Biochem. J. (1968) **106**, 69
Printed in Great Britain

The Purification and Properties of Two Staphylolytic Enzymes from *Streptomyces griseus*

BY J. B. WARD* AND H. R. PERKINS†
Twyford Laboratories, Twyford Abbey Road, London, N.W. 10

(*Received* 6 *June* 1967)

1. Two staphylolytic enzymes have been purified from cultures of a soil isolate of *Streptomyces griseus*. 2. The purified enzymes were shown to be basic proteins of low molecular weight. Each enzyme released *N*-acetylmuramic acid reducing groups from the cell walls of *Staphylococcus aureus*. 3. The enzymes lysed whole staphylococci best at higher pH values and lower ionic strengths than when the substrate was isolated cell walls or purified mucopeptide. 4. Added teichoic acid did not inhibit the enzymes, but it formed an ethanol-precipitable complex with them. 5. The possibility that teichoic acid on the surface of whole cells prevents the access of the enzymes to their mucopeptide substrate is discussed.

The development in recent years of knowledge of the structural chemistry of the bacterial cell wall has been accompanied by an increasing interest in the enzymic lysis of the cell wall (Salton, 1964; Martin, 1966). Such enzymes have also been used for complete removal of the cell wall, making possible quantitative isolation and investigation of the intracellular components of the cell. One of the most frequently used enzymes for this purpose is egg-white lysozyme (McQuillen, 1956, 1960; Weibull, 1956). However, many bacteria are resistant to the lytic action of this enzyme and investigation of their cell-wall and intracellular components requires the isolation of suitable lytic enzymes. *Staphylococcus aureus* has been known for many years to be lysozyme-resistant (Thompson & Khorazo, 1935), and other staphylolytic enzymes have been sought. These have been isolated from bacteria (Kotani, Hirano, Kitaura, Kato & Matsubara, 1959; Schindler & Schuhardt, 1964), *Streptomyces* (Ghuysen, 1957) and fungi (Hash, 1963).

This report, a brief account of which has already been published (Ward & Perkins, 1966), concerns the purification and properties of two staphylolytic enzymes produced by *Streptomyces griseus*.

MATERIALS AND METHODS

Cell walls. Staphylococcus aureus N.C.T.C. 8418 was grown at 37° in shaken flask cultures to stationary phase, in nutrient broth containing 1% glucose added as a sterile solution. Cell walls were prepared essentially as described by Allsop & Work (1963).

Measurement of staphylolytic activity. Stationary-phase cells of *Staph. aureus*, washed and freeze-dried from water, were used as a substrate (Hash, 1963). All assays of an experimental series were performed on the same batch of cells. Lytic activity was measured in 8ml. of 0·01M-phosphate buffer, pH 7·5, containing sufficient cells (2·8–3·2mg. dry wt.) to give an initial turbidity of 3·0 (EEL colorimeter, Evans Electroselenium Ltd., Halstead, Essex; filter OGR1). The tubes were incubated at 37° and readings of the turbidity were made at 5min. intervals over a period of 30min. One unit of lytic activity was defined as the amount of enzyme causing a decrease of 1% in the turbidity/min. under these conditions.

Analytical methods. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) as a standard. *N*-Acetylamino sugars were estimated by the method of Aminoff, Morgan & Watkins (1952) with a heating time of 12min., as described by Perkins (1960). Free amino groups were measured by reaction with 1-fluoro-2,4-dinitrobenzene, as described by Ghuysen & Strominger (1963).

Ultracentrifugation. The purified concentrated enzymes 1 and 2, dissolved in 0·05M-phosphate buffer, pH 7·0 (protein concentration approximately 0·5%), were centrifuged at 42040 and 59780rev./min. at 17·5° and 21·8° respectively in the An-D rotor of a Spinco model E analytical ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.). The sedimentation coefficient of the protein at 20° was calculated and correction made for the effect of the buffer salt.

Electrophoresis. Electrophoresis of the purified enzymes was performed on 8% (w/v) polyacrylamide gel (1·67% cross-linking) with 0·015M-acetate buffer, pH 4·5, 0·05M-phosphate buffer, pH 7·0, and 0·05M-tris–HCl buffer, pH 8·5, at room temperature in the apparatus described by Ornstein & Davis (1962). The gels were equilibrated by electrophoresis with a current of 8mA/tube at 110v for 30min.

* Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo., U.S.A.

† Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

Samples (5–20 μl.), containing 50–100 μg. of protein, were mixed with glycerol (10%, v/v) and layered under the buffer on to the surface of the gel. After further electrophoresis for 45–95 min., the gels were stained with Amido Black 10B (1%, w/v, in 10%, v/v, acetic acid), and destained by washing in methanol–acetic acid–water (5:1:5, by vol.), in which the gels were also stored.

Determination of molecular weight. The molecular weights of the enzymes were estimated by gel filtration as described by Andrews (1964). A column (59 cm. × 1·5 cm.) of Sephadex G-100 [Pharmacia (G.B.) Ltd., London, W. 13] was equilibrated with 0·1 M-KCl in 0·05 M-tris buffer, pH 7·5 (or, in some experiments, pH 9·0). A 1·0 ml. sample containing 2·0 mg. of enzyme protein was eluted at room temperature with the KCl–tris buffer at a flow rate of 20–25 ml./hr. Fractions (2·0 ml.) were collected automatically and assayed for extinction at 280 mμ. Those fractions containing absorbing material were assayed for lytic activity against cell walls. The molecular weights of the enzymes were determined by comparing their elution volumes with those of several proteins of known molecular weight.

