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# Purification, Properties and Mechanism of Action of a Staphylolytic Enzyme Produced by *Aeromonas hydrophila*

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1. An enzyme produced by *Aeromonas hydrophila* and capable of lysing *Staphylococcus aureus* cells was purified 180-fold by gel filtration and chromatography on columns of AG-50 W resin. 2. Physical measurements on the purified enzyme suggest that it is a small basic protein with an isoelectric point between pH 9·0 and pH 9·5. 3. Maximum lytic activity was obtained in 20mM-tris–glycine buffer, pH 8·5, at 45°, with no detectable activity in the absence of a nitrogenous base. 4. The enzyme is active in the above buffer containing 1·5M-sucrose, and is useful for the preparation of protoplasts of *Staphylococcus aureus*. 5. Purified cell wall peptidoglycans of two strains of *Staphylococcus aureus*, differing in amino acid composition, were hydrolysed by the enzyme with the liberation of glycine oligopeptides, principally diglycine and triglycine. 6. Synthetic glycine oligopeptides larger than triglycine, but not polyglycine, were hydrolysed, as were a number of leucine-containing dipeptides and tripeptides, but no proteolytic activity could be demonstrated. 7. It is concluded that the enzyme is lytic towards *Staphylococcus aureus* because it splits the pentaglycine cross-links of the cell-wall peptidoglycan.

Over the past few years enzymes capable of lysing viable cells of *Staphylococcus aureus* have been isolated from a variety of sources, and in a number of cases considerable effort has been made to determine the mechanisms involved in the actual lytic process. [For a list of references, see the account by Strominger & Ghuysen (1967).] We reported (Coles & Gilbo, 1967) that culture supernatant fluids of a species of *Aeromonas* were capable of rapidly lysing live cells of *S. aureus*, and *prima facie* evidence that the active principle was an enzyme was subsequently provided (Gilbo, Beaton & Coles, 1967). The present paper reports the purification and further characterization of the lytic factor. This work was reported in part by Broad, Gilbo & Coles (1967).

## MATERIALS AND METHODS

The strains of *S. aureus* and *Aeromonas* sp. were as described previously (Coles & Gross, 1965; Coles & Gilbo, 1967; Gilbo *et al.* 1967). Further work on the taxonomy of the lytic organism has shown that it may be classified as *Aeromonas hydrophila* (Schubert, 1967).

Sephadex was obtained from Pharmacia AB, Uppsala, Sweden, and AG-50 W resin from Bio-Rad Laboratories, Richmond, Calif., U.S.A. DEAE-cellulose (floc form) was a Whatman product. Pentaglycine and glycyl-L-prolylglycylglycine were obtained from Mann Research Laboratories, New York, N.Y., U.S.A.; hexaglycine was from International Nuclear and Chemical Corp., City of Industry, Calif., U.S.A.; glycyl-L-lysine, L-lysyl-L-alanine, *N*-CBZ*-glycylglycine ethyl ester, *N*-CBZ-glycylglycine, *N*-CBZ-DL-alanylglycine amide and polyglycine were from Yeda Research and Development Co., Rehovoth, Israel. All other synthetic peptides used were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Triethylamine was redistilled before use and trimethylamine hydrochloride was converted into the free base by passing it through a column of Dowex 1 resin ($OH^-$ form).

Protein was determined by the micro biuret method of Ellman (1962) with bovine serum albumin as standard. The isolation of staphylococcal cell walls and peptidoglycan (mucopeptide) was carried out as described by Gilbo *et al.* (1967). Acid hydrolysis of peptides and peptidoglycan before amino acid analysis was performed by heating in either 4N-HCl for 6hr. at 100° or 5·7N-HCl for 16hr. at 110°. The resulting amino acids were identified and quantitatively determined with a Beckman model 120 amino acid analyser (Spackman, Stein & Moore, 1958). With the amino acid analyser glycine oligopeptides up to hexaglycine were easily separated from each other, identified and quantitatively determined. The order of elution was glycine, hexaglycine, pentaglycine, tetraglycine, diglycine and triglycine.

Peptidoglycan was digested with the lytic enzyme by incubating 100mg. in 20ml. of 20mM-trimethylamine–acetate buffer, pH 8·6–9·0, for 22hr. at 28° with $4 \times 10^4$–$10 \times 10^4$ units (as defined below) of enzyme. The digestion mixture was exhaustively dialysed against water in $\frac{18}{32}$ Visking tubing, and the diffusate was concentrated by

* Abbreviation: *N*-CBZ, *N*-benzyloxycarbonyl.

freeze-drying before analysis. The digestion was considered 'complete' when, on the addition of fresh lytic enzyme to the non-diffusible portion of the digestion mixture, no additional ninhydrin-positive compounds became diffusible. Synthetic peptides were tested as possible substrates for the lytic enzyme by incubating a 20mM solution of each peptide in 20mM-tris–acetate buffer, pH 8·6, for 16hr. at 28° with 8000 units of lytic enzyme/ml. Samples were analysed for split products by high-voltage paper electrophoresis, by paper chromatography or with the amino acid analyser.

