM-Sephadex 6B and equilibrated with treatment buffer containing 0.2 M KCl. The column was eluted with a linear concentration gradient of KCl from 0.2 to 0.3 M in treatment buffer. Fractions (2 ml) were collected and dialyzed overnight against treatment buffer. Fractions containing depolymerase activity greater than 30 U/ml (fraction IV) were analyzed individually and pooled. Sodium dodecyl sulfate-(SDS) PAGE of fraction IV depolymerase was performed with 7.5% acrylamide by previously described methods (34, 37).

RESULTS

Release of depolymerase activity with EDTA. A substantial proportion of the total cellular depolymerase activity of strain PM1 was released into the extracellular medium immediately after treatment with EDTA (Fig. 1). Expressed as a percentage of total cellular activity, 53.8, 91.2 and 94.7% of all depolymerase activity appeared in the cell-free supernatant after a 1-min incubation in buffer with 1.5, 3.0, and 10.0 mM EDTA, respectively. Less than 6% of total cellular depolymerase activity appeared in the supernatant treatment buffer without EDTA after 30 min of incubation. At all concentrations of EDTA tested, incubation beyond 5 min did not significantly increase the yield of cell-free depolymerase activity.

In a separate analysis, measurements of depolymerase and glucose-6-phosphate dehydrogenase activity present in the treatment buffer and the treated cell pellets were made to determine if the release of depolymerase by EDTA was a result of whole-cell lysis. Neither enzyme appeared in the supernatant of cells suspended in treatment buffer without EDTA (Table 1). Alternatively, 60% of total cellular depolymerase activity but only 1.9% of total cellular glucose-6-phosphate dehydrogenase activity appeared in the supernatant after treatment with 3.0 mM EDTA.

Ammonium sulfate precipitation of fraction I depolymerase. Fraction I depolymerase was selectively fractionated in
containing EDTA. Treatment depolymerase cellular three - fraction II (fraction total weight of each eluted 0.5 M did 30, 60, depolymerase contained 0.3 percent (102 columns molecular-weight activity fractions retained eluted with 0.2 2 percent (6B). Twenty-seven specific activity of the depolymerase by 65% (Fig. 5). Increasing the concentration of EDTA beyond 1.0 M did not significantly reduce specific activity further.

The effects of increasing concentrations of salts of monovalent cations on reaction kinetics were also examined. Maximum specific activity was observed with 3.0 mM KCl or NaCl (Fig. 5). Concentrations of salt exceeding 10.0 mM were inhibitory. At 50 mM KCl or NaCl, specific activity was reduced to 50 and 60% of salt-free control values, respectively.

Maximum specific activity of the depolymerase coincided with a pH of 8.0.

Analysis of substrate specificity and determination of the apparent \( K_m \) and \( V_{max} \) of fraction IV depolymerase. Maximum rate kinetics of the depolymerase were observed with EPS substrate from the same strain (0.32 to 0.34 nmol min \(^{-1}\)). A reduction in the rate of depolymerization was seen when the O-acyl-substituted polymannuronic acid substrate from strain M9 was used (0.1 to 0.14 nmol min \(^{-1}\)). Little difference in the rate of depolymerization of Mg-EPS or Ca-EPS from either strain was noted (data not shown). Purified preparations of commercial alginic acid reacted poorly as a substrate for the bacterial depolymerase (0.02 nmol min \(^{-1}\)). By using the polymannuronic acid substrate from strain M1, the apparent \( K_m \) and \( V_{max} \) values for the depolymerase were calculated. The \( K_m \) was estimated to be \( 9.6 \times 10^{-5} \) M and the corresponding \( V_{max} \) was 123 nmol min \(^{-1}\) per mg of protein using linear regression (correlation coefficient, 0.859).

### TABLE 1. Release of glucose-6-phosphate dehydrogenase and EPS depolymerase after treatment of strain PM1 with EDTA or after sonic disruption of harvested cells

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>G-6-PDH activity</th>
<th>Depolymerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant of cells treated with buffer without EDTA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant of cells treated with buffer without EDTA</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant of cells treated with EDTA-buffer</td>
<td>1.9</td>
<td>60</td>
</tr>
<tr>
<td>Supernatant of cells after treatment with EDTA-buffer</td>
<td>54.8</td>
<td>28</td>
</tr>
</tbody>
</table>

* Abbreviations: G-6-PDH, glucose-6-phosphate dehydrogenase; % TCA, percentage of total cellular enzyme activity.
Depolymerase activity among mucoid and nonmucoid isolates. Fourteen additional PaM and the nonmucoid revertants of each were examined to determine the number of strains producing EDTA-extractable or cellular depolymerase activity. Three mucoid strains (PM3, PM4, and PM8) were unable to grow in the defined medium. The results, (Table 2) show that (i) 5 of 12 mucoid strains released depolymerase into the cell-free supernatant after treatment with EDTA; (ii) two strains (PM5 and PM9) demonstrated only cell-associated depolymerase activity which was not extracted with EDTA; and (iii) none of the nonmucoid revertants produced detectable depolymerase activity either after treatment with EDTA or sonic disruption of cells (data not shown).