Effect of pH *and ionic strength on lytic activity.* The effect of both pH and ionic strength on lytic activity of the two enzymes against both purified cell walls and fresh whole cells of *Staph. aureus* was determined by a modification of the staphylolytic assay previously described. Cells harvested in the late exponential phase of growth were washed three times in ice-cold water and finally resuspended in water.

The following buffers were used either at constant pH with variable ionic strength or at constant ionic strength and variable pH: *A*, sodium acetate–acetic acid (Boyd, 1945); *B*, K_2HPO_4–KH_2PO_4 (Boyd, 1965); *C*, tris–HCl (Long, 1961). To 2·5 ml. of double-strength buffer were added 2·0 ml. of diluted enzyme solution containing a constant amount of protein and 0·5 ml. of a suspension of either whole cells or purified cell walls, such that the initial turbidity of the mixture was 0·5 when measured in a 1 cm. cell at 650 mμ (Unicam Spectrophotometer SP. 600). The final concentration in μg. of dry material/ml. was 250–300 for whole cells and 550–600 for cell walls. The tubes were incubated in a water bath, at 37° for whole cells and at 25° for purified walls. These temperatures were chosen so that comparable degrees of lysis were achieved in the standard period of 30 min. The temperature used did not affect the optimum pH or ionic strength. Enzymic activity was expressed as the percentage decrease in the turbidity after 30 min. incubation.

Cell-wall composition and characterization of the products of lytic action. Purified cell walls (40 mg.) were incubated at 37° for 24 hr. with each of the lytic enzyme preparations at a concentration of 50 μg./ml. in buffer (4·0 ml.) of optimum pH and ionic strength. Estimations of free amino groups and *N*-acetylhexosamine were performed on the supernatant solutions of samples centrifuged at 10000 *g*. The resultant clear solutions were dialysed against water (two changes of 40 ml.) and the non-diffusible material was retained and freeze-dried; the diffusate, which contained no detectable *N*-acetylhexosamine, was discarded.

Samples of the purified cell walls and non-diffusible material from enzyme digests were resuspended in water and treated with excess of $NaBH_4$, as described by Salton & Ghuysen (1960). Salts were removed from the preparations by dialysis against several changes of water and samples were hydrolysed in sealed tubes with 4 N-HCl for 4 hr. at 105°. The HCl was removed *in vacuo* and the hydrolysates were examined by two-dimensional chromatography on Whatman no. 4 paper with pyridine–water (4:1, v/v) and 2,6-lutidine–water (4:1, v/v). Amino acids and amino sugars were detected with ninhydrin. In addition, hexosaminitols were detected by spraying with periodate followed by benzidine (Viscontini, Hoch & Karrer, 1955).

Muramicitol and glucosaminitol. These were prepared by the reduction with $NaBH_4$ of muramic acid and *N*-acetylglucosamine. The resultant solutions were passed through a column (5 cm. × 0·5 cm.) of Zeokarb 225 (H^+ form). The eluates and column washings (water for *N*-acetylglucosaminitol, dil. NH_3 for muramicitol) were evaporated to dryness, and boric acid was removed by repeated evaporation of added methanol. The *N*-acetylglucosaminitol was deacylated by hydrolysis in 2 N-HCl for 2 hr. at 105°, and the HCl was removed by repeated evaporation *in vacuo* of small quantities of water. The purity of each preparation was checked by paper chromatography.

Culture of Streptomyces griseus. *Streptomyces griseus*, kindly identified by Professor E. Küster, was isolated from a soil sample on agar plates containing staphylococcal cell walls (Salton, 1955). Stock cultures were maintained on nutrient agar slopes stored at 4°. A variety of media supporting growth of the organism were tested but lytic enzymes were only produced in cultures grown on the medium containing feather hydrolysate, casein and salts (Ghuysen, 1957). Growth on media containing staphylococci or their isolated cell walls did not enhance the yield of lytic activity. Cultures were grown on a rotary shaker in 2 l. Erlenmeyer flasks containing 1 l. of medium. These were shaken at 120 rev./min. for 60–72 hr. at 30°; the inocula were each 10 ml. of a 24 hr. culture of the organism growing on the same medium. Larger batches (15 l.) were grown for 60–72 hr. at 30° in a fermenter of 30 l. capacity. The aeration rate was such that it maintained a pressure inside the vessel of 10 lb./in.^{2} and a flow rate of 5 l./min. The vessel was inoculated with 200 ml. of a 24 hr. culture of the organism. Antifoam [70% (v/v) silicone MS antifoam A in silicone fluid MS 200/10CS (Pirt & Callow, 1958)] was added automatically when foaming occurred.

Purification of the lytic enzymes. (1) Concentration. The organisms were removed from 15 l. of culture, after 58 hr. at 30°, by centrifuging at 9000 *g*. in a Sharples centrifuge. The supernatant was filtered, 2% Hyflo Super-Cel (Hopkin and Williams Ltd., Chadwell Heath, Essex) being added as a filter aid, and the filtrate was brought to 4°. Water was removed in a climbing-film evaporator (Q.V.F. Ltd., Stone, Staffs.) at a rate approaching 12 l./hr. and a temperature not exceeding 35°. With precooled culture filtrate the bulk of the liquid in the apparatus remained below 30°. In this step the culture filtrate was concentrated tenfold with only slight loss of lytic activity.