Whatman no. 1 paper was used in descending paper chromatography in either (*a*) butan-1-ol–acetic acid–water (4:1:5, by vol.) or (*b*) butan-2-ol–aq. 90% (w/w) formic acid–water (50:11:6, by vol.). After paper chromatography or high-voltage paper electrophoresis, amino acids and peptides were detected by dipping the dried papers in an acetone–ninhydrin solution and heating them at 80°. Reducing groups were determined by the method of Park & Johnson (1949) and free amino groups by the unpublished procedure of O. H. Lowry (see Ghuysen & Strominger, 1963). Proteolytic activity was measured by the method of Anson (1939) with albumin as substrate.

*Assay of lytic activity.* For reasons that will become apparent below, the previously used method of assay (Coles & Gilbo, 1967) was modified to increase sensitivity. Suspensions (washed three times in water) of *S. aureus* in 20mM buffer (a mixture of 20mM-glycine and 20mM-tris to give final pH 8·5 at 45°) were made to give $E_{660}$ about 0·6 in a Bausch and Lomb Spectronic-20 colorimeter. Samples for assay of lytic activity (up to 0·5ml.) were added to volumes of this suspension to give a final volume of 5·0ml. One unit of lytic enzyme activity in this paper is defined as that amount causing a decrease in $E_{660}$ of 0·001 in this suspension in 15min. at 45°.

*Electrophoresis.* Column electrophoresis was carried out in an apparatus (type 3340C; LKB Produkter AB, Stockholm, Sweden) stabilized by means of a sucrose density gradient. The bottom electrode vessel (cathode) was filled to the level equal to the bottom of the electrophoretic column with 25% (w/v) sucrose in 0·5M buffer (0·5M-tris adjusted with acetic acid for pH 5–9; 0·5M-triethylamine adjusted with acetic acid for pH 9–10). In the anode compartment was placed a similar amount of 0·5M buffer. The remainder of the apparatus contained 0·2M buffer and the electrophoretic column was stabilized with a 5–20% (w/v) sucrose density gradient. The column was cooled by passing an ethylene glycol–water mixture at 0° through the cooling jacket. The enzyme sample ($2\times10^5$–$4\times10^5$ units) dissolved either in 3% (w/v) sucrose in 20mM buffer or in 20mM buffer containing sufficient sucrose to give the required density, was applied either to the top of the column or to a position midway down the column. Initial voltages of 200–1000v (current 20–70mA) were applied for 15–20hr. Fractions were collected at the end of this time and assayed for lytic activity.

High-voltage paper electrophoresis was carried out with a Savant model LT-36 instrument with strips of Whatman 3MM paper in pyridine–acetic acid–water buffer (1:10:89, by vol.), pH 3·5.

*Sedimentation velocity.* Determination of sedimentation velocity was carried out with a Spinco model E ultracentrifuge by the synthetic-boundary technique. Rotor temperature was regulated close to 20° and the rotor speed was 59780rev./min. Protein concentration was 0·58mg./ml. (lytic enzyme assay, $1\cdot8\times10^5$ units/ml.), and the solvent used was 4·5mM-triethylamine–acetate, pH 6·7.

## RESULTS

*Purification. A. hydrophila* was grown in 2% (w/v) Difco yeast extract medium (200ml./21. Erlenmeyer flask) for 20hr. at 28° on a shaker (New Brunswick Scientific Co.) for maximum yield of the lytic enzyme (Coles & Gilbo, 1967). Cells were removed by centrifugation in the cold and the supernatant fluid was adjusted with stirring at 4° to pH 9·0 with 1N-sodium hydroxide. All subsequent operations were carried out at 0–4°. The supernatant fluid was then stirred for 30 min. with DEAE-cellulose ($Cl^-$ form; 10g./l.) and filtered. The filtrate was treated once more with DEAE-cellulose under the same conditions. Treatment of the culture supernatant fluid with DEAE-cellulose under the above conditions removed about half the protein as measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951), but, because this method could not be used in the later stages of purification owing to interference by triethylamine, the determination of protein was carried out by the method of Ellman (1962). The filtrate from the second DEAE-cellulose treatment was concentrated by freeze-drying and further fractionated by gel filtration through Sephadex G-50. Samples of freeze-dried material (20g.) were dissolved in 20mM-glycine buffer (adjusted to pH 9·0 with sodium hydroxide) (30ml.) and applied to a column (125cm. × 5·5cm.) of Sephadex G-50, equilibrated with the same buffer. The column was eluted with 20mM-glycine buffer at a flow rate of approximately 75ml./hr. A typical elution pattern is shown in Fig. 1, which shows a small protein peak eluted near