DISCUSSION

The depolymerase characterized in this study was obtained from a PaM of cystic fibrosis origin and demonstrated preferential hydrolysis of the polymannuronic acid EPS from the same strain. The depolymerase was less specific for the O-acyl-substituted polymannuronic acid EPS from a different PaM and showed little activity against commercial alginic acid from brown algae (containing mannuronic and guluronic acids). In an earlier study, Linker and Jones (31) have demonstrated that alginolysase activity was markedly reduced versus that of O-acyl-substituted alginites. The substrate specificity of alginolyses for either polymannuronic acid or polyguluronic acid has also been demonstrated with other bacterial species including Pseudomonas maltophilia and Pseudomonas putida (2, 3, 10, 14, 32, 36). In many of these studies, bacterial alginolysis synthesis was induced in the presence of sodium alginate (from brown algae) as a sole carbon source. Hansen et al. (20) were able to induce synthesis of a polymannuronic acid depolymerase in Bacillus circulans with EPS from a PaM of cystic fibrosis origin as a sole carbon source.

There are obvious similarities between the depolymerase from strain PM1 and the alginic acid depolymerases described by Preiss and Ashwell (33) and Davidson et al. (10). Each enzyme was selectively precipitated by similar (NH₄)₂SO₄ concentrations and demonstrated similar binding affinities for cationic and anionic exchange resins. The mechanism of action of these enzymes on polyuronic acid substrates appears to be similar based on the formation of thiobarbituric acid-reactive end products and pH optima.

Our data suggest that the depolymerase of strain PM1 is (i)
located in the periplasmatic space or associated with the outer cell membrane, and (ii) released from the cell rather than activated after a reduction in available Mg\(^{2+}\). This is supported by the following. First, the extracellular release of depolymerase activity by EDTA was not accompanied by a simultaneous discharge of cytoplasmic glucose-6-phosphate dehydrogenase. This is similar to the periplasmic alkaline phosphatase of *P. aeruginosa* described previously (7, 8).

Second, the concentration of Mg\(^{2+}\) (3.0 mM) required to prevent hydrolysis of high-molecular-weight EPS in cultures of PM1 (Dunne and Buckmire, in press) was found to increase the specific activity of the purified depolymerase. Therefore, inactivation of extracellular depolymerase by concentrations of Mg\(^{2+}\) equal to or greater than 3.0 mM appears unlikely.

The rate of hydrolysis of EPS substrate from strains PM1 or PM9 was not influenced by the divalent cation content of the growth medium from which EPS was obtained. Mg-EPS and Ca-EPS from the same strain were depolymerized at equivocal rates. This suggests that Ca\(^{2+}\) in the growth medium does not, in this system, influence the chemical composition (guluronic acid content) of the EPS as it does with *A. vinelandii* (23, 28, 29).

It is interesting that 60% of the PaM from patients with cystic fibrosis which we examined were capable of depolymerizing the EPS from strain PM1. The ability of these strains to degrade their own EPS was not examined, however. It is possible that all PaM are capable of producing EPS depolymerases but that these enzymes are only expressed under certain conditions and also show substrate specificity.

From these data it is tempting to suggest a strategy for treating patients with cystic fibrosis chronically infected with PaM with agents capable of causing the extracellular release of EPS depolymerases. These hypothetical compounds may augment the efficiency of antimicrobial therapy by promoting degradation of the viscous EPS, thus allowing greater antibiotic access to the bacterial cell wall. In this regard, Hancock et al. (19) have shown that standard anti-

![Figure 4](image1.png)

**FIG. 4.** Specific activity of fraction IV depolymerase as a function of increasing concentrations of CaCl\(_2\) (■), MgCl\(_2\) (□), MgSO\(_4\) (○), and EDTA (●). Brackets indicate the standard error of the mean for three determinations of enzyme activity.

![Figure 5](image2.png)

**FIG. 5.** The effects of increasing KCl (●) and NaCl (○) concentrations on the specific activity of fraction IV depolymerase. Brackets indicate the standard error of the mean for three determinations of enzyme activity.

| TABLE 2. Total cellular and EDTA-released depolymerase activity among PaM |
|---------------------------------|---------|---------|---------|---------|
| **Mucoïd strains** |
| EDTA (U/ml) | Total cell (U/ml) | EDTA (U/mg of protein) | Total cell (U/mg of protein) |
| PM1 | 2.05 | 3.45 | 3.79 | 1.96 |
| PM5 | 0 | 1.10 | 0 | 0.56 |
| PM6 | 2.55 | 4.48 | 8.50 | 2.08 |
| PM7 | 1.79 | 3.93 | 9.73 | 2.38 |
| PM9 | 0 | 2.28 | 0 | 1.63 |
| PM14 | 1.52 | 3.66 | 3.30 | 2.52 |
| PM15 | 0.76 | 0.90 | 10.54 | 12.12 |

* Strains PM3, PM4, and PM8 were unable to grow in the chemically defined V-B medium. Strains PM2, PM10, PM11, PM12, and PM13 produced no detectable cellular or EDTA-extracted depolymerase activity.
Pseudomonas agents such as gentamicin compete with Mg²⁺ for binding sites on the outer cell wall of gram-negative bacteria. Recently, Dunne (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A79, p. 14) has shown that sublethal concentrations of gentamicin do promote the extracellular release of the polymanuronic acid depolymerase from strain PM1 in vitro. The aminoglycosides may, therefore, act in a dual fashion in vivo when used in the treatment of pulmonary infections caused by PaM.

LITERATURE CITED