(2) Precipitation with ammonium sulphate. Solid $(NH_4)_2SO_4$ was added to the concentrated culture filtrate to 60% saturation. After the mixture had stood overnight at 4°, 1% Hyflo Super-Cel was added as a filter aid and the precipitate was collected by filtration. The resulting dark-brown filter-cake was suspended with stirring in 300 ml. of cold 0·2 M-phosphate buffer, pH 7·0. After 30 min., the suspension was filtered through a coarse sintered-glass filter and the Celite was washed with a small amount of the buffer. The combined filtrate and washings were dialysed

against 0·2 M-phosphate buffer (three changes of 10 l. over a 36–48 hr. period). Insoluble material in the non-diffusible portion was removed by centrifuging at 10000*g* for 20 min. The supernatant contained 78% of the original lytic activity.

Chromatography on CM-cellulose. A column (25 cm. × 2 cm.) of CM-cellulose was washed with 0·02 M-phosphate buffer, pH 7·0. The redissolved and dialysed $(NH_4)_2SO_4$ precipitate (375 ml.) was applied to the column. The column was eluted with 0·02 M-phosphate buffer, followed by 0·05 M-phosphate buffer and finally with this buffer containing 0·2 M-NaCl. The column effluent was collected in fractions (25 ml.) and samples were tested for extinction at 280 mμ and staphylolytic activity. Enzymically active fractions were eluted in both the 0·05 M-phosphate buffer eluate and the 0·2 M-NaCl eluate. These fractions, which contained 14% and 29% respectively of the lytic activity applied to the column, were combined and designated enzyme 1 (0·05 M-phosphate eluate) and enzyme 2 (0·2 M-NaCl eluate).

The combined active fractions from CM-cellulose columns were dialysed against dry Carbowax 4000 (Union Carbide Chemicals Co. Ltd., London, W. 1) until the volume had decreased fivefold. This solution was dialysed in fresh tubing against 3 l. (three changes of 10 l.) of 0·02 M-phosphate buffer. A small quantity of insoluble material present in the concentrate of enzyme 1 was removed by centrifuging at 25000*g* for 30 min. No loss of staphylolytic activity occurred during this treatment.

The concentrated and dialysed solution of enzyme 1 (68 ml.) was rechromatographed on a column of CM-cellulose (20 cm. × 1·5 cm.). The column was eluted with 0·02 M-phosphate buffer, pH 7·0, and then successively with 0·03 M-, 0·04 M- and 0·05 M-phosphate buffers. Fractions (25 ml.) of the column effluent were assayed as described above. The bulk of the lytic activity was eluted with 0·03 M-phosphate buffer. Rechromatography of enzyme 2 was carried out on a column of CM-cellulose (10 cm. × 1·5 cm.) washed with 0·05 M-phosphate buffer, pH 7·0. The concentrated and dialysed solution of enzyme 2 (12 ml.) was applied to the column. The column was eluted with 0·05 M-phosphate buffer and then with the same buffer containing 0·2 M-NaCl. Fractions (5 ml.) were collected and assayed for lytic activity. For each enzyme, the enzymically active fractions were combined, dialysed against water and freeze-dried to yield a white powder. Rechromatography of enzyme 2 gave only marginal increases (1–7%) in specific activity and was usually omitted from the purification procedures.

RESULTS

A strain of *Streptomyces griseus* that produced staphylolytic enzymes was isolated. Two of these enzymes were purified by ammonium sulphate precipitation followed by column chromatography on CM-cellulose (Table 1). Electrophoresis on polyacrylamide gels suggested that each enzyme was a pure basic protein.

Synergistic action has been reported between lytic enzymes purified from *Streptomyces albus* G (Salton & Ghuysen, 1957) and from the digestive juice of *Helix pomatia* (Takeda, Strasdine, Whitaker & Roy, 1966). However, Hash (1963) found only an additive effect with the two purified enzymes from *Chalaropsis* sp. In view of the low recovery of lytic activity in the two purified fractions from CM-cellulose chromatography the possibility that some similar synergistic action also occurred in the lytic enzymes of *Streptomyces griseus* was considered.

Purified enzymes 1 and 2 were assayed singly and as a mixture, and the rates of lysis were additive. When an appropriate proportion of the protein not absorbed by the CM-cellulose column was also added to the mixture, the lytic activity was 47% greater than that of the sum of the activities of the three components. Under these conditions the total activity recovered from the CM-cellulose column would represent about 60% of that applied. It

Table 1. *Steps in the purification of staphylolytic enzymes from* Streptomyces griseus

Details of each step are given in the Materials and Methods section. Enzyme activity was estimated by the decrease in turbidity of a suspension of freeze-dried *Staph. aureus* cells. The purification factors quoted are based on the assumption that the whole of the initial activity was due to each enzyme separately. If the initial activity could be proportioned in the ratio 1:2 between enzyme 1 and enzyme 2 respectively then the final purification for each enzyme becomes 28-fold for enzyme 1 and 76-fold for enzyme 2.

