PARTIAL PURIFICATION OF THE EXTRACELLULAR HEMOLYSIN OF PSEUDOMONAS AERUGINOSA

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ABSTRACT

BERK, RICHARD S. (Wayne State University, College of Medicine, Detroit, Mich.). Partial purification of the extracellular hemolysin of Pseudomonas aeruginosa. J. Bacteriol. 88:559-565. 1964.—Through a series of chemical fractionation steps, the extracellular hemolysin of Pseudomonas aeruginosa was purified 126-fold with a recovery of 49%. Hemolytic activity of crude preparations was irreversibly lost upon contact with anionic exchange materials such as diethylaminoethyl Sephadex or ECTEOLA-Cellulose, but traveled with the solvent front during passage through Sephadex G-200 and carboxymethyl Sephadex. The hemolysin was soluble in water and ethanol, and was partially extractable with ether, but not with trichlorotrifluoroethane (Freon). Although normal serum and serum albumin blocked hemolytic activity, it was unaffected by trypsin, deoxyribonuclease, or ribonuclease. Partially purified hemolysin was studied in vivo, but did not exert dermonecrotic activity in mice or rabbits in the concentrations tested. Although preparations were toxic to mice, lethality appeared to be more a reflection of the nonhemolytic protein content of the preparations rather than of hemolytic activity.

Previous studies by Berk and Nelson (1961, 1962) suggested that various fractions of disrupted Pseudomonas cells exerted a variety of toxic effects on the metabolism of mammalian cells; however, the role of the various extracellular agents in the pathogenesis of Pseudomonas infections has not been accurately examined to any great extent. This is primarily owing to the lack of studies in which individually purified toxin preparations are used. Some of the agents known to be produced are a hemolysin (Liu, 1957; Berk, 1962), lecithinase (Esselmann and Liu, 1961), lipases (Mustie, 1954; Liu, Abe, and Bates, 1961), protease (Morihara, 1963), deoxyribonuclease (Streitfeld, Hoffmann, and Janklow, 1962), elastase (Mandl, Keller, and Cohen, 1962; Mull and Callahan, 1963), mucolytic enzyme (Bergamini, 1952), gelatinase-fibrinolytic enzymes (Kourilsky and Richou, 1954), and a toxic extracellular slime (Liu et al., 1961; Eagon, 1962). Recent interest in the Pseudomonas hemolysin has been renewed, owing to the discovery of an intracellular hemolytic agent which appears sometime after elicitation of the extracellular hemolysin when cultures are grown on solid media (Berk, unpublished data). In addition, broth-grown cells do not elicit extracellular hemolysin, but do contain an intracellular agent which accumulates in the growth medium after 2 to 3 weeks as a result of cellular autolysis (Berk, 1963). The relationship of the various hemolysins to each other as well as to the disease processes is presently obscure, and cannot be resolved until each agent is purified and characterized. Consequently, the main purpose herein is to describe procedures used to purify partially the extracellular hemolysin, and to describe some of its in vivo and in vitro properties.

MATERIALS AND METHODS

Organism. The culture of P. aeruginosa used in these studies was obtained from a patient and was previously described (Berk, 1962).

Medium. The composition of the growth medium consisted of 2% Tryptone, 1% glucose, 0.5% sodium chloride, and 1.5% agar.

Cellophane technique for hemolysin production. The method of Birch-Hirschfeld (1934) was used for the harvesting of Pseudomonas hemolysin. A sheet of autoclaved dialyzing membrane was placed on the surface of the agar medium and was inoculated with a few drops of P. aeruginosa from a broth culture. The organisms were then streaked over the surface of the membrane with a sterile cotton swab. After incubation at 37 C for 48 hr, the membranes were placed in a beaker, and the organisms were washed off with 3 to 5 ml of 0.1 M phosphate buffer (pH 5.9). The cells were centrifuged at 30,000 × g.
for 30 min, and the resultant supernatant fluid containing hemolysin was decanted and refrigerated; the remaining cells were discarded. Approximately 25 to 30 membranes were harvested for each purification attempt.

**Membrane.** Routine harvesting of bacterial cells and hemolysin was performed by use of dialyzing membrane s 300 PT 62 purchased from Technicon Co., Chauncey, N.Y.