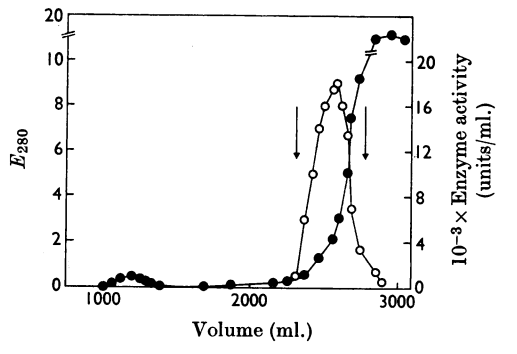


Fig. 1. Purification of the lytic enzyme on Sephadex G-50. Experimental details were as given in the text. ○, Lytic activity; ●, $E_{280}$. $E_{260}$ was slightly less than $E_{280}$ until about 2900ml. of buffer had passed through the column. The arrows indicate the active fractions that were pooled.

the solvent front, and the lytic activity, which moved only slightly ahead of the bulk of the low-molecular-weight compounds and yellow material.

Fractions between the arrows (Fig. 1) from two Sephadex G-50 columns were pooled and concentrated by freeze-drying to give a solution containing $8 \times 10^6$–$9 \times 10^6$ units of lytic activity in a maximum volume of 50ml. This solution was then applied to a Sephadex G-10 column (52cm. × 5cm.) and eluted with water. Active fractions, which were eluted near the solvent front, were pooled, diluted with water to 350ml. and adjusted to pH 7·5 with a few drops of 1N-sodium hydroxide. This step rapidly removed glycine and other salts from the enzyme, and was necessary for satisfactory performance of the following fractionation step. The enzyme solution (350ml.) was then passed through a column (130cm. × 2cm.) of AG-50W resin (X8, 100–200 mesh, triethylammonium form). The enzyme was adsorbed on the column, which was washed with water until the extinctions of the washings at 260mμ and 280mμ were negligible. A concentration gradient of triethylammonium acetate, made by pumping at 50ml. of 0·5M-triethylammonium–acetate, pH 8·5/hr. into a closed mixing chamber containing 2l. of water, was used to elute the enzyme from the column. The peak of activity was eluted after about 550–650ml. of solution had been passed through the column (Fig. 2). The concentration of triethylammonium–acetate at this point was approx. 0·15M. Fractions pooled as indicated by the arrows in Fig. 2 were either stored at −25° or, in some cases, concentrated by freeze-drying and subjected to filtration through Sephadex G-10 as described above to remove excess of triethylammonium–acetate. This latter Sephadex G-10 filtration also separated material with a high extinction at 260mμ, and the final product then had $E_{280}/E_{260} > 1$. The properties of the lytic enzyme described below were determined with enzyme eluted from AG-50W resin. A summary of the degree of purification and overall recovery of the enzyme is given in Table 1.

*Assay of lytic activity.* The turbidity of the standard *S. aureus* suspension used for assay of lytic enzyme, measured after 15min., decreased linearly with increasing amounts of enzyme only over a certain range (Fig. 3). For a quantitative assay, 50–175 units (i.e. an amount causing a change in extinction of 0·050–0·175 in 15min.) was required

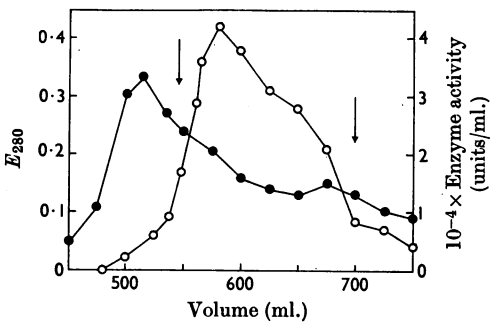


Fig. 2. Purification of the lytic enzyme on AG-50 W resin. Experimental details were as given in the text. ○, Lytic activity; ●, $E_{280}$. $E_{260}$ was slightly greater than $E_{280}$ in all fractions. The arrows indicate the active fractions that were pooled.

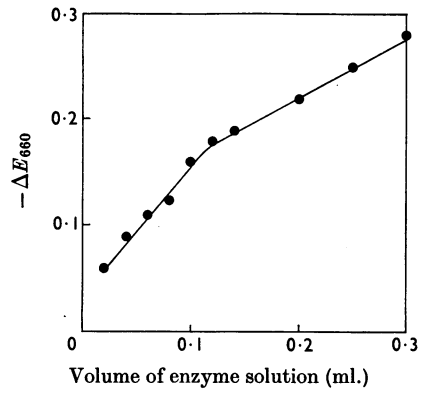


Fig. 3. Relationship between decrease of turbidity of *S. aureus* suspensions ($-\Delta E_{660}$) and amount of the lytic enzyme. Suspensions (5ml.) of *S. aureus* (8325) in 20mM-tris–glycine buffer, pH 8·5, initial $E_{660}$ 0·6, were incubated at 45° for 15min. with the volumes of enzyme solution indicated, and the decrease in $E_{660}$ was measured. The enzyme solution contained 92μg. of protein/ml.

