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Bacteriolytic Enzymes from *Staphylococcus aureus*

PROPERTIES OF THE ENDO-β-*N*-ACETYLGLUCOSAMINIDASE

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An extracellular bacteriolytic endo-β-*N*-acetylglucosaminidase has been purified and its specificity of action has been investigated (Wadström & Hisatsune, 1970*a*,*b*). Some enzymic properties, such as optimum pH for enzyme activity on whole cells and cell walls of *Micrococcus lysodeikticus* and *Staphylococcus aureus* and optimum pH for stability, have been studied. The activity was maximum in 0.05M-tris–hydrochloric acid buffer, pH 7.0. A higher ionic strength inhibited cell-wall hydrolysis. Since the crude and purified enzymes were found to be unstable on storage, the stabilizing and inhibiting effects of several compounds were investigated. Several heavy metal ions inactivated the enzyme at very low concentrations. Thiol compounds stabilized and thiol-reacting compounds partly inhibited the activity. Crude and purified glucosaminidase was found to be heat-stable at acidic pH and unstable at alkaline pH as previously found for several lysozymes (endo-β-*N*-acetylmuramidases). Other properties of the staphylococcal enzyme and hen's-egg-white lysozyme have been compared, since the modes of action of the two are quite similar (Wadström & Hisatsune, 1970*b*).

Strains of *Staphylococcus aureus* produce peptidases, amidases and hexosaminidases that can hydrolyse the peptidoglycan of the cell wall (Tipper, 1969; Wadström & Hisatsune, 1970*a*,*b*). They are probably active in both cell-wall synthesis and autolysis (Tipper, 1969). An extracellular hexosaminidase was purified (Wadström & Hisatsune, 1970*a*) and characterized as an endo-β-*N*-acetylglucosaminidase (Wadström & Hisatsune, 1970*b*). Whereas endo-β-*N*-acetylmuramidases (EC 3.2.1.17) are common, endo-β-*N*-acetylglucosaminidases are rare (Strominger & Ghuysen, 1967). However, a streptococcal bacteriolytic glucosaminidase called muralysin has previously been purified and some properties of the enzyme have been presented (Barkulis, Smith, Boltralik & Heymann, 1964). Since there is probably no extensively purified endo-β-*N*-acetylglucosaminidase available except the enzyme from *S. aureus* strain M18, some of its enzymic properties have been studied and compared with those of muralysin and hen's-egg-white lysozyme.

Studies on structure, function and properties of hen's-egg-white lysozyme have revealed several new aspects of enzyme action and indicated a number of useful approaches to the study of enzyme mechanisms in general (Chipman & Sharon, 1969). The similarity between hen's-egg-white lysozyme and the staphylococcal glucosaminidase in several aspects, such as molecular net charge, specificity of action, bacteriolytic spectrum and heat-stability at different pH values have stimulated further characterization of the properties of the staphylococcal enzyme. The higher molecular weight and difference in stability on storage, probably related to free thiol groups in the staphylococcal enzyme, are important differences between the two hexosaminidases. The drastic loss of activity often noticed on purification and storage of the staphylococcal enzyme was found to be related to inactivation by very low concentrations of heavy metal ions. Addition of Cleland's reagent (dithioerythritol) protected the enzyme and greatly facilitated further studies on the enzymic properties, whereas EDTA and other chelators destroyed the activity.

MATERIALS AND METHODS

Materials. A detailed description of the materials used was given by Wadström & Hisatsune (1970*a*,*b*). Lysozyme (EC 3.2.1.17), trypsin (EC 3.4.4.4), deoxyribonuclease I (EC 3.1.4.5) stock no. DN-C and ribonuclease type III A were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Soya-bean trypsin inhibitor type IS, deoxyribonucleic acid type I from calf thymus, ribonucleic acid type XI from yeast, dithioerythritol, dithiothreitol, DL-penicillamine, 2-mercaptoethanol and β-mercaptoethylamine were also obtained from Sigma. Perhydrol (30% H_2O_2), 1,10-phenanthroline, 2,2′-bipyridyl (Dipyridyl) and cupferron were purchased from Merck AG,

Darmstadt, Germany. Bovine serum albumin was kindly supplied by KABI, Stockholm, Sweden, Freund's complete adjuvant was purchased from Difco Laboratories, Detroit, Mich., U.S.A.

