Specificity of a Bacteriolytic Enzyme from *Pseudomonas aeruginosa*¹

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A bacteriolytic enzyme isolated from shake-flask cultures of *Pseudomonas aeruginosa* and capable of lysing cells of *Staphylococcus aureus* was purified approximately 500-fold by passage through diethylaminoethyl cellulose and chromatography on carboxymethyl-cellulose. The purified enzyme was shown to act as an endopeptidase, cleaving the pentaglycine cross-bridges of the cell wall peptidoglycan at D-alanyl-glycine and glycyl-glycine linkages with the release of di-, tri-, and tetra-glycine fragments. Release of NH₂-alanine indicated weak *N*-acetylmuramyl-L-alanine amidase activity, but most of the residual peptide remained attached to the glycan. No hydrolysis of the glycan occurred. The lytic spectrum of the enzyme toward a variety of other cell walls of known peptidoglycan composition indicated relatively high specificity for peptidoglycans with polyglycine bridges.

In 1965, Zyskind et al. (23) reported the production of a staphylolytic enzyme system by a species of *Pseudomonas*. The enzyme has been purified over 300-fold by chromatography on phosphonic acid cellulose and Bio-Gel P2 and has been partially characterized (2). This report describes an alternative method of purification of the staphylolytic activity and determination of the chemical specificity of the purified enzyme. A preliminary report of this work has already appeared (M. Lache, J. W. Zyskind, and W. R. Hearn, Fed. Proc. 25:752, 1966).

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain X (22) was used for production of the lytic enzyme. Strain 655 of *Staphylococcus aureus* (15) was used as a substrate for assaying the lytic activity of a given enzyme sample. Strain Copenhagen of *S. aureus* was used to prepare cell walls for determining the chemical specificity of the lytic enzyme. Other cultures used in this study for determination of the lytic spectrum were supplied by D. D. Focht (Department of Bacteriology, Iowa State University), with the exception of *Micrococcus roseus* strain R27, which was kindly pro-

vided by J. F. Petit (Institut de Biochimie, Orsay, France). All microorganisms were maintained at 4 C on Brain Heart Infusion Agar slants.

Enzyme assays. Staphylolytic activity was measured by determining the rate of lysis at 25 C of a fresh suspension of lyophilized whole cells of *S. aureus* strain 655 in 0.01 M sodium phosphate buffer (pH 8.5). Initial turbidity of the suspension (total volume, 10.0 ml) was adjusted to an absorbancy of 0.50 at 535 nm, by using a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) with 18-mm cuvettes. Turbidity (*A*₅₃₅) was determined at various intervals, and one lytic unit (LU) was defined as the amount of activity necessary to cause a turbidity decrease of 0.001 absorbancy unit per min, by using the best straight line drawn after an initial lag period (Fig. 1). Specific activity of an enzyme preparation was defined as the number of LU per milligram of protein. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin (Armour) as standard.

Protease (caseinase) activity was determined by a modification of the procedure of Kunitz (10). The enzyme was added to 8.0 ml of 1% casein (Hammarsten, Merck) in 0.01 M sodium phosphate buffer (pH 8.5). The assay mixture was made to a volume of 10.0 ml with water and incubated at 25 C. After exactly 1 hr, a 2-ml sample was withdrawn, added to 3 ml of 10% trichloroacetic acid, placed in a boiling-water bath for 15 min, and then cooled to room temperature. The flocculated casein was removed by filtration through Whatman no. 1 paper, and the absorbancy of the filtrate at 280 nm was determined

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by using a Beckman DU spectrophotometer. One caseinase unit was defined as the amount of activity giving an increase of 0.010 absorbancy unit. The useful range of this assay was 10 to 60 caseinase units.

Production and purification of the enzyme. Stock cultures of *P. aeruginosa* strain X were transferred to fresh Brain Heart Infusion Agar slants and grown for 12 hr at 37°C in loosely capped vials. A loop of this culture was inoculated into each of four 2-liter flasks containing 1 liter of a Casitone-yeast extract broth (2% Difco Casitone, 0.2% yeast extract, 0.92% K_2HPO_4 , and 0.2% KH_2PO_4). The flasks were agitated on a gyratory shaker at 37°C. After 14 hr, the flasks were removed and quickly cooled in an ice bath. All subsequent operations were carried out at 4°C or below, including the initial centrifugation to remove the cells. To 4 liters of supernatant fluid cooled to 0°C, 3 volumes of precooled acetone was added dropwise over a 3-hr period with constant stirring. The mixture was allowed to stand an additional 1 hr to allow the precipitate to settle. Most of the supernatant fluid was removed by decantation, and the remainder was removed by centrifugation.