Step no.	Preparation tested	Total vol. (ml.)	Total protein (mg.)	Lytic activity (units)	Specific activity (units/mg.)	Recovery	Purification factor
1	Crude culture filtrate	15000	16125	31500	1·95	100	1
2	Concentrated culture filtrate	1510	14670	27150	1·85	86·3	0·95
3	60%-satd. $(NH_4)_2SO_4$ precipitate	375	4515	24600	5·45	78·0	2·79
4	CM-cellulose eluate						
	Enzyme 1 (0·05 M-phosphate buffer)	300	365	3585	9·85	11·4	5·05
	Enzyme 2 (+0·2 M-NaCl)	100	71	6800	96·0	21·6	48·8
5	Rechromatography; CM-cellulose eluate						
	Enzyme 1 (0·03 M-phosphate buffer)	150	179	3290	18·4	10·5	9·45
	Enzyme 2 (+0·2 M-NaCl)	50	66	6500	98·5	20·6	50·5

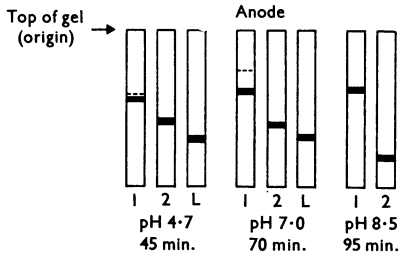

Fig. 1. Electrophoresis of lytic enzymes on polyacrylamide gel. The samples were: (1) enzyme 1; (2) enzyme 2; and (L) egg-white lysozyme.

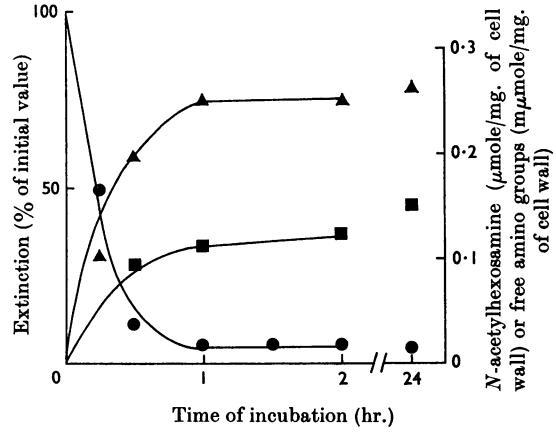

Fig. 2. Action of enzyme 2 on the isolated cell walls of *Staph. aureus*. The cell walls (1 mg./ml.) were incubated at 37° with enzyme (50 μg./ml.) in acetate buffer, pH 4·7 and *I* 0·1. The decrease in turbidity (●) was followed, together with the release of material reacting as *N*-acetylhexosamine (▲) (μmole/mg. of cell wall) and free amino groups (■) (mμmole/mg. of cell wall).

seems likely that the culture filtrate contained enzymes capable of attacking the staphylococcal cell wall that are not absorbed by CM-cellulose under the conditions used.

Properties of the purified enzymes

The electrophoretic properties of the two purified enzymes were compared at pH 4·7, 7·0 and 8·5, in polyacrylamide gels (Fig. 1). At these pH values each enzyme ran as a cationic component. There were faint traces of contaminating protein in enzyme 1, but none in enzyme 2. When the experiment was performed with the electrodes reversed, no protein bands appeared at any of the above pH values. At pH 10, enzyme 1 was negatively charged, but the position of enzyme 2 was difficult to determine. Thus enzyme 2 is the more basic of the two *Streptomyces* enzymes, but both enzymes are less basic than egg-white lysozyme.

In the ultracentrifuge the purified enzymes each gave only a single peak, the material in the peaks having a sedimentation coefficient, $S_{20,w}$, of 1·32s for enzyme 1 and 2·59s for enzyme 2.

The molecular weights of the enzymes were determined by comparing the elution volume of the enzyme from Sephadex G-100 with those of several proteins of known molecular weights: cytochrome *c* (from horse heart) (Margoliash, 1962); ribonuclease (Hirs, Moore & Stein, 1956); cytochrome *c* dimer (prepared from horse heart cytochrome *c*) (Margoliash & Lustgarten, 1962); and ovalbumin (Warner, 1954). A linear relationship exists between the elution volume and the logarithm of the molecular weight of the proteins tested (Andrews, 1964). Calculations of the molecular weights of the purified enzymes from the elution volumes either at pH 7·5 or at pH 9·0 gave values of 11800 for enzyme 1 and 13000 for enzyme 2.

Unexpectedly, calculation of the molecular weight of egg-white lysozyme from the elution volume gave a molecular weight of 7600, contrasting with the established value of 14307 (Canfield, 1963). Similar results were obtained at both pH values and over a range of concentrations of the eluting buffer up to 1·0 M-potassium chloride. These higher concentrations would be sufficient to counteract any simple ionic association of lysozyme and the acidic groups of the gel (Whitaker, 1963). Indeed, the result did not seem to be attributable to the gel properties, since similar results were obtained with columns of polyacrylamide (Bio-Gel P-30, Calbiochem Ltd., London, W. 1) or agar (Sagarose S-10, Seravac Laboratories Ltd., Maidenhead, Berks.).

Identification of the lytic enzymes as muramidases. Enzymes 1 and 2 were tested for quantitative release of *N*-acetylhexosamines and free amino groups from cell walls of *Staph. aureus*. The results obtained with enzyme 2 are given in Fig. 2; those obtained with enzyme 1 were essentially the same. Hence both enzymes are *N*-acetylhexosaminidases. Enzyme 2 consistently released more *N*-acetylhexosamine per mg. of cell wall than did enzyme 1. The very small increase in free amino groups can be accounted for in both cases by the dissolution of the cell wall, since the assay measured all free amino groups present in the final supernatant. However, the products of lysis by either enzyme were polymeric, since it was found that all the material reacting as *N*-acetylhexosamine was non-diffusible.