Table 1. *Summary of the purification of the lytic enzyme*

| Stage of preparation | $10^{-2}$ × Concn. of enzyme (units/ml.) | $10^{-6}$ × Total enzyme (units) | Recovery (%) | Specific activity (units/μg. of protein) |
|---|---|---|---|---|
| Culture supernatant | 110 | 22 | 100 | 1·8 |
| Treatment with DEAE-cellulose | 99 | 19·8 | 90 | 1·8 |
| Filtration through Sephadex G-50 | 147 | 11·0 | 50 | 13·4 |
| Elution from AG-50 W resin | 180 | 2·7 | 12 | 330 |

Table 2. *Comparison of the activity of the lytic enzyme in various buffers*

*S. aureus* suspensions (5 ml.) of $E_{660}$ approx. 0·6 were prepared in the following buffers and incubated at 28° for 15 min. with 500 units of lytic enzyme. The buffers were 20 mM and were adjusted to pH 9·0 with either sodium hydroxide or acetic acid.

| Buffer | $10^3 \times -\Delta E_{660}$ |
|---|---|
| Tris | 120 |
| Glycine | 110 |
| Leucine | 110 |
| Collidine | 100 |
| Ethylamine | 50 |
| Diethylamine | 55 |
| Triethylamine | 55 |
| Trimethylamine | 65 |
| Benzylamine | 50 |
| Lutidine | 60 |
| Pyridine | 70 |
| 2-Amino-2-methylpropane-1,3-diol | 60 |
| Ammonium acetate | 20 |
| Ethanolamine | 10 |
| Sodium veronal | 15 |
| Sodium carbonate | 5 |
| Sodium borate | 0 |
| Sodium phosphate | 0 |

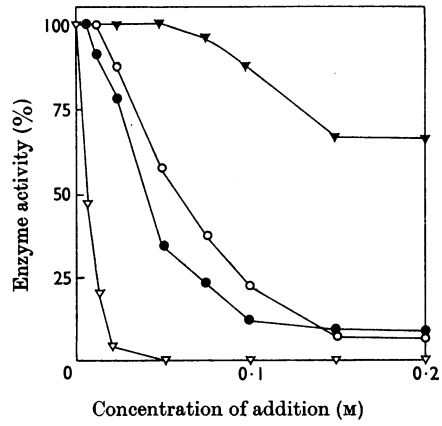


Fig. 4. Influence of concentration of various compounds on lytic activity. Suspensions (5 ml.) of *S. aureus* (8325), initial $E_{660}$ 0·6, either in water with the addition of glycine–glycine sodium salt buffer, pH 8·5, ○, or tris–acetate buffer, pH 8·5, ●; or in 20 mM-tris–glycine buffer, pH 8·5, with the addition of sucrose, ▼, or NaCl, ▽, were incubated for 15 min. at 45° with 100–200 units of the lytic enzyme. The maximum change in $E_{660}$ for each addition was taken as 100% enzyme activity.

in the assay system. Within this limitation the decrease in turbidity of a suspension of *S. aureus* under the stated conditions was found to be a satisfactory assay for our purpose. As has been found with other bacteriolytic systems (Kato *et al.* 1962; Whitaker, 1965; Zyskind, Pattee & Lache, 1965) the rate of decrease in turbidity of a bacterial suspension was not linear with time. However, with the amount of enzyme as indicated above, near-linearity is obtained during the initial 15 min. reaction period.

*Influence of buffer composition.* The composition of the medium has a pronounced effect on the activity of the lytic enzyme. Of a number of substances that buffer in the range pH 8·5–9·0, only nitrogenous bases permitted the lytic reaction to proceed, and even some of these did not support lysis (Table 2). The activity of the lytic enzyme was also strongly dependent on the concentration of the incubation medium, as shown in Fig. 4. Activity was at a maximum at comparatively low ionic strengths. The nature of the anion may also have an important effect. Thus tris–chloride and tris–acetate were much superior to tris–phosphate. Polyamino compounds such as lysine, spermine, spermidine and putrescine did not support the lytic process; they were actually strong inhibitors. In 20 mM-lysine–acetate buffer (20 mM-lysine neutralized to pH 8·5 with acetic acid), the enzyme exhibited only 25% of the activity shown in 20 mM-tris–glycine buffer at the same pH.

*Optimum temperature and heat stability.* Although the optimum temperature for production of the lytic enzyme by *A. hydrophila* was 28–30°, and almost none was produced at 37° (Coles & Gilbo, 1967), the optimum temperature for the activity of the enzyme was much higher (Fig. 5); it was 45–50°. When heated at 45° at pH 8·5 in the absence of substrate, the enzyme lost about 20% of its activity after 15 min., 50% after 1 hr. and 70% after 2 hr. The activity of the enzyme was completely destroyed in 15 min. when it was heated under the above conditions at 60°.