The acetone precipitate was dissolved in a minimum volume of chilled buffer (pH 8.5; 0.01 M sodium phosphate, 10^{-4} M in $MgCl_2$) and dialyzed against 10^{-5} M $MgCl_2$. In contrast to the results of Burke and Pattee (2), we found that the presence of Mg^{++} ion greatly stabilized lytic activity during all stages of purification. Hence $MgCl_2$ was incorporated in all solutions of the enzyme throughout our purification procedures. A small amount of material precipitating during dialysis was removed by centrifugation. At this stage, the enzyme could be lyophilized and conveniently stored as a crude preparation.

A 2-g portion of the crude lyophilized preparation was dissolved in 50 ml of water, and the solution was rapidly passed through a column (3 by 15 cm) of diethylaminoethyl (DEAE)-cellulose (Bio-Rad) pre-equilibrated with buffer (pH 8.5; 0.01 M sodium phosphate, 10^{-5} M in $MgCl_2$). The enzyme was quickly washed through the column with two 50-ml portions of water; we found that lytic activity began to decline when the enzyme stayed on a DEAE-cellulose column for more than 3 min. The column effluent was dialyzed against 10^{-5} M $MgCl_2$ and then lyophilized.

A column (1.2 by 16 cm) of carboxymethyl (CM)-cellulose (Bio-Rad) was equilibrated with 10^{-4} M $MgCl_2$. A 100-mg sample of the lyophilized DEAE-cellulose effluent was dissolved in 2 ml of buffer (pH 5.9; 0.04 M sodium phosphate, 10^{-4} M in $MgCl_2$) and applied to the CM-cellulose column. Elution was carried out with a continuous sodium phosphate buffer (10^{-4} in $MgCl_2$), going from pH 5.9 (0.04 M) to pH 8.9 (0.1 M). The flow rate varied from 15 to 36 ml/hr during a run. Protein content was estimated by continuous monitoring of absorbancy at 280 nm. Fractions of 5 ml were collected, and lytic activity was assayed on suitably diluted samples of each fraction.

Cell wall preparations. Cell walls of *S. aureus* strain Copenhagen were prepared by disrupting the post-log-phase cells with glass beads by standard pro-

cedures (5, 6, 19). The preparations were heated at 100°C for 20 min to destroy any autolytic enzymes (13) before being treated with trypsin and ribonuclease. The same procedures were used to prepare cell walls of *M. roseus* strain R27 and *Gaffkya tetragena* ATCC 10875. A similar preparation of cell walls from *M. radiodurans* was kindly provided by J.-M. Ghuysen (Liege).

Certain preparations of *S. aureus* cell walls were further treated with mild alkali to remove labile alanine ester moieties from the nonpeptidoglycan portion. Cell walls (38.5 mg) were suspended in 0.1 M sodium pyrophosphate buffer (pH 9.2) and held at 37°C for 4 hr. The walls were collected by centrifugation, washed three times with water, and suspended in 3.5 ml of water. The final suspension contained 10 mg of alanine ester-free cell walls per ml. Repeated treatment failed to remove any further alanine from the walls.

Analytical procedures. Solubilized reducing power was determined by a modification (8) of the ferricyanide procedure of Park and Johnson (14), by using *N*-acetylglucosamine as a standard. Precipitation during color development tends to occur with polymeric glycan and was prevented by the addition of 12 μ liters of 10 N H_2SO_4 after 10 min (18). Total solubilized amino groups were determined after treating samples with fluoro-2,4-dinitrobenzene by a modification (8) of an unpublished procedure of O. H. Lowry. A solution of D-alanine was used as a standard. Determination and quantitation of N-terminal amino acids were carried out on hydrolysates (6 N HCl, 16 hr, 110°C) of dinitrophenylated digests by a quantitative thin-layer chromatography technique (8). Samples were dinitrophenylated for 60 min at 60°C in 0.1 M triethylamine in 50% ethyl alcohol containing 0.05 M fluorodinitrobenzene. This procedure gives better yields of dinitrophenylglycine from oligoglycyl peptides (18). Standards were a mixture of free amino acids and N^{α} -(glycyl)-L-lysine. Determination and quantitation of C-terminal amino acids were carried out by hydrazinolysis (100°C, 8 hr), followed by removal of hydrazides with benzaldehyde, dinitrophenylation of the free amino acids, and quantitative thin-layer chromatography (8). Standards were a mixture of free amino acids, glycyl-D-alanine, and D-alanyl-glycine. Electrophoresis was carried out on a machine (Gilson Medical Electronics, model E) on Whatman no. 3MM paper, and peptides were detected by dipping in 0.2% ninhydrin in 95% ethyl alcohol containing 4% collidine, followed by heating for 10 min at 70°C (18).