Polyvalent staphylococcal antisera were generously supplied as follows: CPP 76/63, CPP 68/36 and EX 1480 from Wellcome Research Laboratories, Beckenham, Kent, U.K. and serum from Commonwealth Serum Laboratories, Melbourne, Vic., Australia (batch 011).

Bacterial strains were obtained as described by Wadström & Hisatsune (1970*b*). Preparation of cell walls, peptidoglycan and teichoic acid is also described in that paper.

If not otherwise stated all chemicals were of analytical grade. Crude and purified endo-β-*N*-acetylglucosaminidase (steps 1–6, Table 1, specific activity 1300 units/mg, in Wadström & Hisatsune, 1970*a*) were used in the experiments described below. Almost salt-free samples were obtained after dialysis against a volatile buffer and after repeated freeze-drying.

Assay. The decrease in E_{640} for stable suspensions of whole cells, cell walls and staphylococcal peptidoglycan was recorded as described by Wadström & Hisatsune (1970*a*,*b*). One unit of enzyme activity is defined as the amount of enzyme that gives $\Delta E_{640}^{1.0\,cm}$ 0.1/min. If not otherwise stated in standard assay conditions whole cells of *M. lysodeikticus* (Sigma) were used as substrate for endo-β-*N*-acetylglucosaminidase activity.

Proteolytic enzymes. A purified enzyme preparation (0.5 mg/ml) was incubated with trypsin (1 mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.7, containing $CaCl_2$ (final concentration 1 mM). After incubation at 37°C the proteolytic reaction was stopped by addition of soya-bean trypsin inhibitor (final concentration 1 mg/ml).

Heat-stability. The heat-stability of the glucosaminidase was investigated at different temperatures and pH values in a thermostatically controlled water bath. Portions (0.5 ml) of crude material (40 units/ml; protein content 1.1 mg/ml) and purified enzyme (150 units/ml) were heated and chilled in an ice–water bath and after different time-intervals. The pH of the incubation mixture was measured at 4°C before and after the incubation. In no case was a change of more than 0.1 pH unit noticed.

Antisera. Antiserum against crude exoproteins of strain M18 was obtained by two subcutaneous injections into rabbits of 5 mg of crude sterile-filtered supernatant in adjuvant with 1 month's interval, followed by one intravenous injection and bleeding after 4 days.

An 0.5 ml sample containing 50 units of purified glucosaminidase/ml was mixed with the same volume of the different antisera: undiluted, diluted 1:10, 1:20, 1:50 and 1:100 (v/v) with 0.05 M-tris–HCl buffer, pH 7.0, incubated at 20°C for 15 min and shaken every 5 min.

RESULTS

Effect of pH *on enzyme activity.* Staphylococcal glucosaminidase (isoelectric point 9.5) was assayed under standard conditions except that all buffers used had the same final concentration of 0.1 M. In the pH range 6.3–7.2, 80–100% of the maximum decrease in turbidity of whole cells of *M. lysodeikticus* was obtained (Fig. 1*a*).

The optimum pH for the bacteriolytic activity of *Streptomyces* F_1 muramidase varied greatly according to the bacterial substrates (Muñoz, Ghuysen, Leyh-Bouille, Petit & Tinelli, 1966). No such extreme variation in the pH profile was noticed for