**Protein.** Determination of protein was based on a modification of the Lowry method (Zak and Cohen, 1961). Bovine serum albumin was used in the construction of the standard curve.

**Determination of hemolytic activity.** Sheep cells were collected once a week in heparinized saline and refrigerated. Daily cell suspensions of 1% were prepared by washing cells three times with 0.85% saline prior to use. The tests were carried out in 8-ml spectrophotometer tubes. Cells (0.5 ml) were added to 1 ml of saline, 1 ml of phosphate buffer (pH 5.9), and 0.5 ml of hemolysin. After mixing the suspension, the tubes were incubated at 37 C for 1 hr, the unlysed cells were centrifuged down at 4,000 × g (4 C), and the amount of hemoglobin in the supernatant fluid was determined with a Bausch & Lomb spectrophotometer at 540 mμ. The per cent hemolysis was determined by comparison with a standard curve of varying concentrations of red cells lysed with water to give 100% hemolysis.

In addition, an alternate method employing turbidimetric measurement of intact erythrocytes at 700 mμ was used for determination of activity over several time intervals.

**Titration of hemolysin.** Hemolytic titers were determined by twofold dilutions in buffered saline (pH 5.9); 0.5 ml of a 1% suspension of red cells was added, and the final readings were made after 1 hr of incubation at 37 C. The reciprocal of the highest dilution showing complete hemolysis was taken as an approximation of hemolytic units per milliliter present in the preparations. Subsequently, quantitative estimation of the true titer was then determined by performing closely spaced dilutions between the last positive and negative twofold dilution.

**In vivo activity.** Determination of dermonecrotic activity of purified preparations was performed by injecting various dilutions subcutaneously into sets of six mice for each dilution. Control mice received an equal amount of saline. The lethal effect of various preparations was determined by injecting the material (0.9% saline) intraperitoneally and subcutaneously. Injected mice were observed at several time intervals over a 72-hr period. Dermonecrotic activity was determined in rabbits by injection of several dilutions of purified hemolysin intradermally, whereas lethality was determined by intravenous injection.

**Column chromatography.** Fractionation of hemolytic preparations was attempted through the use of diethylaminoethyl (DEAE) Sephadex A-25, carboxymethyl (CM) Sephadex C-25, and Sephadex G-200. All materials were purchased from Pharmacia Fine Chemicals, Inc., New York, N.Y. After washing and activation of the various column materials, 3.5-ml sample volumes were passed through prepared columns and collected as 3.0-ml effluents. The flow rate was approximately 3.0 ml per 10 min, and most column dimensions were 1 by 16 cm. In the case of CM-Sephadex and Sephadex G-200, hemolytic activity traveled with the solvent front (0.01 m phosphate buffer, pH 6.6) and did not require gradient elution. The absorption spectrum of raw and purified hemolysin preparations was determined with a Beckman DB spectrophotometer.

**Enzymes.** Solutions of 0.1% trypsin (pH 7.6), ribonuclease (pH 7.0), and deoxyribonuclease (pH 7.0) were prepared in 0.1 m phosphate buffer and incubated with undiluted, partially purified hemolysin (1:1) for 60 min at 37 C. The commercial enzymes were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The titer of hemolytic activity both before and after treatment was compared to determine whether hemolytic activity had been affected by the enzyme supplement.

**Results**

A summary of the purification procedure is shown in Table 1.

The first step in the purification procedure was to place a flask of the pooled cellophane washings (100 to 200 ml) in a boiling-water bath for 15 to 30 min. The purpose of this step was to inactivate previously demonstrated heat-labile enzymes which were potentially capable of lysing erythrocytes, such as lecithinase and possibly lipase (Liu et al., 1961). Thus, hemolytic activity of purified preparations reflected the activity of only the heat-stable hemolysin (Berk, 1962). Occasionally, a 10 to 15% reduc-
Concentration, owing to preparation of hemolytic action accomplished in heat. An activity of the material was used, the less chance of precipitation. In addition, heated preparations occasionally yielded an increase in hemolytic titer. For example, one preparation containing a total of 3,000 hemolytic units (HU) yielded 3,400 HU after the heat treatment, suggesting an unmasking of activity due to destruction of inhibitors or activation of the hemolysin moiety itself.

