Purification and Properties of a Bacteriophage-induced Cell Wall Peptidase from Staphylococcus aureus

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Received for publication 8 December 1966

A phage-induced cell wall solubilizing enzyme isolated from phage-infected Staphylococcus aureus phage type 80 was purified 588-fold. The pH optimal activity was 6.8 to 7.3, and pH optimal stability, 6.5 to 7.5. It was inhibited by p-hydroxymercuribenzoate, ethylenediaminetetraacetic acid, and specific rabbit antiserum. The cell wall lytic reaction is a peptidase resulting in cleavage of the cell wall peptide at N-terminal alanine, glutamic acid, and glycine. Electron micrographs are shown of cell wall "ghosts" remaining after the enzymatic digestion of cell walls.

A bacteriophage-elaborated lysin for staphylococcal cells has been described by Ralston et al. (4–7). Their findings indicated that virolysin acts on whole staphylococcal cells only after the cells have been subjected to damaging treatment with such agents as heat, ultraviolet rays, or chemicals, or after "sensitization" of the staphylococcal cells with bacteriophage. C. C. Doughty and M. A. Werckle (Bacteriol. Proc., p. 32, 1964) reported a similar bacteriophage-elaborated lysin in Staphylococcus aureus phage type 80. This lysin acts directly on isolated cell walls to effect cell wall solubilization without the "sensitization" with bacteriophage. Ralston (7) has also shown their virolysin acts directly on isolated S. aureus cell walls. The present paper describes the purification and properties of the cell wall solubilizing enzyme (CWSE-80), elaborated after infection of S. aureus phage type 80 with its homologous bacteriophage.

MATERIALS AND METHODS

Production of CWSE-80. S. aureus phage type 80 and the type 80 bacteriophage were kindly supplied by Margaret Mellody. For turbidity measurements, the bacterial cultures were grown in Brain Heart Infusion (Difco) at 30°C in Erlenmeyer flasks with 18-mm side-arm tubes. When the bacterial turbidity reached an optical density of 0.10 (660 m), 1 ml of phage stock (10^9 phage) was added for each 10 ml of bacterial broth culture. Calcium chloride was added to a final concentration of 4 \times 10^{-4} M. After 90 min of incubation, the bacterial turbidity cleared, owing to bacterial lysis. A sample of the lysate was removed and saved as phage stock. The remaining lysate was rapidly cooled in ice to 4°C. Cellular debris and virus were removed by centrifugation at 37,000 \times g for 30 min at 4°C. The supernatant fluid was saved and was designated as crude CWSE-80. To preserve the enzyme activity, either dithiothreitol (Cleland's reagent) to a concentration of 10^{-3} M or p-hydroxymercuribenzoate (p-HMB) to 10^{-4} M was added. The p-HMB was a more effective preservative, and at a 1,000-fold lesser concentration than either cysteine or dithiothreitol. The crude CWSE-80 was stored at -40°C.

Staphylococcal cells for making cell walls were grown at 37°C on Tryptose Phosphate Broth (Difco) enriched by the addition of 0.7% glucose and 0.1 M potassium phosphate (pH 7.0). Cell walls were prepared from S. aureus phage type 80 by grinding with 0.11-mm glass beads in a mechanical homogenizer. Suspensions [30% (w/v) wet weight] were made in either 0.10 M potassium phosphate (pH 7.0) or 0.10 M tris(hydroxymethyl)aminomethane (Tris) chloride (pH 7.0). Glass beads were added to 60% (w/v). Either a Sorval Omni-mixer or a Braun homogenizer was employed for breaking the cells. During grinding, the temperature was maintained below 10°C by submerging the omni-mixer chamber in an ice bath at 0°C, and by employing alternate grinding periods with cooling periods (10 sec of grinding to 20 sec of cooling) for a total of 1.5 hr. The Braun homogenizer chamber was cooled with carbon dioxide as described by Bliweis, Karakawa, and Krause (1). Cell breakage was complete in 8 min in the Braun homogenizer. Glass beads were removed by use of a sintered-glass filter or by centrifugation at 500 \times g for 10 min. The glass beads were washed with the buffer (pH 7.0), and the washings were combined with the cell homogenate fraction. The cell walls were sedimented by centrifugation for 20 min at 37,000 \times g, washed three times in buffer (pH 7.0), and resuspended in buffer. They were then incubated for 3 hr with \alpha-chymotrypsin (0.2 mg per ml of suspension), washed three times with buffer, and stored at -40°C.

