Generation of Purpura-Producing Principle from Pneumococcal Cell Walls

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The in vitro kinetics of muramic acid-alanine bond hydrolysis and pneumococcal purpura-producing principle generation by incubation of Streptococcus pneumoniae cell wall preparations with the bacterial autolysin N-acetylmuramyl-1-alanine amidase were similar. The generated purpura-producing principle preparation had a weight-average molecular weight of ca. 2.6 × 10^5 and possessed the glycan and teichoic acid constituents of the pneumococcal cell wall. The results support the idea that the pneumococcal purpura-producing principle is a high-molecular-weight, glycan-teichoic acid fragment released by hydrolysis of the muramic acid-alanine bonds in the bacterial cell wall.

The production of purpura in mice, rabbits, and guinea pigs injected with cell extracts of Streptococcus pneumoniae was first described by Julianelle and Reimann (7, 8, 11), who used the term “purpura-producing principle” (PPP) to denote the active constituent in the crude preparations. Julianelle and Reimann (8) claimed that PPP activity was associated with protein break down products (primary proteoses) of the pneumococcus; however, Sickle and Shaw (13) and Wadsworth and Brown (15) reported that activity was associated with an uncharacterized carbohydrate of the bacterium. Recently, we isolated the PPP from pneumococcal cell extracts and cell wall preparations and proposed that activity was associated with a water-soluble, high-molecular-weight peptidoglycan-teichoic acid complex generated by the action of the pneumococcal autolysin N-acetylmuramyl-1-alanine amidase on the bacterial cell wall (2, 3). In addition, we found that purified PPP could be obtained more easily from autolysin-digested cell wall preparations than from cell extracts (3). In this communication we present additional data on the kinetics of enzymatic generation and the chemical nature of the pneumococcal PPP.

*S. pneumoniae* R61 was grown in brain heart infusion broth as previously described (2), and purified cell wall preparations were obtained by the method described by Mosser and Tomasz (10) for strain R36A cell walls. Partially purified preparations of pneumococcal autolysin were obtained as described by Holtje and Tomasz (6) from *S. pneumoniae* R61 cells grown in ethanolamine-containing M medium (3), except that the purification procedure was performed only through the second step of the scheme (ammonium sulfate precipitation). PPP activity was not detected in specimens (1 mg) of the partially purified autolysin preparation, in contrast to its detection in the crude autolysin preparations used in our previous studies (3).

The kinetics of cell wall digestion and generation of PPP by the autolysin were determined with a mixture consisting of 500 mg of cell wall preparation and 50 mg of autolysin preparation in 200 ml of 50 mM potassium phosphate buffer (pH 6.9). The mixture was kept at 4°C for 5 min to activate the autolysin and then was incubated at 37°C with gentle mixing on a gyratory shaker. At various times postincubation, samples (1 ml) of the incubation mixture were examined for turbidity at 650 nm in cuvettes (light path, 1 cm) in a DB-GT spectrophotometer (Beckman Instruments, Inc.) and were heated (100°C for 5 min) to inactivate the autolysin. The heated specimens were centrifuged (12,000 × g for 20 min at 4°C), and the supernatant fluids were assayed for an increase in total free amino groups (5) (used as a measure of hydrolysis of the muramic acid-alanine bonds in the cell wall) and for PPP activity (3). After incubation for 6 h, the mixture was centrifuged (25,000 × g for 20 min at 4°C), and the supernatant fluids were dialyzed for 1 day against 8 liters of cold, glass-distilled water and lyophilized. The PPP in the preparation was further purified by gel filtration with Sepharose 6B in 100 mM ammonium bicarbonate as previously described (3), and the active fractions were pooled and lyophilized. The preparation was stored at 0°C until the bioassay, chemical analyses, and molecular weight determination were performed.

The turbidity of the cell wall-autolysin incubation mixture markedly and progressively decreased during incubation at 37°C for 45 min to 2 h, and the maximal reduction in turbidity was observed after 2 to 6 h postincubation (Fig. 1). In addition, the kinetics of muramic acid-alanine bond hydrolysis, as determined by the release of free amino-group-containing reaction products, and the kinetics of PPP generation paralleled one another. During control experiments, cell wall preparations incubated without autolysin for 6 h did not release free amino groups or generate PPP activity, and autolysin preparations incubated without cell walls did not generate PPP activity and released only 6 to 7% of the free amino groups released by the autolysin-cell wall incubation mixture. Thus, the data support the idea that hydrolysis of pneumococcal cell wall muramic acid-alanine bonds by the bacterial autolysin N-acetylmuramyl-1-alanine amidase releases a water-soluble cell wall fragment possessing PPP activity.