The *N*-acetylhexosamine estimated could be either *N*-acetylmuramic acid or *N*-acetylglucos-

amine depending on the specificity of the enzyme. Hence lysates reduced with sodium borohydride and acid-hydrolysed should yield either muramicitol or glucosaminitol. Two-dimensional paper chromatography of hydrolysates of cell wall before treatment with enzyme 2 revealed only the amino sugars and amino acids found in the staphylococcal cell wall. After lysis and reduction much of the muramic acid had disappeared, to be replaced by a new spot running in the position of authentic muramicitol. Chromatography of walls treated with enzyme 1 gave the same result.

These results indicate that both enzymes liberate reducing end groups of *N*-acetylmuramic acid residues. Thus both enzymes cleave the amino sugar backbone of the mucopeptide at the same position that egg-white lysozyme hydrolyses the mucopeptide of *Micrococcus lysodeikticus* (Salton & Ghuysen, 1959; Perkins, 1960).

Ionic-strength and pH *optima.* The inhibitory effects of high ionic strength on the lytic activity of enzymes from a variety of sources have been described (Ghuysen, 1957; Kato *et al.* 1962; Hash, 1963). Initial experiments on the assay of lytic activity in crude culture filtrates had shown inhibition of lysis by a variety of buffers of different concentrations. Their subsequent removal by dialysis prevented this inhibition. However, prolonged dialysis of the culture filtrate against water resulted in a marked decrease in lytic activity against freeze-dried cells of *Staph. aureus*. This activity could be restored by the presence of various salts at approximately neutral pH. Restoration of the lytic activity appeared to be independent of the nature of the salts and was most probably a function of ionic strength.

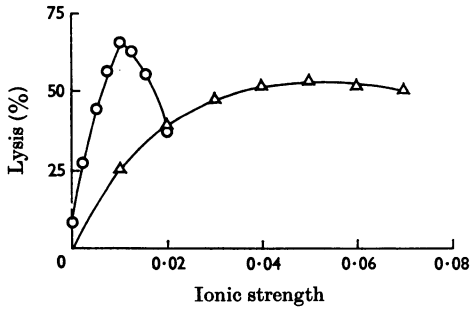

Fig. 3. Ionic-strength optimum of enzyme 1 for lytic activity against whole cells and isolated cell walls. Enzyme protein concentration was 25 μg./ml. for lytic activity against whole cells (○), incubated at 37° in phosphate buffer, pH 7·5, and 15 μg./ml. for activity against isolated cell walls (△), incubated at 25° in phosphate buffer, pH 6·5. The ordinate represents the percentage of lysis after 30 min. incubation.

The effects of ionic strength and pH on the lysis of *Staph. aureus* cells and cell walls by the purified enzymes were determined. Samples of the two enzymes were exhaustively dialysed against water before being assayed against one or other of the substrates. As indicated in Figs. 3 and 4, lysis of whole cells by either enzyme occurred only at low ionic strengths, the optima being *I* 0·011 for

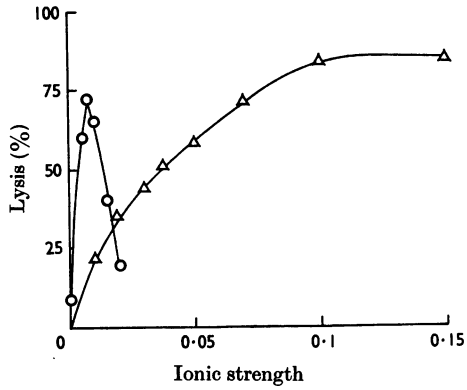

Fig. 4. Ionic-strength optimum of enzyme 2 for lytic activity against whole cells and isolated cell walls. Enzyme protein concentration was 10 μg./ml. for activity against whole cells (○), incubated at 37° in phosphate buffer, pH 7·5, and 5 μg./ml. for activity against isolated cell walls (△) incubated at 25° in acetate buffer, pH 4·5. The ordinate represents the percentage of lysis in 30 min.

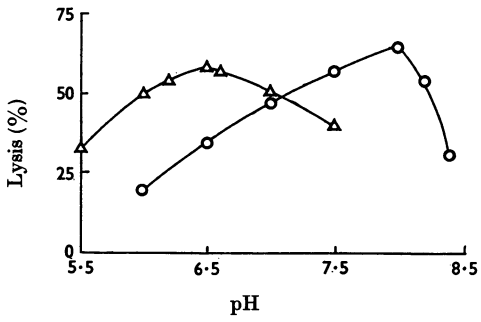

Fig. 5. pH optimum of enzyme 1 for lytic activity against whole cells and isolated cell walls. Enzyme protein concentration and experimental conditions were the same as those given in Fig. 3, except that the buffers used were of constant ionic strength, *I* 0·01 for whole cells (○) and *I* 0·05 for isolated cell walls (△). Acetate, phosphate or tris buffer was used according to the pH value required, results in overlapping regions being repeated with both buffers. The rate of lysis was unaffected by the type of buffer, only the ionic strength being important.