A turbidity decrease assay was used to measure solubilization of cell walls by the phage lysozyme (CWSE-80). Cell walls were diluted to an optical density of 0.4 to 0.5 (1-cm cuvettes, at 660 m) in either 0.1 M...
Tris chloride or 0.1 M potassium phosphate buffer containing 10⁻³ M calcium chloride. In the one experiment in which whole cells were used (Fig. 1), the cells were likewise diluted to an optical density of 0.5. The reaction was started by addition of enzyme to the cuvette.

The peptidase assay used for measurement of increased free amino acids has been described by Ghuy-sen and Strominger (2). For each determination, a control containing cell walls and a control containing enzyme were run along with the samples of the enzyme-cell wall digestion mixture. Samples of the digestion mixture were removed at intervals until lysis was completed. They were pipetted into a tube and placed in a boiling-water bath for 3 min. Samples of boiled digestion mixture containing from 25 to 300 μmole of free amino groups were diluted with water to 0.1 ml. To this were added 0.5 ml of 1% NaB₄O₇·10H₂O and 0.06 ml of 0.1 M 2-nitrophenylfluorobenzene (DNFB) in ethyl alcohol. The tubes were immediately mixed and placed in a 60 C. water bath for 30 min. After cooling with tap water, 1.0 ml of 5 N HCl was added. Absorbancy was read at 420 mμ, and results were calculated according to the extinction A₄₂₀μm = 4,400 cm⁻¹/mole.

The N-terminal amino acids liberated during the enzyme digestion were determined by use of thin-layer chromatography as described by Ghuy-sen et al. (3). Control tubes contained (i) cell walls only, (ii) enzyme only, and (iii) a standard mixture of the amino acids alanine, glutamic acid, glycine, and lysine. The enzyme was incubated with cell walls until lysis was completed. A sample of the digested cell walls containing 20 to 80 μmole of free amino groups was treated with DNFB in 1% borate for 30 min at 60 C. This was then acidified to 6 N HCl and hydrolyzed overnight in sealed tubes at 100 C. After acid digestion, the 2,4-dinitrophenyl (DNP) derivatives were extracted several times with ether. The ether was evaporated, and the DNP amino acids were redissolved in water and chromatographed on thin-layer Silica Gel G plates. Two solvent systems were used: (I) n-butanol-0.15 N ammonium hydroxide (1:1, upper layer), 2 hr at room temperature; (II) chloroform-methanol-acetic acid (85:14:1) in an ice bath at 0 C for 1 hr.

RESULTS

The activity of CWSE-80 on whole staphylococcal cells was compared with that on isolated staphylococcal cell wall preparations. Partially purified CWSE-80 (0.3 mg) produced no significant clearing of turbidity in the suspension of whole cells at 1 hr (Fig. 1). By contrast, a similar amount of enzyme produced a linear decrease in optical density of cell wall suspensions from 0.50 to 0.25. Clearing continued to less than 0.05 after 2 to 3 hr of incubation. Preincubation of cell walls with bacteriophage type 80 resulted in no change in rate. Hence, it appeared that bacteriophage was not required for CWSE-80 activity with cell walls as substrate. With purified cell walls, lysis proceeded without a lag and with a linear decrease in turbidity. Ready accessibility of substrate sites on the cell walls to CWSE-80 was indicated. The accessibility of the enzymatic sites on intact bacterial cells possibly could have been altered by bacteriophage, thus rendering them more susceptible to the virolysin than "unsensitized" cells.

The pH optimal activity of CWSE-80 in 0.1 M potassium phosphate buffer was from 6.8 to 7.3 (Fig. 2). The pH stability of CWSE-80 was determined by storage of the enzyme for 2 hr in buffer ranging in pH from 5 to 8.5. After storage, the pH of CWSE-80 was readjusted to 7.0 with phosphate and assayed. The pH optimal stability was found between 6.5 and 7.5 in either potassium phosphate or Tris chloride. Buffers used included 0.01 M sodium acetate from pH 5 to 6.1, 0.01 M potassium phosphate from 6.1 to 7.4, and 0.01 M Tris chloride from 7.4 to 8.5. The same stability curve was observed when the storage buffer contained acetate, phosphate, and Tris in the same buffer mixtures.