*Optimum* pH. Optimum activity of the enzyme in 20 mM-tris–glycine buffer at 45° was obtained at pH 8·0–8·6, with activity falling more sharply at lower rather than at higher pH values.

*Electrophoretic mobility.* Electrophoresis of the lytic enzyme at pH 5–10 showed that the active principle behaved as a cation at pH 9·0 and below and as an anion at pH 10. The mobility at pH 9·5 was slightly towards the anode and hence the isoelectric point must lie between pH 9·0 and pH 9·5.

*Sedimentation coefficient.* A sample of lytic enzyme eluted from AG-50W resin was concentrated by freeze-drying to a small volume, and the excess of triethylamine–acetate was decreased by passage through Sephadex G-10. This process was repeated and on ultracentrifugation of the material obtained, a single peak was observed with a $S_{20,w}$ of 1·1s.

*Effect of metal ions.* At 1 mM, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Fe^{2+}$ and $Mn^{2+}$ inhibited the lytic enzyme by

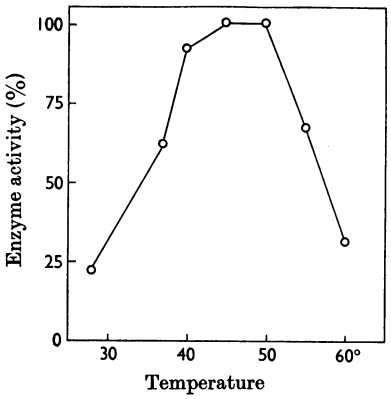


Fig. 5. Optimum temperature for enzyme activity. Suspensions (5ml.) of *S. aureus* (8325) in 20mM-tris–glycine buffer, pH 8·5 at 45°, initial $E_{660}$ 0·6, were incubated at the temperatures indicated for 15min. with 100–600 units of the lytic enzyme and the decrease in $E_{660}$ measured. Activity at the optimum temperature was taken as 100%.

60–70%. Inhibition by $Zn^{2+}$ at this concentration was over 90%, whereas $Co^{2+}$ inhibited by only 30%. At 0·1mM, $Zn^{2+}$ inhibited by 70%, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ inhibited by 30–40%, and $Co^{2+}$ and $Fe^{2+}$ increased the lytic rate by 10–20%. Mercuric chloride caused complete inhibition at 0·1mM.

*Gel filtration.* Fig. 1 shows that the lytic enzyme was considerably retarded on gel filtration through Sephadex G-50. Considerable retardation was also shown when the enzyme was filtered through Sephadex G-10, G-15 and G-25. Table 3 shows the volume of buffer required to elute the peak of activity through these gels and compares this volume with that required to elute some high- and low-molecular-weight materials. It has been observed previously (Miranda, Rochat & Lissitzky, 1962; Glazer & Wellner, 1962) that basic proteins are adsorbed to Sephadex gels from solutions of low ionic strength, and a similar situation evidently prevails with the lytic enzyme. Thus, with Sephadex G-15, the lytic enzyme was eluted by only 49ml. of 20mM-glycine containing 0·1M-NaCl, suggesting the involvement of ionic forces in the binding of the enzyme to the gel. However, considerable inhibition of enzyme activity was observed under these conditions.

*Inhibitors.* Incubation of the lytic enzyme for 30min. at 4° in 8M-urea at pH 8·5 caused only about 15% loss in activity. After dilution of the sample for assay the final concentration of urea was 20mM, which caused no loss of enzyme activity. The polyamines spermine and spermidine, as their phosphate salts, caused complete inhibition of activity at 1mM, whereas putrescine at the same concentration

Table 3. *Behaviour of the lytic enzyme on different Sephadex gels*

Sephadex gels were equilibrated with 20mM-glycine, pH 9, and poured into columns (35cm. × 2cm.) at 4°. Samples (0·5ml.) of blue dextran, maltose, glucose and the lytic enzyme ($2 \times 10^3$–$5 \times 10^3$ units) were applied separately to the top of the columns and eluted with the equilibration buffer. Fractions (1ml.) were collected and assayed by extinction (blue dextran), ability to reduce Fehling's solution (maltose and glucose) or lytic activity against suspensions of *S. aureus*.

| Substance | Elution volume (ml.) | | |
|---|---|---|---|
| Sephadex ... | G-10 | G-15 | G-25 |
| Blue dextran | 54 | 47 | 43 |
| Glucose | 75 | 86 | 128 |
| Maltose | 64 | 80 | 128 |
| Lytic enzyme | 64 | 80 | 123 |

caused about 50% inhibition. The enzyme was not inhibited by sodium azide, sodium iodoacetate, sodium arsenite or sodium *p*-chloromercuribenzoate at 1mM concentrations, whereas about 30% inhibition of activity was observed in the presence of 1mM-EDTA (tris salt).