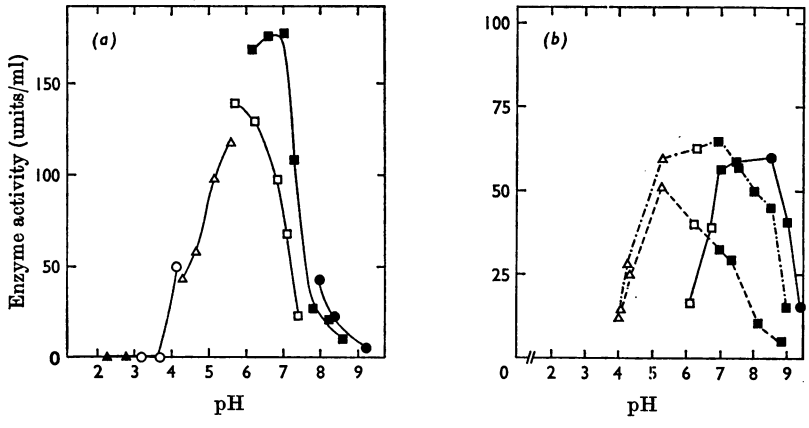

Fig. 1. (*a*) Effect of pH on enzymic activity. Purified staphylococcal glucosaminidase (180 units/ml) was assayed under standard conditions on whole cells of *M. lysodeikticus* except that the following buffers were used (all at a final concentration of 0.1 M): glycine–HCl (▲), sodium citrate (○), sodium acetate (△), sodium phosphate (□), tris–HCl (■) and glycine–NaOH (●). Double experiments were performed and the final pH in the incubation mixture was determined at 37°C and plotted against the enzymic activity. (*b*) Effect of pH on lysis of *S. aureus* strain 3528 whole cells (——), cell walls (– – – –) and peptidoglycan (–·–·–). Symbols are as for (*a*).

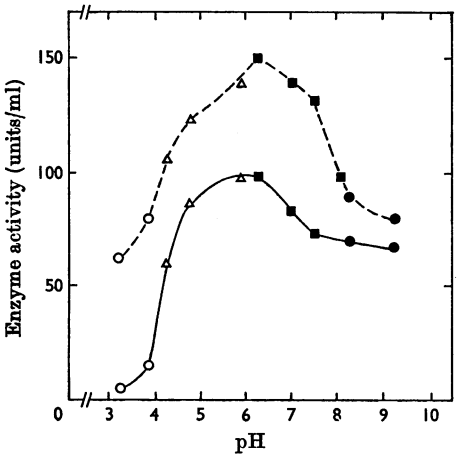

Fig. 2. Stability of purified staphylococcal glucosaminidase at different pH values. A freeze-dried sample of the enzyme (180 units/ml) was dissolved in the following buffers (all at a final concentration of 0.1 M): sodium citrate (○), sodium acetate (△), tris–HCl (■) and glycine–NaOH (●). The final pH in the incubation mixture was measured and used in the figure. After storage at 4°C for 2h (----) and 24h (——) the samples were tested against whole cells of *M. lysodeikticus*.

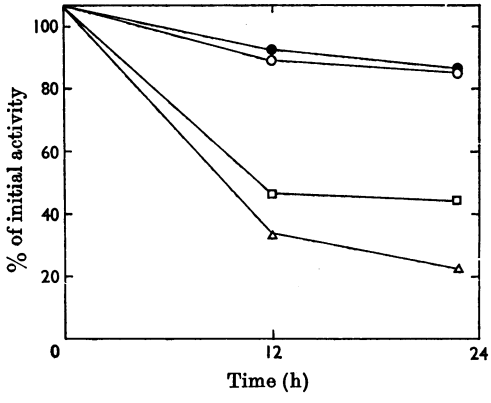

Fig. 3. Stability of staphylococcal glucosaminidase at alkaline pH. Purified enzyme (100 units/ml) stored at 4°C for 24h in ○, Ampholine solution (carrier ampholytes 8–10, 1%, w/v, final concentration); ●, the same, but with the addition of 40% (w/v) sucrose; □, 0.05 M-glycine–NaOH buffer, pH 9.5; △, water adjusted to pH 9.5 with conc. NH_3.

lysis of *M. lysodeikticus*, whole cells, cell walls or peptidoglycan from *S. aureus* strain 3528 (Fig. 1*b*) or whole cells of *Bacillus megaterium* and *Staphylococcus epidermidis*.