Figure 3 shows 100% inhibition of CWSE-80 with 10⁻³ M p-HMB. This inhibition was reversed when 10⁻³ M cysteine was added after 30 min of incubation. The rate of cell wall digestion then proceeded at the same rate as when no inhibitor was added. When both 10⁻³ M p-HMB and...
and 10^{-3} \text{ M} \text{ cysteine} were added at zero-time, cell wall digestion occurred at the same rate as when no inhibitor was added. Even after 1 year of storage at -40 \text{ C} in 10^{-4} \text{ M} \text{ p-HMB}, the enzyme was fully active when 10^{-3} \text{ M} \text{ dithiothreitol} or 10^{-4} \text{ M} \text{ cysteine} was added to the assay cuvette. On prolonged storage, addition of 10^{-5} \text{ M} \text{ p-HMB} was more effective than addition of sulphydryl compounds. To preserve the activity of CWSE-80 with dithiothreitol, it was necessary to add as much as 10^{-2} \text{ M} \text{ dithiothreitol}.

The rate of CWSE-80 digestion of dialyzed cell walls was increased twofold by addition of either 3 \times 10^{-4} \text{ M} \text{ calcium chloride} or magnesium chloride when the reaction was in either Tris chloride or potassium phosphate (pH 7.0). As shown in Fig. 3, cell wall digestion is 100\% inhibited by 10^{-3} \text{ M} \text{ ethylenediaminetetraacetic acid (EDTA).}

The effects of specific rabbit antisera are illustrated in Fig. 4. Preimmune rabbit sera produced no significant inhibition of CWSE-80. Likewise, antisera prepared to bacteriophage type 80 produced no inhibition. The use of specific antisera to CWSE-80 resulted in over 90\% inhibition of the rate of lysis. Antisera to cell walls showed a partial inhibition in the rate of lysis, as did antisera to cell homogenates which contained cell wall material. Inhibition of lysis by antisera to cell walls may suggest a competition for sites on the cell wall between CWSE-80 and antibody to cell walls. The lack of inhibition by specific antisera to bacteriophage type 80 supports our finding that the phage was not necessary for action of CWSE-80 on cell walls.

Fractional purification of CWSE-80 was followed by using turbidity decrease assay of cell walls as the criterion for purification. In addition, fractions were assayed for increases in the amount of N-terminal amino acids by the method of Ghysen and Strominger (2). In each case, the fractions which contained the CWSE-80 showed a significant increase in amounts of N-terminal amino acids.
amino acids (Fig. 5). Sedimentation of 30% of the CWSE-80 activity occurred with ammonium sulfate between 20 and 50% saturation at 0°C in 0.01 M potassium phosphate at pH 7.0. After 30 min of stirring at 0°C, the precipitate was redissolved in 5.0 × 10⁻³ M Tris (pH 7.0). This step resulted in a 13-fold purification (Table 1). After dialysis against two changes of distilled water, this fraction was then applied to a diethylaminoethyl (DEAE) Sephadex A50 column (25 by 340 mm, pH 7.0) which had been equilibrated for 24 hr with 5 × 10⁻³ M Tris chloride (pH 7.0) and 10⁻³ M p-HMB. The protein was eluted by linearly increasing the ionic strength of the eluting buffer with sodium chloride to a final concentration of 2 M sodium chloride in 0.1 M Tris chloride (pH 7.0). The protein elution is illustrated in Fig. 5. Both the cell wall solubilizing activity and the peptidase activity were in the first protein peak. The fractions were assayed in 10⁻³ M dithiothreitol to reverse the effects of p-HMB. A purification of 588-fold was achieved (Table 1). A gain in total units of activity was observed after the DEAE Sephadex column step relative to the ammonium sulfate precipitation step. This may have been due to removal of some inhibitory substance during the purification.