Digestion procedures. For determination of chemical specificity of the lytic enzyme, alanine ester-free cell walls of *S. aureus* (5.51 mg) were suspended in sodium phosphate buffer (pH 7.0; 0.01 M, 10^{-5} M in $MgCl_2$), and enough enzyme was added to give a final concentration of 182 LU per ml. Total volume of the digest was 2.1 ml. The mixture was incubated at 25°C. Periodically, 204- μ liter samples were withdrawn, added to 784 μ liters of water, and boiled for 4 min to destroy lytic activity. Turbidimetric readings at 535 nm were made on the diluted and boiled sam-

ples after cooling, by using a Zeiss PMQ II spectrophotometer with microcuvettes having a 1-cm light path. Each sample was then centrifuged, and the supernatant fluid was used for determination of solubilized reducing power and solubilized amino groups. Separate samples were used without centrifugation for determination of C- and N-terminal amino acid residues liberated during digestion.

For determination of the lytic spectrum of the enzyme, washed cells of the various organisms were suspended in 10.1 ml of 0.01 M sodium phosphate (pH 8.5) to give an absorbance at 535 nm of 0.500, under the same conditions employed for the turbidimetric assay of the enzyme against *S. aureus*. Enzyme (240 LU) was added and turbidity readings were taken at appropriate intervals.

RESULTS

Purification of the lytic enzyme. Figure 1 shows that the assay for staphylolytic activity was satisfactory for amounts of 10 to 50 LU of enzyme, the rate of lysis being a linear function of the amount of enzyme added. Figure 2 shows a typical profile of elution of the activity from CM-cellulose, the final step in our purification scheme. Results at various stages of the purification procedure are presented in Table 1.

Chemical specificity of the enzyme. Table 2 shows the liberation of various functional groups during digestion of alanine ester-free walls of *S. aureus* by the purified enzyme. The data are presented as moles of functional group liberated per mole of lysine residues in the sample analyzed. Lysine residues are present in amounts of 0.47 μ mole per mg of alanine ester-free wall. It has been shown for *S. aureus* that there is one lysine residue per repeating structural unit of cell wall peptidoglycan (12) and that it is the peptidoglycan

FIG. 1. Influence of enzyme concentration on the rate of lysis of whole cells of *S. aureus* strain 655. The enzyme sample (IIIpd) assayed here was an eluate from DEAE-cellulose.

FIG. 2. Purification of the lytic enzyme by chromatography on CM-cellulose. Gradient elution by sodium phosphate containing 10^{-4} M $MgCl_2$, from pH 5.9 (0.04 M) to pH 8.9 (0.1 M).

TABLE 1. Summary of purification data for the lytic enzyme from *P. aeruginosa* strain X

Step	Specific activity ^a	Ratio of caseinase to LU ^b	Purification (fold)	Yield (% recovery)
Initial growth liquor	40	1.4	1	100
Acetone ppt.	185	1.4	4.6	107
Effluent from DEAE-cellulose	640	1.4	16.0	90
Active fractions from CM-cellulose	20,800	0.013	520	23

^a LU per milligram of protein. One LU is the amount of enzyme required to reduce the absorbance at 535 nm of a suspension of cells of *S. aureus* strain 655 by 0.001 absorbance unit per min.

^b One caseinase unit is the amount of enzyme required to increase the absorbance at 280 nm of trichloroacetic acid-solubilized casein by 0.01 absorbance unit in 60 min.

that confers structural rigidity upon the cell wall. Therefore, expressing our results relative to lysine is a convenient means of describing the extent of disruption of the cell wall peptidoglycan network in molecular terms.

The data presented show that solubilization of *S. aureus* alanine ester-free walls is accompanied by a parallel liberation of N-terminal amino groups.