Precipitation of the hemolysin at 4 C was accomplished by addition of a saturated solution of ammonium sulfate (pH 5.0) to the raw material to give a final concentration of 0.60 saturation. After standing overnight at 4 C, the precipitate was removed by centrifugation at 30,000 × g for 30 min, and the remaining supernatant fluid was discarded. The precipitate was suspended in 10 to 30 ml of absolute alcohol. The hemolysin remained in solution, while a great deal of protein immediately precipitated out of solution and was removed by low-speed centrifugation. The alcohol solution was then concentrated to 2 to 8 ml by dialysis against solid Aquacide (Calbiochem). Additional protein precipitated out of solution during concentration and was also removed. The alcohol step was then repeated, and the final concentrated alcohol solution was dialyzed against a 0.9% solution of sodium chloride. A 128-fold purification with a 49% recovery in activity was obtained (Table 1). Although this fractionation procedure routinely yielded preparations of increased purity ranging from 90- to 140-fold, one preparation achieved a 333-fold purification with a recovery of 46%.

It was noted that with increased success in purification the water solubility tended to decrease, whereas alcohol solubility increased. Spectral comparison of raw, unpurified hemolytic extracts with partially purified preparations was performed (Fig. 1). Extracts of each preparation were diluted to contain 2 HU/ml prior to spectral examination. Untreated preparations exhibited a great deal of protein and nucleic acid, whereas a purified preparation having a specific activity of 2.0 exhibited a peak at 230 and possibly at 260, with little or no peak at 280 μ. However, examination of a preparation having a specific activity of 10.0 exhibited a slight peak at 260 μ only.

To avoid the possibility of splitting off the hemolysin from a toxic, nonhemolytic moiety which could conceivably be bound to it, an alternate method of purification was attempted by avoiding the use of organic solvents. Preliminary study with various ion-exchange materials and gel-filtration systems indicated that

<table>
<thead>
<tr>
<th>Step</th>
<th>Total vol</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pooled washings</td>
<td>163</td>
<td>16</td>
<td>200</td>
<td>0.08</td>
<td>2,608</td>
</tr>
<tr>
<td>2. Boiling for 15 to 30 min</td>
<td>163</td>
<td>16</td>
<td>200</td>
<td>0.08</td>
<td>2,608</td>
</tr>
<tr>
<td>3. 0.6 (NH4)2SO4-alcohol</td>
<td>30</td>
<td>65</td>
<td>40</td>
<td>1.62</td>
<td>1,950</td>
</tr>
<tr>
<td>4. Concentration, dialysis, and centrifugation</td>
<td>7.0</td>
<td>240</td>
<td>80</td>
<td>3.0</td>
<td>1,680</td>
</tr>
<tr>
<td>5. Absolute alcohol (1:1), concentration, and dialysis</td>
<td>8.0</td>
<td>100</td>
<td>15.8</td>
<td>10.1</td>
<td>1,250</td>
</tr>
</tbody>
</table>

**Fig. 1. Absorption spectrum of raw and purified hemolysin; material contained 2 hemolytic units per ml suspended in 0.1 M phosphate-0.09% sodium chloride (pH 6.0). Samples of different purity exhibited a specific activity (SA) of 8 and 10, respectively.**
both raw and partially purified hemolytic preparations rapidly passed through CM-
Sephadex and Sephadex G-200 without the use of
gradient elution techniques (pH 6.6). The
ehemolysin traveled with the solvent front, asso-
ciating with fractions containing the highest
concentrations of protein (Fig. 2). Use of gradient
elution, with the use of saturated sodium chloride,
yielded a good deal of nonhemolytic protein
which was eluted from the CM-Sephadex well
after recovery of hemolysin. On the basis of these
results, this step was later employed batchwise
in the alternate purification procedure.

Column chromatography with anion ex-
changers such as DEAE-Sephadex or Ecteola-
Cellulose at pH 7.6 indicated a binding of
hemolytic activity, although a great deal of
nonspecific protein passed through the column.
However, attempts to remove the hemolysin
by gradient elution techniques with the use of
saturated sodium chloride, saturated phosphate
buffer, or a solution of both, proved to be un-
successful. Negative results were also obtained by
washing the column material with 0.5 N sodium
hydroxide or 0.5 N hydrochloric acid. Because
high salt concentrations inhibited hemolytic
activity, all eluates were dialyzed before assay.