*Protoplast formation.* Concentrations of sucrose below 50mM do not affect the activity of the lytic enzyme (Fig. 4). Higher concentrations up to 0·15M cause increasing inhibition up to about 30%, but further increases in sucrose concentrations to as high as 1·5M cause no further inhibition of lytic activity. In the absence of sucrose, the maximum concentration of cells in the lytic medium to obtain complete lysis in the minimum time is about 250–300$\mu$g./ml. ($E_{660}$ 0·6 in the Bausch and Lomb colorimeter), whereas in the presence of 1·5M-sucrose, cell concentrations as high as 2mg./ml. may be used. Osmotic lysis was tested for by dilution in 20mM-tris–glycine buffer. It was thus possible to prepare osmotically fragile protoplasts of *S. aureus* by incubating suspensions with lytic enzyme in 20mM-tris–glycine containing 1·5M-sucrose.

*Action of lytic enzyme on staphylococcal peptidoglycan.* Table 4 shows the amino acid composition of acid hydrolysates of purified peptidoglycan isolated from the two strains of *S. aureus* used. When peptidoglycan samples were incubated with lytic enzyme there was a rapid increase in the number of free amino groups with no change in the total reducing capacity of the digestion mixture, indicating peptidase activity rather than carbohydrase activity as the enzymic basis for the lytic process.

It was found that on dialysis most of the free amino groups newly liberated during digestion of peptidoglycan by the lytic enzyme were readily diffusible. The principal compounds in the diffusate were identified as glycine oligopeptides (Table 5),

Table 4. *Amino acid composition of the peptidoglycans of two strains of* S. aureus.

Peptidoglycan samples from *S. aureus* 8325 were hydrolysed in 5·7 N-HCl for 16 hr. at 110°. Samples from strain 2237 were hydrolysed in 4 N-HCl for 6 hr. at 100°. The results given are the means ± S.E.M. of three determinations on each sample of the composition in μmoles of amino acid/100 mg. of peptidoglycan. The ratio of each amino acid to glutamic acid is also given.

| | Amino acid composition | | | |
|---|---|---|---|---|
| *S. aureus* strain ... | 8325 | | 2237 | |
| Amino acid | (μmoles/100 mg.) | (ratio) | (μmoles/100 mg.) | (ratio) |
| Glutamic acid | 66·1 ± 1·7 | 1·0 | 53·4 ± 1·5 | 1·0 |
| Lysine | 86·5 ± 0·5 | 1·3 | 55·1 ± 2·6 | 1·0 |
| Alanine (total) | 170·5 ± 0·7 | 2·6 | 126·6 ± 3·6 | 2·4 |
| Glycine | 176·4 ± 5·8 | 2·7 | 230·1 ± 5·9 | 4·3 |
| Serine | 5·1 ± 0·5 | 0·1 | 13·3 ± 1·0 | 0·2 |

Table 5. *Liberation of glycine oligopeptides from peptidoglycan of* S. aureus *by digestion with the* Aeromonas *lytic enzyme*

Peptidoglycan from the two strains of *S. aureus* was incubated with the lytic enzyme, as described in the text. The digestions in Expts. 1 and 2 were 'complete'. In Expt. 3, after the first digestion (stage i), on the addition of fresh lytic enzyme as described in the text, further glycine oligopeptides became diffusible (stage ii). At the end of stage ii, it was shown that the digestion was 'complete'. Analytical values for each peptide are expressed as equivalent μmoles of free glycine, e.g., in Expt. 1, 7·7 μmoles of triglycine were recovered. The values quoted as 'Total after acid hydrolysis' refer to glycine recovered after acid hydrolysis (5·7 N-HCl for 16 hr. at 110°) of a sample of the total diffusate.

| | Peptides released (μmoles of glycine equivalent/100 mg. of peptidoglycan) | | | |
|---|---|---|---|---|
| *S. aureus* strain ... | 2237 | 8325 | | |
| | Expt. 1 | Expt. 2 | Expt. 3 | |
| Peptide | | | Stage i | Stage ii |
| Glycine | 1·6 | 1·0 | 0·6 | 0·8 |
| Diglycine | 49·0 | 65·6 | 30·5 | 21·7 |
| Triglycine | 23·1 | 27·3 | 20·9 | 5·6 |
| Tetraglycine | 17·2 | 2·8 | 25·1 | 1·5 |
| Pentaglycine | 6·2 | 4·0 | 1·1 | 3·3 |
| Total from each stage | | | 78·2 | 32·9 |
| Total | 97·1 | 100·7 | 111·1 | |
| Total after acid hydrolysis | 104·0 | 102·4 | 108·7 | |

Table 6. *Amino acid composition of the diffusate from the digestion of peptidoglycan of* S. aureus *by the lytic enzyme*

Conditions were as for Table 5; the amino acids were analysed after total acid hydrolysis. The results are expressed in μmoles of amino acid/100 mg. of peptidoglycan, and as percentages of the total amino acids.