A sample of the same preparation of alanine ester-free *S. aureus* cell walls was solubilized with the *Myxobacter* AL-1 peptidase, which is devoid of glycanase activity (21). The product had 0.06 mole of reducing power per mole of lysine, corresponding to a chain length of 15 to 20 hexosamines for the undegraded glycan (21). After 18 hr, the *Pseudomonas* enzyme digest also contained 0.06 mole of reducing power per mole of lysine,

TABLE 2. Liberation of functional groups during digestion of alanine ester-free cell walls of *S. aureus* strain Copenhagen by the *Pseudomonas* lytic enzyme

Time hr	Turbidity %	Total solubilized amino group ^a	N-terminal groups ^a		C-terminal groups ^a	
			gly	ala	gly	ala
0	100	0.14	0.20	0.09	0.05	0.24
1	30.6	0.22	— ^b	—	—	—
3	3.3	0.56	0.33	0.20	—	—
6	1.0	0.70	0.61	0.20	0.40	—
18	—	1.9	1.70	0.18	0.98	0.66

^a Expressed as moles per mole of lysine residues in cell walls.

^b Not determined.

showing that this enzyme is also devoid of glycanase activity.

S. aureus Copenhagen post-log-phase cell walls have a 75% average degree of peptide cross-linking (18), and this is reflected by the initial 20 to 24% N-terminal glycine and C-terminal alanine (Table 2). Solubilization was accompanied by the release of N-terminal glycine and an equivalent amount of C-terminal alanine plus glycine. Since no N-terminal lysine or glutamic acid was liberated, this indicates hydrolysis of D-alanyl-glycine and glycyl-glycine linkages. An average of 1.5 peptide bonds was hydrolyzed in each pentapeptide cross-bridge, giving a total of 1.7 free N- and C-terminal groups per bridge (Table 2), which corresponds to an average of 0.66 open D-alanyl-glycine and 0.94 open glycyl-glycine bonds in each cross-bridge. The original cell walls also contained 0.2 to 0.24 open (uncross-linked) D-alanyl-glycine bonds and small amounts of N-terminal alanine, possibly a residue of the ester-linked residues of the teichoic acid. N-terminal alanine showed a small increase during hydrolysis with the *Pseudomonas* enzyme, probably indicating weak amidase action. The results of these hydrolytic activities should be to release oligoglycine peptides, but to leave the bulk of the residual peptide attached to the glycan. Electrophoresis at pH 3.9 of the products of 18 hr of hydrolysis with the *Pseudomonas* enzyme showed spots of di-, tri-, and tetraglycine with their characteristic mobilities and initial yellow color with ninhydrin reagent containing collidine (18). After elution, acid hydrolysis (6 N HCl, 16 hr, 110 C), and thin-layer chromatography, these spots were found to contain only glycine. Determination of N-terminal glycine before and after hydrolysis confirmed the size of these peptides and showed the amounts of di-, tri-, and tetraglycine to be approximately

0.1, 0.4, and 0.25 mole per mole of peptidoglycan subunit, respectively. Most of the rest of the peptidoglycan remained near the origin during electrophoresis.

Lytic spectrum. Cells of *S. aureus* strain 655 or strain Copenhagen were lysed most rapidly, the turbidity being reduced to 10% its initial value within 30 min.

The enzyme was somewhat less active against whole cells of *M. roseus* ATCC 418, *G. tetragena* ATCC 10875, and *M. lysodeikticus* ATCC 4698; suspensions of these cells required 3 hr to be reduced to 20% of their initial turbidity.

The enzyme was inactive against whole cell suspensions of *Sarcina lutea* ATCC 272, *Streptococcus faecalis*, *S. bovis*, *S. durans*, *M. luteus*, *Bacillus subtilis*, *B. megaterium* strain KM, *B. cereus* strain T, *Lactobacillus casei* ATCC 334, *Escherichia coli* strain B, *P. aeruginosa* ATCC 10145, *Erwinia caratovora* ATCC 495, *Proteus vulgaris* ATCC 13315, *Shigella boydii* ATCC 9207, *Salmonella choleraesuis* ATCC 13312, *Serratia marcescens* strain Nima, *S. kilensis* ATCC 8101, *Leuconostoc mesenteroides* ATCC 12291, *Aeromonas liquefaciens* ATCC 14715, *Aerobacter aerogenes* ATCC 13048, and *Achromobacter viscosus* ATCC 12448.

A direct comparison was made of digestion by the enzyme of isolated cell walls of *S. aureus* strain Copenhagen, *G. tetragena* ATCC 10875, *M. radiodurans*, and *M. roseus* strain R27. Cell wall preparations at a concentration of 20 mg/ml were incubated at 25 C with 24 LU of enzyme in a total volume of 1.0 ml of 0.01 M sodium phosphate (pH 8.5). Turbidity readings at 535 nm were taken at intervals. Suspensions of the first three were reduced to 10% or less of their original turbidity readings within 10 min. The cell wall suspension from *M. roseus* exhibited no appreciable decrease in turbidity, even after 2 hr.