Gel-filtration studies with Sephadex G-200
and solubilized hemolysin obtained from two
ammonium sulfate precipitations (0.6, 0.4 satu-
ration) were initiated with a 1 by 36 cm column at
pH 6.6. At least 95% of the input protein was re-
covered without the use of gradient elution, but
less than 60% of the hemolytic activity was re-
covered. Normally, partially purified prepa-
ations which were not alcohol-treated always
exhibited a cloudy opalescence, which was most
likely due to the presence of lipoprotein. How-
ever, the opalescence passed through the column
along with the solvent front, while the clear-
appearing hemolysin fractions trailed imme-
diately behind, exhibiting a slight amount of
overlapping (Fig. 3). All clear-appearing frac-
tions exhibiting hemolytic activity were pooled
and were passed through Sephadex G-200 again.
This procedure was later employed as the final
step in the alternate purification procedure.

In an attempt to remove nucleic acid from
hemolytic preparations, studies with protamine
sulfate were initiated; however, despite the
removal of a great deal of material, a concomi-
tant decrease in hemolytic activity was also
obtained. Examination of the soluble and sedi-
mentable fractions indicated the presence of
activity in both, suggesting that the hemoly-sin
moiety was firmly bound to the nucleic acid.
In addition, studies with various adsorbents,
such as calcium phosphate gels and alumina
C₇⁺, were relatively unsuccessful.

The following procedure was employed in the
alternate purification study: boiling for 15 to 30
min, precipitation with saturated ammonium
sulfate (0.6 saturation), batchwise treatment

![Figure 2](image1.png)

**FIG. 2.** Fractionation of raw hemolysin on
carboxymethyl Sephadex C-25 equilibrated at pH 6.6 with 0.01 M phosphate buffer; 3-ml fractions were collected.

![Figure 3](image2.png)

**FIG. 3.** Filtration of partially purified hemolysin on Sephadex G-200 equilibrated at pH 6.6 with 0.01 M phosphate buffer; 3-ml fractions were collected.
with CM-Sephadex, precipitation with ammonium sulfate (0.4 saturation), and gel filtration through Sephadex G-200. The overall purification was poor, and yielded a 29-fold increase in specific activity with a 30% recovery in activity. In general, it appears quite difficult to extensively purify the hemolysin without resorting to the use of some organic solvent.

In vivo studies were performed with partially purified hemolysin obtained by both purification procedures. No dermonecrosis was noted in mice or rabbits with either preparation when injections ranging from 1 to 40 HU (0.5 to 20 \( \mu g \) of protein) were administered. In addition, nonhemolytic, opalescent protein samples (1 to 40 \( \mu g/\mathrm{ml} \)) separated from hemolysin by gel filtration through Sephadex G-200 were also inactive. Results of intraperitoneal injections of hemolysin purified by procedures in which alcohol was employed were variable, and depended on the purity of the sample. The results suggest that toxicity is dependent upon the protein concentration rather than upon the hemolytic activity (Table 2). The data also clearly demonstrate that hemolytic activity is independent of toxicity, and can be attributed to a molecule other than protein.

Although hemolytic samples obtained from the alternate purification procedure were very toxic to mice, they contained substantial quantities of protein, making it difficult to assess accurately the role of hemolysin in these results. In addition, since 10 to 20 \( \mu g \) of nonhemolytic protein per ml obtained from gel-filtration studies exhibited a mouse LD50, the data further suggest that the lethality is due primarily to exocellular toxic factors of \( P. \ aeruginosa \) other than the hemolysin per se. Rabbit lethality experiments were not extensively performed, owing to the need for large amounts of purified material necessary for accurate assessment of the results.

Characterization of partially purified hemolysin (alcohol method) was initiated by determining the effect of pH on activity. With diluted preparations, activity occurred over a wide range from above 8.5 to below 4.0 as previously noted (Berk, 1962). The maximal rate of activity seemed to be centered between 4.5 and 5.0 when 0.1 m phosphate buffer was used. Since phosphate does not exhibit adequate buffering capacity below 4.5, a 0.1 m citrate buffer was used for determination of activity at lower pH values. It was found that, as the pH was lowered from 5.6 to 3.0, a concomitant increase in activity was noted. The optimal pH in citrate could not be accurately determined owing to spontaneous lysis below pH 4.0. It should be pointed out that similar changes in pH optima have been described with the intracellular hemolysin of group A streptococci when various buffers were used to determine hemolytic activity (Schwab, 1955).