After enzyme digestion of cell walls with CWSE-80, the resulting N-terminal amino acids were reacted with DNFB. After overnight digestion in 6 N HCl, the DNP amino acid derivatives were extracted with ether as described by Ghuyse et al. (3). After the extracts were evaporated to dryness and redissolved in water, chromatograms were run on thin-layer silica gel plates, first in butanol-ammonium hydroxide and then in chloroform-methanol-acetic acid.

The chromatograms revealed three spots present in enzyme digests which were not visible in the cell wall or enzyme controls. These spots had Rf values identical to those of control preparations of DNP alanine, DNP glutamic acid, and DNP glycine as reported previously (C. C. A. N. AND J. B. MANN.

![Figure 4](http://jb.asm.org/)

**Fig. 4. Inhibition of CWSE-80 with specific antisera.** Cuvette contents: 2.7 ml of cell walls (type 80) suspended in 0.1 M potassium phosphate and 0.15 M sodium chloride. Preincubated for 5 min with 0.1 ml of antisera before addition of 0.2 ml (2 mg of protein) of CWSE-80 to initiate enzyme reaction.

![Figure 5](http://jb.asm.org/)

**Fig. 5. Column purification of CWSE-80 on DEAE Sephadex A-50.** Column was 25 by 340 mm; flow rate was 0.8 to 1.0 ml/min. CWSE-80 (55 mg of protein in 5 ml) was applied in the starting buffer of 0.1 M Tris chloride-10⁻³ M p-HMB (pH 7.0) with elution by a linear increasing ionic strength with sodium chloride in 0.1 M Tris chloride-10⁻³ M p-HMB (pH 7.0). Fractions collected were 5.0 ml and the temperature of the column and the fractions was 4°C.
Doughty, N. L. Shoemaker, and J. A. Mann, Bacteriol. Proc., p. 84, 1966). The spots were eluted from the chromatograms and estimated by use of spectrophotometry. The results are shown in Table 2. These results are expressed as moles of amino terminal amino acids released per mole of glutamic acid in cell walls free from ester-alanine. The values for glutamic acid, alanine, and glycine were significantly higher than the cell wall controls. Hence, we concluded that the CWSE-80 acts on cell walls to liberate these three terminal amino acids from the glycopeptide of the cell wall.

Another assay for the cell wall solubilizing enzyme was devised. The peptide cleavage was titrated with 0.010 N sodium hydroxide. Because of the limited range of pH optimum of this enzyme, pH 7.0 was selected. As is shown in Fig. 6, the reaction remained linear for a sufficient time to be used as a method for enzymatic assay, although only a small fraction of the cleavage products were titrated at pH 7.0. The reaction was maintained at 37 C in a nitrogen atmosphere with constant stirring. The pH was held at 7.0 by use of a Radiometer TTT-1 pH stat. Cell walls were diluted to an optical density of 0.10 (light path, 1 cm at 660 mu). The cell wall suspensions and the enzyme were preadjusted to pH 7.0. The reaction was initiated by addition of enzyme (0.03 to 0.2 ml) to 3.0 ml of cell wall suspensions. A small amount of alkali (usually less than 0.05 mu mole) was consumed by the cell wall suspensions alone in the first few minutes of stirring. It is thought that this is related to physical factors involved in stirring particulate cell wall suspensions. With enzyme alone, no alkali consumption occurred. No increase in alkali consumption was observed when previously boiled enzyme was added to cell wall suspensions. When active enzyme was added, consumption of alkali increased at a linear rate until lysis was completed. The rate was dependent upon the amount of enzyme added. Sufficient enzyme was added so that time of consumption of 6 mu moles of alkali ranged from 5 min to 1 hr.

After digestion of cell walls with CWSE-80, a small residual turbidity remained. Electron micrographs (Fig. 7) revealed cell wall ghostlike structures which may partially explain this residual turbidity.