| | Amino acid composition | | | |
|---|---|---|---|---|
| *S. aureus* strain ... | 2237 | | 8325 | |
| Amino acid | (μmoles/100 mg.) | (%) | (μmoles/100 mg.) | (%) |
| Glutamic acid | 6·4 | 4·7 | 4·8 | 3·6 |
| Lysine | 6·7 | 4·9 | 5·1 | 3·8 |
| Alanine | 13·0 | 9·5 | 9·2 | 6·9 |
| Glycine | 104·0 | 76·0 | 108·7 | 82·0 |
| Serine | 5·4 | 4·0 | 3·5 | 2·6 |
| Aspartic acid | 1·4 | 1·0 | 1·0 | 0·8 |
| Total | 136·9 | 100·1 | 132·3 | 99·7 |

mainly diglycine, triglycine and tetraglycine. These three compounds, together with pentaglycine and free glycine, accounted for all the glycine in the diffusate, and no other glycine peptides were detected. In Expt. 2 the digestion was 'complete' and it is evident that little more hydrolysis of glycine peptides could take place (see below). In Expt. 1, although the digestion was 'complete' in that no further liberation of diffusible ninhydrin-positive compounds took place, it is probable that further hydrolysis of the tetraglycine and pentaglycine would have occurred had the reaction mixture remained in contact with the lytic enzyme. In Expt. 3, after the first dialysis (stage i) and reincubation of the non-diffusible fraction with lytic enzyme, further diffusible ninhydrin-positive compounds were liberated (stage ii). Thus the content of each peptide in the digestion mixture varied with time of digestion.

Glycine is the principal amino acid component in the diffusate, accounting for about 80% of the total amino acid content (Table 6). Of the remaining amino acids found in the diffusate, some, notably alanine, were free, but most were in the form of small unidentified peptides, indicated by the pattern observed with the amino acid analyser.

*Action of lytic enzyme on other peptides.* Synthetic polyglycine and glycine oligopeptides from diglycine to hexaglycine were incubated with the lytic enzyme, and the products were examined either by paper chromatography or on the amino acid analyser. Polyglycine, diglycine and triglycine were not attacked, but pentaglycine was split to equimolar amounts of diglycine and triglycine. Both tetraglycine and hexaglycine were hydrolysed more slowly than pentaglycine. A mixture of glycine, diglycine and triglycine was produced from tetraglycine, and, in addition, tetraglycine was formed from hexaglycine. The proportions of each of the components varied during the incubation.

The following peptides were incubated with lytic enzyme under the conditions described, and no evidence of hydrolysis was obtained: glycyl-DL-serine, glycyl-DL-leucine, glycyl-DL-alanine, glycyl-DL-methionine, glycyl-DL-phenylalanine, glycyl-L-tryptophan, glycyl-L-tyrosine, glycyl-DL-valine, glycyl-L-prolylglycylglycine, DL-alanyl-DL-valine, DL-alanyl-DL-alanine, DL-alanyl-DL-asparagine, DL-alanyl-DL-leucine, DL-alanyl-DL-methionine, DL-alanyl-DL-phenylalanine, DL-alanylglycylglycine, L-lysyl-L-alanine, D-leucylglycine, D-leucyl-L-tyrosine, benzoyl-DL-alanine, *N*-CBZ-DL-alanylglycine amide, *N*-CBZ-glycylglycine, *N*-CBZ-glycylglycine ethyl ester.

When incubated with lytic enzyme L-leucylglycine was hydrolysed to leucine and glycine. DL- and D-Leucylglycylglycine were hydrolysed to leucine and diglycine, but the rate with the DL mixture was much faster than that with the D isomer. Leucine and tyrosine were formed from L-leucyl-L-tyrosine. No evidence of peptide-bond hydrolysis was found when gelatin, a protein rich in glycine, or albumin was incubated with lytic enzyme.

## DISCUSSION

The low sedimentation coefficient together with the results on the electrophoretic mobility and the small amount of inhibition caused by urea are taken as evidence that the lytic enzyme is a small basic protein with little secondary structure. Although they proved to be of great use in the purification of the enzyme, Sephadex gels proved of little value in determining its molecular weight (Andrews, 1964), because of interference by adsorption phenomena. Part of this adsorption is apparently due to ionic forces, as evidenced by the result obtained with Sephadex G-15 in the presence of 0·1M-sodium chloride, a concentration which, according to Andrews (1964), is sufficient to overcome ionic-adsorption problems. However, because of the high degree of inhibition of enzyme activity under these conditions, it was not possible to determine molecular size by carrying out chromatography in the presence of 0·1M-sodium chloride.