Other properties were examined and found to be similar to those previously described for the raw, unpurified hemolysin (Berk, 1962). These consisted of the following: rate of activity temperature-dependent; lysis of sheep, human, rabbit, and mouse erythrocytes; activity unaffected by a saturated solution of ethylenediaminetetra-acetate, inorganic ions, reducing agents, acid, alkali, or dialysis. In addition, activity was unaffected by treatment with 0.1% solutions of trypsin, deoxyribonuclease, and ribonuclease. However, normal serum, as well as bovine serum albumin, suppressed hemolytic activity. Solu-

### Table 2. Toxicity of samples having varying degrees of hemolytic activity and purity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity</th>
<th>Activity</th>
<th>Protein</th>
<th>Mortality of mice at*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HU</td>
<td>( \mu g )</td>
<td>12 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>I</td>
<td>1.7</td>
<td>40</td>
<td>23</td>
<td>4/6</td>
</tr>
<tr>
<td>II</td>
<td>5.6</td>
<td>40</td>
<td>7.90</td>
<td>0/6</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>160</td>
<td>16</td>
<td>4/6</td>
</tr>
<tr>
<td>IV</td>
<td>30</td>
<td>5/6</td>
<td>6/6</td>
<td></td>
</tr>
</tbody>
</table>

* The denominators indicate the number of mice used and the numerators indicate the number of mice that died. Statistical evaluation of mortality at 36 hr (samples 1 to 3) indicates the correlation \( r \) between per cent mortality and protein content to be 87%, and the relationship between per cent mortality and hemolytic content to be zero.
bility studies indicated that the hemolysin could be partially extracted from saline with ether, but not with trichlorotrifluoroethane (Freon).

**Discussion**

The main problem in studying the extracellular hemolysin of *P. aeruginosa* has been the inability of the organism to secrete significant amounts of hemolytic material into a broth growth medium. Because hemolysin is secreted extracellularly only when cultures are grown on solid media, harvesting of hemolysin was performed by use of the cellophane-agar overlay technique of Birch-Hirschfeld (1934). Unfortunately, use of this procedure is laborious, and makes it difficult to initiate fractionation procedures which would lead to large volumes of purified hemolytic material necessary for the determination of its molecular weight by ultracentrifugal studies. Another difficulty has been the inability to utilize ion-exchange materials which selectively bind the active agent without alteration of its properties. Although anionic exchangers such as DEAE or ECTEOLA both bound the hemolysin, extreme difficulty was encountered in elution of the material in an active form.

One of the interesting features of the extracellular hemolysin is that its in vitro properties were not significantly altered after partial purification. The ability to withstand boiling for 60 min, extreme pH values, and dialysis without a marked reduction in activity is a reflection of an unusual biologically active moiety. Consequently, it is difficult to believe that the hemolysin portion of the molecule is enzymatic or even protein in nature, despite its dependence on pH, temperature, and partial loss in activity upon storage at −20 C. On the basis of spectral examination, protamine studies, stability to various enzymes, nonspecific masking of activity by normal serum, decreasing water solubility as purity increased, and difficulty in achieving a specific activity exceeding 10 to 13, it would appear that small amounts of nucleic acid and protein are bound to the hemolysin, which is most likely a lipid material behaving as a surface-active agent.

It should be pointed out that the advantage of the purification procedure described herein is that a significant increase in purity was obtained with a concomitant retention of water solubility. Although samples of greater purity can be obtained by extraction procedures with the use of organic solvents other than ethanol, the samples in these cases were no longer watersoluble, making it difficult to quantitate both in vitro and in vivo reactions.

In general, in vivo comparison of purified and unpurified hemolysin preparations is of little value because of the presence of extraneous toxic substances present in untreated preparations. Consequently, the absence of dermonecrotic activity and reduction in lethality in highly purified preparations is reminiscent of the studies of Robinson, Thatcher, and Gagnon (1958), who noted the loss of both activities after purification of staphylococcal hemolysins. Although some partially purified *Pseudomonas* preparations exhibited lethal activity in mice, it was always related to the high protein content of the samples rather than to the degree of hemolytic activity present.

**Acknowledgments**

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