The yield of PPP obtained by Sepharose 6B gel filtration of the supernatant fluids from the cell wall-autolysin incubation mixture was 233 mg (ca. 47% of the starting weight of the cell...
Galactosamine
5.2 1.61
Glucose
Threonine
Arginine
0.4 0.07
Aspartic acid
Glutamic acid
1.3
Alanine
Isoleucine
0.5
Valine
Muramic acid
Phosphate
11.9

Fluids sample after Wagner as sample hydrolysis of PPP by peptidoglycan and cell wall contained amino acids; ratios (10) associated enzymatic digestion of contaminant amino acids. The molar ratios (compared to glucosamine) of the amino acids normally associated with intact pneumococcal cell wall peptidoglycan (alanine, glutamic acid, and lysine) were reported (10) to be ca. 3:1:1, respectively. The purified PPP preparation contained markedly reduced amounts of those amino acids; however, this finding was expected because of the known enzymatic specificity of the pneumococcal autolysin, which normally would result in the release of the peptides from the glycan backbone. The components listed in Table 1 accounted for approximately one-half of the weight of the purified glycan-teichoic acid complex. This can be partially explained because the ribitol and diaminopimelate of teichoic acid and the N-acetyl groups of glucosamine and muramic acid were not determined. In addition, significant losses of the components analyzed could occur during the hydrolysis step before analysis. Also, we do not exclude the possibility that minor contaminants might still be present in the preparation.

We previously reported that the PPP eluted at the void volume of Sepharose 6B (2, 3) and later found (unpublished observation) that the PPP also eluted close to the void volume of Sepharose 2B. Thus, the molecular weight of the PPP may be estimated, by gel filtration, to be \( \approx 2 \times 10^7 \). In the current communication, the weight-average molecular weight of the PPP, as estimated by the light scattering procedure of Carr et al. (1), was ca. \( 2.6 \times 10^7 \) (Fig. 2).

It should be emphasized that the autolysin preparation used in this study was partially purified and therefore may have contained small quantities of other classes of bacterial enzymes capable of acting on cell walls. However, the size and chemical nature of the cell wall fragments released during incubation of cell wall-autolysin mixtures indicates that the predominant bacteriolytic activity is that of \( N \)-acetylmuramyl-L-alanine amidase.

In summary, the results of the kinetic studies and physicochemical analyses described in this communication support the idea that the pneumococcal PPP is a water-soluble, high-molecular-weight, glycan-teichoic acid fragment or complex generated or released by the action of the pneumococcal autolysin on the bacterial cell wall. Our previous observation that the PPP is inactivated by lysozyme (2, 3) showed that activity requires the intact \( \beta-1,4 \)-glucosidic linkages of the glycan backbone; however, at present the minimal glycan chain length required for PPP activity has not been determined. In addition, it is not known whether the teichoic acid moiety is required for activity.

![FIG. 1. Kinetics of *S. pneumoniae* cell wall hydrolysis and generation of PPP by pneumococcal autolysin. Strain R61 cell wall-autolysin mixture was prepared, incubated, and assayed for cell wall hydrolysis and PPP activity as described in the text.](image1)

![FIG. 2. Estimation of the weight-average molecular weight (\( M_w \)) of PPP by light scattering studies. The measurements were performed in an apparatus developed by Carr et al. (1), which contained a laser (632.8 nm) as a light source and a cylindrical cell with a light path of 2.5 cm. The weight-average molecular weight was estimated with a PPP preparation (0.05 mg per ml of 0.15 M NaCl buffered with 0.04 M phosphate [pH 7.2]) by using a refractive index increment (dn/dc) of 0.139.](image2)

### Table 1. Chemical composition of PPP generated by digestion of *S. pneumoniae* R61 cell wall preparation by pneumococcal autolysin

<table>
<thead>
<tr>
<th>Component</th>
<th>% Composition (wt/wt)</th>
<th>Molar ratio (to glucosamine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>5.2</td>
<td>1.61</td>
</tr>
<tr>
<td>Phosphate</td>
<td>11.9</td>
<td>4.00</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>10.1</td>
<td>1.81</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5</td>
<td>0.45</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>4.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>5.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.4</td>
<td>0.32</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.0</td>
<td>0.26</td>
</tr>
<tr>
<td>Valine</td>
<td>0.8</td>
<td>0.23</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.7</td>
<td>0.29</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.8</td>
<td>0.19</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.4</td>
<td>0.07</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.3</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* The PPP was isolated by Sepharose 6B gel filtration (3) of the supernatant fluids obtained from the cell wall-autolysin incubation mixture.
* Choline was assayed by the method of Smits (14), and phosphate was determined as described by De Sertvi (4). Galactosamine was assayed, after sample hydrolysis for 1 h at 120°C with 4 N hydrochloric acid, by the method of Wagner (16). Glucose, muramic acid, and glucosamine were determined, after sample hydrolysis under vacuum for 3 h at 100°C with 2 N sulfuric acid, as their alditol acetates (12). Amino acid analyses were performed after sample hydrolysis under vacuum for 24 h at 110°C with 6 N hydrochloric acid, as described by Klapper (9).
We thank S. Lacks for giving us a specimen of *S. pneumoniae* R61 and J. Schwab for the use of his laboratory facilities during the preparation of the purified cell wall and autolysin. In addition, we thank D. Klapper and R. Hantgan for assistance with the amino acid analyses and molecular weight determination, respectively. This investigation was supported by Public Health Service grant HL-16769 from the National Heart, Lung and Blood Institute.

**LITERATURE CITED**