Enzymes capable of lysing bacteria do so by acting on the rigid peptidoglycan located within the cell wall. The enzymes so far studied fall into three classes (Strominger & Ghuysen, 1967): (1) glycosidases that hydrolyse the polysaccharide chains; (2) endopeptidases that hydrolyse the peptide cross-links; and (3) acetylmuramyl-L-alanine amidases that split the bond between polysaccharide and peptide. The enzyme produced by *A. hydrophila* clearly falls into class (2), as it is an endopeptidase capable of splitting the pentaglycine bridges of the cell-wall peptidoglycan. In this respect the *Aeromonas* enzyme is similar to those isolated from *Flavobacterium* (Kato *et al.* 1962), *Sorangium* (Gillespie & Cook, 1965; Whitaker, 1965; Tsai, Whitaker, Jurasek & Gillespie, 1965) and *Staphylococcus* (Schindler & Schuhardt, 1964; Browder, Zygmunt, Young & Tavormina, 1965), but the enzyme that perhaps bears the closest resemblance to the one described in the present paper is the lytic peptidase isolated from *Myxobacter* (Ensign & Wolfe, 1965, 1966; Jackson & Wolfe, 1968). In common with the *Myxobacter* enzyme (Jackson & Wolfe, 1968), the *Aeromonas* enzyme hydrolyses a number of glycine oligopeptides, with the notable exceptions of diglycine, triglycine and polyglycine. In contrast with the *Myxobacter* enzyme, however, the enzyme from *A. hydrophila* exhibits no proteolytic activity and is hydrolytic towards a number of short leucine-containing dipeptides and tripeptides.

Most of our knowledge on the composition and structure of the peptidoglycan of *S. aureus* has been gained from studies with the strain Copenhagen carried out by Strominger and Ghuysen and their co-workers (Strominger & Ghuysen, 1967). The molar ratios of the amino acid components of the cell walls of this strain (Mandelstam & Strominger, 1961) are similar to those reported for a number of other strains (Salton, 1964) and so may be considered typical of the species *S. aureus*. However, little comparative data on the amino acid composition of purified peptidoglycan of other strains is available. The peptidoglycan of strain Copenhagen contains (residues/residue of D-glutamic acid):1·16 D-alanine, 0·98 L-alanine (total alanine 2·14), 0·85 L-lysine and 4·61 glycine (Mandelstam & Strominger, 1961). This ratio is not very different from that obtained in the present study with strain 2237 (Table 4), which is considered therefore to be a typical *S. aureus* strain. Marked differences in the amino acid composition of the purified peptidoglycan from strain 8325 are apparent, however, particularly in the higher lysine and alanine contents coupled with the low glycine content. Despite these differences in amino acid composition, the lytic enzyme liberates very similar products from the peptidoglycans of both strains, and to the same extent (Tables 5 and 6): e.g. approx. 100 $\mu$moles of glycine are liberated from 100 mg. of peptidoglycan of both strains. However, this amount of glycine accounts for only 57% and 43% of the total glycine of the peptidoglycan of strains 8325 and 2237 respectively (Tables 4 and 5), and about half the glycine remains attached to a large molecular species.

The fundamental structure of the peptidoglycan of *S. aureus* strain Copenhagen is composed of polysaccharide chains consisting of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues (Strominger, Izaki, Matsuhashi & Tipper, 1967). A tetrapeptide sub-unit composed of L-alanyl-D-$\gamma$-isoglutaminyl-L-lysyl-D-alanine is substituted on the acid group of the *N*-acetylmuramic acid, and interpeptide bridges of pentaglycine link the sub-units through the $\epsilon$-amino group of L-lysine and the carboxyl group of D-alanine. The available data on the structure of the peptidoglycans of the two strains used in the present study are consistent with the existence of a similar fundamental structure in strain 2237. If we assume that this unit is the substrate for the enzymic action of the lytic enzyme on the peptidoglycan of *S. aureus* strain 2237, then the most prominent feature of the lytic reaction is the splitting of the pentaglycine bridges. A similar unit evidently forms part of the structure of the peptidoglycan of strain 8325. However, there is insufficient glycine present to link every tetrapeptide sub-unit by a pentaglycine bridge, and an additional type of bridge appears to exist in the peptidoglycan of this strain. From the results presented in Table 5, Expt. 1 and Expt. 3, stage i, it appears that tetraglycine (or two diglycine) units were split from the bridges at a faster rate than pentaglycine (or one diglycine and one triglycine) units. Triglycine and tetraglycine units could not have arisen from tetraglycine and pentaglycine units respectively, as insufficient free glycine was formed. However, from these results it cannot be concluded whether diglycine and triglycine units were split out of the bridges independently of tetraglycine and pentaglycine. Also, further work is needed to decide whether different types of glycine bridges occur in the peptidoglycan or whether the 50% residual glycine is the remaining part of the pentaglycine bridges after diglycine and triglycine units have been removed. Ghuysen, Tipper, Birge & Strominger (1965) also suggested, on the basis of the differential action of peptidases from *Streptomyces*, the possibility of at least two types of glycine cross-bridges in the peptidoglycan of *S. aureus* Copenhagen.

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