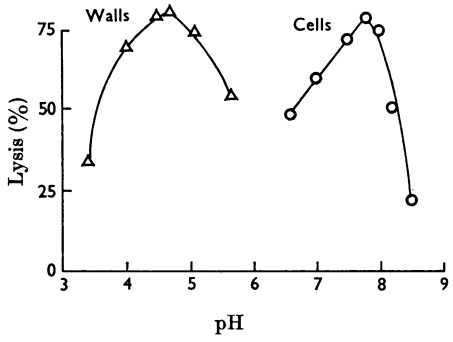

Fig. 6. pH optimum of enzyme 2 for lytic activity against whole cells and isolated cell walls. Enzyme protein concentrations and experimental conditions were the same as those given in Fig. 4, except that the buffers used (acetate, phosphate or tris, see Fig. 5) were of constant ionic strength, *I* 0·01 for whole cells (○) and *I* 0·1 for isolated cell walls (△).

enzyme 1 and *I* 0·008 for enzyme 2. The pH optima for the two enzymes were determined at constant ionic strengths closely approximating to the optima previously established. These were found to be again similar, being 8·0 for enzyme 1 and 7·8 for enzyme 2 (Figs. 5 and 6).

In contrast, maximum dissolution of isolated cell walls was found to occur at much higher ionic strengths. With enzyme 1 activity reached a maximum at *I* 0·05, decreasing only slightly as *I* increased to 0·1. An even higher optimum was found for enzyme 2, maximum dissolution occurring at *I* 0·1 with no significant decrease in activity as the ionic strength was doubled (Figs. 3 and 4). The pH optima found at these optimum ionic strengths were 6·5 and 4·7 for enzymes 1 and 2 respectively (Figs. 5 and 6). The pH and ionic-strength optima of enzyme 2 were also determined with purified mucopeptide of *Staph. aureus*, prepared by the method of Park & Hancock (1960). In both cases the optima determined were almost identical with those obtained with purified cell walls.

Difference in the lytic action of enzyme 2 on whole cells and isolated cell walls of Staph. aureus

The above results showed that the optimum conditions for the lytic action of both enzymes depended on whether the substrate was whole cells or cell walls. These results present something of a paradox since the actual substrate of the enzymes, the mucopeptide, must surely have been the same in both materials. A possible explanation for these observations was that even in whole cells maximal dissolution of the mucopeptide occurred at the optima established for isolated cell walls, but that under these conditions lysis of the cells was prevented by the high ionic strength of the medium.

Whole cells were incubated at 37° in acetate buffer (*I* 0·1, pH 4·7) with enzyme 2 (10 μg./ml.). At 10 min. intervals samples (0·2 ml.) were removed and diluted 20-fold either with water or with phosphate buffer (*I* 0·005, pH 7·5). Under neither of these conditions was lysis observed when the mixture was compared with a control from which enzyme was omitted. Under similar conditions a suspension of cell walls (1 mg./ml.) was completely solubilized in 20 min.

Evidently the above hypothesis was incorrect, since neither lowering the ionic strength, nor, in the second experiment, simultaneously increasing the pH to conditions approximating to those found to be optimum for lysis of whole cells, caused lysis of the cells under test.

Effect of teichoic acid on the lytic action of enzyme 2. The marked differences found in the ionic-strength and pH optima of the two enzymes may point to differences in the accessibility of the substrate to the enzyme. Since walls of *Staph. aureus* have been shown to contain 25–40% of teichoic acid (Baddiley, Buchanan, RajBhandary & Sanderson, 1962), it seems possible that the acidic polymer might in some way be responsible for the observed difference. Rogers (1965) suggested that highly acidic polymers of this type would have a considerable effect on the behaviour of positively charged substances in the vicinity of the cell surface. The following experiments were performed in an attempt to clarify the role of teichoic acid in staphylolysis.

Teichoic acid was prepared from an overnight culture of *Staph. aureus* as described by Baddiley *et al.* (1962). Solutions of purified teichoic acid were made by dissolving a known weight in 0·1 M-sodium hydrogen carbonate and adjusting the pH to 4·7 with acetic acid. The simultaneous presence of teichoic acid, up to a maximum of 500 μg./ml., had no significant effect on the dissolution of isolated cell walls by enzyme 2. However, chemical association between the enzyme and teichoic acid could be shown by precipitation with ethanol. A mixture of enzyme 2 and teichoic acid (both at a final concentration of 1 mg./ml.) in acetate buffer (*I* 0·1, pH 4·7) was allowed to stand for 15 min. at 0°, after which the mixture was treated with ethanol (2 vol.). The precipitate was removed by centrifuging and the lytic activity of the supernatant was measured against cell walls. It was completely without activity. Enzyme 2 alone was not precipitable from solution (1 mg./ml.) by ethanol, and subsequent assay of the ethanol–enzyme mixture gave complete recovery of the lytic activity initially used. The teichoic acid precipitate obtained on centrifuging was redissolved in dilute sodium hydrogen carbonate and the pH adjusted

to 4·7; samples assayed gave recoveries of 83% of the lytic activity initially used. Attempts to show whether co-precipitation occurred less readily under the conditions found to be optimum for the lysis of whole cells (phosphate buffer, *I* 0·01, pH 7·8) were not successful, since the teichoic acid alone could not be completely precipitated.

DISCUSSION

A number of staphylolytic enzymes have been isolated from the culture filtrates of *Streptomyces albus* G (Ghuysen *et al.* 1964). In addition, the culture filtrates of *Chalaropsis* sp. were shown to contain two staphylolytic enzymes (Hash, 1963). The two staphylolytic enzymes purified in the present study appear to be single proteins. However, the synergistic response obtained in staphylolysis on mixing the various fractions obtained from CM-cellulose column chromatography points to the presence of other staphylolytic enzymes in the culture filtrate. The purified enzymes themselves revealed only an additive effect. Similar synergistic action between lytic enzymes present in culture filtrates of *Streptomyces albus* G has been reported (Salton & Ghuysen, 1957).