**DISCUSSION**

The purification of CWSE-80 was followed by measuring turbidity clearing. The cell wall digestion proceeded without a lag and was linear during the early phases of cell wall digestion. The presence of bacteriophage was not required for digestion of cell walls by CWSE-80. This is in contrast to the requirement of "sensitization" with bacteriophage or chemical or physical alteration of whole staphylococcal cells with bacteriophage before digestion with virolysin as reported

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**Table 1. Purification of CWSE-80**

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Specific activity (units/ per mg of protein)</th>
<th>Total activity*</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude CWSE-80</td>
<td>3.9 x 10^-4</td>
<td>0.94</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate 20 to 50% saturated</td>
<td>5 x 10^-3</td>
<td>0.28</td>
<td>13</td>
</tr>
<tr>
<td>DEAE Sephadex A-50 peak no. 1</td>
<td>0.23</td>
<td>0.60</td>
<td>588</td>
</tr>
</tbody>
</table>

* One unit = change in optical density of 1.0 per min.

**Table 2. DNP amino acids extracted from thin-layer chromatograms**

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Glutamic acid</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWSE-80 digested cell walls</td>
<td>0.36</td>
<td>0.25</td>
<td>0.53</td>
<td>0.09</td>
</tr>
<tr>
<td>Wall control</td>
<td>0.09</td>
<td>0.08</td>
<td>0.15</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Expressed as moles of amino terminal amino acids released per mole of glutamic acid in cell walls free from ester-alanine.
The CWSE-80 may provide insight into the events occurring in phage lysis as well as serve as a useful tool in the study of cell wall structures. It differs from other cell lysins described in that N-terminal glutamic acid is a cleavage product.

The structures which were observed in the electron micrographs after digestion of cell walls with CWSE-80 may explain the small residual turbidity (less than 10%) after lysis is completed. The possibility of the presence of some membrane material has not been excluded. We have designated these structures which appear as hollow craters of collapsed cell walls as cell wall "ghosts." Ghuysen and Strominger (2) have described the cell wall as consisting of carbohydrate polymeric chains interconnected by peptide bridges. If the peptides are cleaved, one can visualize the breakdown of a three-dimensional cell wall structure. The evidence presented here indicates that the peptides are cleaved by CWSE-80 and that the carbohydrate polymeric chains are not cleaved, as there is no significant increase in reducing sugars with cell wall lysis. The cell wall "ghosts" may represent a tangle of polysaccharide chains after the cleavage of the peptide cross bridges.

ACKNOWLEDGMENTS

This investigation was supported by grant GB 3573 from the National Science Foundation.

We are grateful to J. L. Strominger and D. J. Tipper for help in identifying the N-terminal amino acids as cleavage products and for their interest in

Fig. 7. Electron micrographs of cell walls before and after treatment with CWSE-80. (left) Staphylococcus aureus cell walls from strain 80 (X 8,000). (right) After treatment with CWSE-80 (X 8,000).

by Ralston et al. (4-7). With purified cell walls, the CWSE-80 appears to be readily accessible to the substrate site of the cell wall.

The CWSE-80 was purified 588-fold. The cell wall solubilizing activity (turbidity decrease) and the peptidase activity (release of free amino groups) occurred in the same fractions of the DEAE Sephadex eluate (Fig. 5). These findings along with the observation that little or no reducing sugar was detected during cell wall digestion indicated that the peptidase is the cause of cell wall lysis.

Enzymatic digests of cell walls were treated with dinitrofluorobenzene, followed by digestion in acid to free amino acids and DNP amino acids. The DNP amino acids were extracted and chromatographed by use of a procedure described by Ghuysen et al. (3). Similar amounts of cell walls and of enzyme controls were run along with the enzymatic digests. The chromatogram of the enzymatic digests of cell walls revealed spots having $R_f$ values identical to DNP alanine, DNP glutamic acid, and some DNP glycine, whereas similar amounts of cell walls and enzyme in the controls showed no visible DNP amino acid spots on the chromatograms. The areas of the spots were eluted, and the absorbancy at 420 mÅ was measured in the spectrophotometer.

Although CWSE-80 was purified 588-fold, it may consist of either one peptidase or several peptidases, e.g., alanine aminopeptidase, glutamic aminopeptidase, and a bridge-splitting enzyme.
PHAGE-INDUCED CELL WALL PEPTIDASE

the problem. We express our appreciation to M. A. Werckle, Nadja Shoemaker, and Paul Han for their help in performing some of the experiments.

LITERATURE CITED