DISCUSSION

Bacteriolytic enzyme systems have been isolated from a large number of sources. Those characterized as specific enzymes have been found to act either by splitting the glycosidic linkages of N-acetylmuramic acid and N-acetylglucosamine in the glycan portion of the cell wall peptidoglycan or by splitting amide bonds of the peptide portion of the peptidoglycan. The latter group of enzymes can be divided into the endopeptidases, which split peptide bonds within the peptide network, and the amidases, which split a particular peptide bond between N-acetylmuramic acid of the glycan and the N-terminal L-alanine of the peptide portion of the peptidoglycan. This subject has been reviewed in detail (8, 17).

Until the isolation of the enzyme described in

this paper (2, 23), no bacteriolytic system had been isolated from the genus *Pseudomonas*. An earlier report of inhibition of *S. aureus* growth in mixed culture with a species of *Pseudomonas* ascribed the inhibition to competition for nutrients between the two microorganisms (22).

The lytic enzyme from *Pseudomonas* appears to exhibit a rather high specificity for polyglycine-type cross-bridges, which are a genus characteristic of the cell wall peptidoglycans of the staphylococci. The only other walls solubilized at rates comparable to that of *S. aureus* were those of *M. radiodurans* and *G. tetragena*. The cell wall peptidoglycan of *M. radiodurans* has been shown to contain intratetrapeptide diglycine bridges (17) in place of the pentaglycine bridges found in walls of *S. aureus*. The cell wall peptidoglycan from *G. tetragena* appears to have its peptide subunits cross-linked by bridges containing one serine and three glycine residues (M. Lache, unpublished data). The fact that the peptidoglycan from *M. radiodurans* contains ornithine in place of the lysine of the peptidoglycans of *S. aureus* and *G. tetragena* seems to have no effect on susceptibility to lysis by the *Pseudomonas* enzyme. It is noteworthy that *M. roseus* R27 walls, which have an intrapeptide bridge of the type L-alanyl-L-alanyl-L-alanyl-L-threonine (16), were not susceptible to lysis under the conditions employed. These observations are consistent with the observed mode of action of the *Pseudomonas* enzyme on *S. aureus* cell walls, its primary site of attack being the pentaglycine cross-bridges.

With respect to its specificity for polyglycine bridges, the *Pseudomonas* enzyme resembles the peptidase of the lysostaphin system (1). The pH requirements of the two enzymes are in the same range, but the activity of the lysostaphin peptidase is unaffected by changes of ionic strength, whereas the lytic enzyme from *Pseudomonas* requires very low ionic strength (2). The *Pseudomonas* enzyme, however, resembles most closely the L₁₁ system from *Flavobacterium* (9), which hydrolyzes D-alanyl-glycine and glycyl-glycine linkages in *S. aureus* Copenhagen cell walls with the release of di-, tri-, and tetraglycine, and also has weak amidase activity (9). However the pH optimum of the *Flavobacterium* system against *S. aureus* cell walls is a sharp 6.5, in contrast to the broad pH range of 6.5 to 8.5 exhibited by the *Pseudomonas* enzyme (2). A glycine bridge-splitting enzyme isolated from culture filtrates of *Streptomyces albus* G and designated "S. A. endopeptidase" (4, 7) differs from the *Pseudomonas* enzyme in having a pH optimum of 8.8 and in splitting D-alanyl-glycine but not glycyl-glycine linkages in *S. aureus*. It also hydrolyzes D-alanyl-L-alanine linkages in *M. roseus* R27 cell walls. Finally, an-

other enzyme that splits the polyglycine bridges of *S. aureus* has been isolated from culture filtrates of a *Myxobacter* species (21). This enzyme hydrolyzes all of the D-alanyl-glycine linkages in these bridges and then splits the opened chains a second time with the release of 1 mole of tri- or tetraglycine per bridge. It slowly hydrolyzes tetraglycine to diglycine and has a weak but highly effective amidase activity (18, 21). The *Myxobacter* enzyme, however, has protease activity and hydrolyzes cell walls of many organisms including *Arthrobacter crystallopoietes* (18), *S. epidermidis* (19), *M. lysodeikticus* (3), *L. casei*, and *B. sphaericus* (D. Tipper, unpublished data), all of which are not susceptible to the *Pseudomonas* enzyme.

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