The ionic-strength and pH optima of the two purified enzymes against whole staphylococci and their isolated cell walls have been determined. Lysis of staphylococci by either enzyme required a low ionic strength and at these optima the enzymes exhibited maximal activity at a slightly alkaline pH. These findings are comparable with those of Ghuysen (1957) for the F_1 enzyme isolated from *Streptomyces albus* G. However, in contrast with Ghuysen's findings on the lysis of isolated walls, where the optima showed little change, the optima for the staphylolytic enzymes under investigaton showed significant differences. Maximum dissolution of isolated cell walls occurred at much higher ionic strengths, the pH optima then being lower (enzyme 1, 1·5 pH units lower; enzyme 2, 3·0 pH units lower). Hash (1963) reported a similar lowering of the pH optimum of a staphylolytic enzyme of *Chalaropsis* sp. when the activities of the enzyme on staphylococci and on their isolated cell walls as the substrate were compared, but did not show similar changes in the ionic-strength optimum. The finding of marked differences in the present investigation may point to differences in the accessibility of the substrate to the enzyme in whole cells and isolated cell walls. It is almost certain that the enzyme substrate in both cases is the same, being the mucopeptide. In Gram-positive bacteria the mucopeptide appears solely responsible for the structural integrity of the cell wall and consequently of the bacterium (Salton, 1964). The lytic action of enzyme 2 on whole cells, under conditions for maximal dissolution of the isolated cell wall, was markedly reduced. Also, the effect of the enzyme on isolated cell walls was very slight under conditions optimum for cell lysis.

The observation that enzyme 2, though retaining its lytic activity, would co-precipitate with teichoic acid at pH 4·7 and high (*I* 0·1–0·2) ionic strength may have some bearing on these results. It was not possible to determine whether a similar phenomenon occurred at pH 7·8 and low ionic strength, the conditions optimum for cell lysis. Under these conditions teichoic acid alone could not be precipitated completely from solution. The actual anatomical relationship of ribitol teichoic acid to the mucopeptide of the staphylococcal cell wall is still open to speculation. It has been suggested that teichoic acid is covalently linked through the terminal phosphate group to the mucopeptide (Ghuysen, Tipper & Strominger, 1965; Hay, Archibald & Baddiley, 1965). However, such linkages do not preclude the possibility that the teichoic acid is present on the surface of the cell as a layer over the mucopeptide. Such a position has been suggested by Salton (1964) as being in accord with the findings of Haukenes, Ellwood, Baddiley & Oeding (1961) and Sanderson, Juergens & Strominger (1961). If this were so, the relatively high pH and low ionic strength determined as optimum conditions for lysis of whole cells may represent the optimum conditions for accessibility of the substrate to the enzyme in the presence of the teichoic acid layer, rather than the optimum for the dissolution of the mucopeptide. It is noticeable that the more basic of the enzymes, enzyme 2, showed the greater differences in optimum pH and ionic strength when the substrate was changed from whole cells to cell walls.

Evidence from the dissolution of purified mucopeptide, free from teichoic acid, points to the true optimum for the dissolution of the substrate being that previously established with isolated cell walls. Under these conditions the enzyme is free to approach the mucopeptide layer from what, in the whole organism, would be the inside of the cell. In other respects the two purified enzymes showed many properties characteristic of the lysozyme group of enzymes. The results obtained on ultracentrifugation and the behaviour of the two enzymes on gel filtration are indicative of a molecular weight for the enzymes around 15000. However, because the enzyme proteins were basic, as revealed on electrophoresis, the results obtained by the latter experiments are open to question. Egg-white lysozyme was found to behave anomalously, being eluted much later than expected from Sephadex G-100, but before the total volume of the column had been exceeded. Irrespective of the pH and ionic strength of the eluting solvent

used, it consistently behaved as if it had a molecular weight of about 7600. These findings agree closely with those of Whitaker (1963) but are in contrast with those of Ensign & Wolfe (1966), who reported lysozyme to be eluted in the correct place from the column. Whitaker (1963) suggested that since the elution volume for egg-white lysozyme was independent of ionic strength, thus ruling out the ion-exchange adsorption previously reported (Miranda, Rochat & Lissitzky, 1962; Glazer & Wellner, 1962), it was possible that the enzyme had formed a complex with the dextran gel through its active site. This seems most unlikely, since similar results were obtained with columns of polyacrylamide (Bio-Gel P-30) and agar (Sagarose). The possibility exists that both our purified enzymes also behaved in a similar anomalous fashion on gel filtration, giving a false low molecular weight.

Each enzyme has been shown to be a muramidase by examination of the digestion products produced by action on isolated cell walls, resembling other staphylolytic enzymes that have been described (Salton & Ghuysen, 1960; Ghuysen, Leyh-Bouille & Dierickx, 1962; Hash, 1963; Tipper, Strominger & Ghuysen, 1964). The substrate specificity of the purified enzymes has not been fully determined, but the finding of small quantities of muramic acid in borohydride-treated digests of staphylococcal cell wall points to some of the products of digestion being larger than disaccharides of glucosamine and muramic acid (cf. Salton & Ghuysen, 1960; Ghuysen *et al.* 1962; Dierickx & Ghuysen, 1962).

We are grateful to Dr J. P. Thornber for ultracentrifuge analysis and to Mrs P. Zatz for technical assistance.

REFERENCES

Allsop, J. & Work, E. (1963). *Biochem. J.* **87**, 512.

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.

Andrews, P. (1964). *Biochem. J.* **91**, 222.

Baddiley, J., Buchanan, J. G., RajBhandary, U. L. & Sanderson, A. R. (1962). *Biochem. J.* **82**, 439.

Boyd, W. C. (1945). *J. Amer. chem. Soc.* **67**, 1035.

Boyd, W. C. (1965). *J. biol. Chem.* **240**, 4097.

Canfield, R. E. (1963). *J. biol. Chem.* **238**, 2698.

Dierickx, L. & Ghuysen, J. M. (1962). *Biochim. biophys. Acta*, **58**, 7.

Ensign, J. C. & Wolfe, R. S. (1966). *J. Bact.* **91**, 524.

Ghuysen, J. M. (1957). *Arch. int. Physiol. Biochim.* **65**, 173.

Ghuysen, J. M., Leyh-Bouille, M. & Dierickx, L. (1962). *Biochim. biophys. Acta*, **63**, 286.

Ghuysen, J. M., Leyh-Bouille, M., Dierickx, L., Simons, A., Strominger, J. L., Tipper, D. J. & Birge, C. (1964). *Proc. 3rd Symp. Fleming's Lysozyme, Milan*, vol. 1, p. 30.

Ghuysen, J. M. & Strominger, J. L. (1963). *Biochemistry*, **2**, 1110.

Ghuysen, J. M., Tipper, D. J. & Strominger, J. L. (1965). *Biochemistry*, **4**, 474.

Glazer, A. N. & Wellner, D. (1962). *Nature, Lond.*, **194**, 862.

Hash, J. H. (1963). *Arch. Biochem. Biophys.* **102**, 379.

Haukenes, G., Ellwood, D. C., Baddiley, J. & Oeding, P. (1961). *Biochim. biophys. Acta*, **53**, 425.

Hay, J. B., Archibald, A. R. & Baddiley, J. (1965). *Biochem. J.* **97**, 723.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1956). *J. biol. Chem.* **219**, 623.

Kato, K., Kotani, S., Matsubara, T., Kogami, J., Hashimoto, S., Chimori, M. & Kazekawa, I. (1962). *Biken's J.* **5**, 155.

Kotani, S., Hirano, T., Kitaura, T., Kato, K. & Matsubara, T. (1959). *Biken's J.* **2**, 143.

Long, C. (ed.) (1961). *Biochemists' Handbook*, p. 33. London: E. and F. Spon Ltd.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McQuillen, K. (1956). *Symp. Soc. gen. Microbiol.* **6**, 127.

McQuillen, K. (1960). In *The Bacteria*, vol. 1, p. 249. Ed. by Gunsalus, I. C. & Stanier, R. Y. London and New York: Academic Press Inc.

Margoliash, E. (1962). *J. biol. Chem.* **237**, 2161.

Margoliash, E. & Lustgarten, J. (1962). *J. biol. Chem.* **237**, 3397.

Martin, H. H. (1966). *Annu. Rev. Biochem.* **35**, 457.

Miranda, F., Rochat, H. & Lissitzky, S. (1962). *J. Chromat.* **7**, 142.

Ornstein, L. & Davis, B. J. (1962). *Disc Electrophoresis*. Rochester, N.Y., U.S.A.: Distillation Product Industries (preprint).

Park, J. T. & Hancock, R. (1960). *J. gen. Microbiol.* **22**, 249.

Perkins, H. R. (1960). *Biochem. J.* **74**, 182.

Pirt, S. J. & Callow, D. S. (1958). *J. appl. Bact.* **21**, 211.

Rogers, H. J. (1965). *Symp. Soc. gen. Microbiol.* **15**, 186.

Salton, M. R. J. (1955). *J. gen. Microbiol.* **12**, 25.

Salton, M. R. J. (1964). *The Bacterial Cell Wall*. Amsterdam: Elsevier Publishing Co.

Salton, M. R. J. & Ghuysen, J. M. (1957). *Biochim. biophys. Acta*, **24**, 160.

Salton, M. R. J. & Ghuysen, J. M. (1959). *Biochim. biophys. Acta*, **36**, 552.

Salton, M. R. J. & Ghuysen, J. M. (1960). *Biochim. biophys. Acta*, **45**, 355.

Sanderson, A. R., Juergens, W. G. & Strominger, J. L. (1961). *Biochem. biophys. Res. Commun.* **5**, 472.

Schindler, C. A. & Schuhardt, V. T. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 414.

Takeda, H., Strasdine, G. A., Whitaker, D. R. & Roy, C. (1966). *Canad. J. Biochem.* **44**, 509.

Thompson, R. & Khorazo, D. (1935). *Proc. Soc. exp. Biol.*, N.Y., **33**, 299.

Tipper, D. J., Strominger, J. L. & Ghuysen, J. M. (1964). *Science*, **146**, 781.

Viscontini, M., Hoch, D. & Karrer, P. (1955). *Helv. chim. Acta*, **38**, 642.

Ward, J. B. & Perkins, H. R. (1966). *Biochem. J.* **101**, 37P.

Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 435. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Weibull, C. (1956). *Symp. Soc. gen. Microbiol.* **6**, 111.

Whitaker, J. R. (1963). *Analyt. Chem.* **35**, 1950.