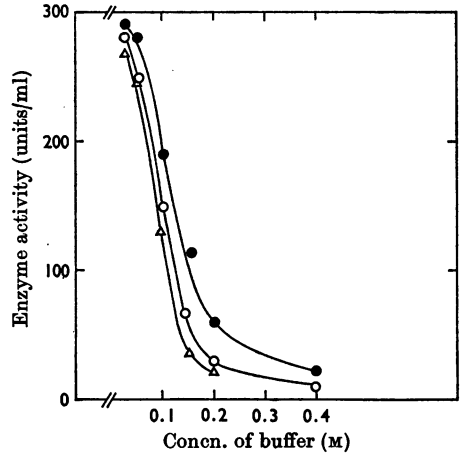

Fig. 4. Effect of ionic strength. Purified glucosaminidase was assayed under standard conditions (300 units/ml) on *M. lysodeikticus* except that the final concentration of the tris–HCl buffer (○) was varied, or the 0.05 M buffer was supplied with sodium chloride (△) or sodium citrate (●).

Effect of pH *on stability.* To investigate the enzymic stability at different pH values, preparations of crude and purified enzyme were dissolved in various buffers and stored at 4 and 37°C. A marked instability was noticed for both preparations at pH values above 7.5 and below 5 (Fig. 2). Carrier ampholytes and glycine were found to stabilize the enzyme to a certain extent at higher pH values (Fig. 3). Attempts to reactivate the enzyme by dialysis against 0.1 M-sodium phosphate buffer, pH 6.5, were not successful.

Effect of ionic strength. The rate of lysis of *M. lysodeikticus* whole cells was found to be very sensitive to ionic strength (Fig. 4). Different concentrations of the tris and phosphate buffers, pH 7.0, used under standard conditions were investigated. In other assays the standard buffer system was also supplied with sodium chloride, potassium chloride, sodium sulphate and sodium citrate. All compounds influenced the activity only as a function of the ionic strength. Similar results were obtained when cell walls of *M. lysodeikticus* or *S. aureus* (3528) were used as substrate.

The highest activity was obtained in water and at an ionic strength of less than 0.05. The dipolar ion of glycine with a net charge of zero does not contribute to the ionic strength and did not produce any inhibition at concentrations up to 0.1 M. Addition of 0.1 M-urea or glycerol to the test system did not influence the activity. On storage in different buffer systems an ionic strength lower than 0.05 and higher than 0.3 seemed to accelerate the inactivation.

Table 1. *Effect of metal salts on bacteriolytic activity* (M. lysodeikticus) *of purified staphylococcal glucosaminidase*

Agent tested	Concentration (mM)	Bacteriolytic activity
None (control)	—	225
Ca^{2+}	1	100
	0.1	110
	0.01	200
Mg^{2+}	1	120
	0.1	120
	0.01	180
Mn^{2+}	1	80
	0.1	70
	0.01	180
Ba^{2+}	1	120
	0.1	130
	0.01	190
Co^{2+}	1	130
	0.1	120
	0.01	190
Zn^{2+}	1	80
	0.1	80
	0.01	160
Cu^{2+}	1	20
	0.1	20
	0.01	70
	0.001	150
Fe^{2+}	1	30
	0.1	50
	0.01	100
	0.001	180
Fe^{3+}	1	70
	0.1	90
	0.01	180
Pb^{2+}	1	60
	0.1	100
	0.01	140
	0.001	180
Ag^{+}	1	40
	0.1	40
	0.01	70
	0.001	160

Attempts to restore the activity of the enzyme by dialysis against 0.1M-sodium phosphate buffer, pH 6.5, were not successful.

Effect of bivalent cations. The possible activating or inhibiting effects of bivalent cations on the crude and purified lytic activity were investigated. Metal salts in a final cation concentration of 0.1M–0.1 μM were added to the assay system. This was the standard system, except that 67mM-sodium phosphate buffer, pH 7.0, was used instead of tris–hydrochloric acid buffer to prevent precipitation of heavy metals with chloride. In other experiments the effect of preincubation was investigated. It was necessary to mix salt and enzyme (100 units/ml; 4°C, 15min) before the addition of substrate to obtain the maximum inhibitory effect. Several heavy-metal ions were found to destroy the activity completely at a concentration of 0.1mM and exhibited a definite inhibitory effect even at 1 μM (Table 1). The most potent inhibitors at a final concentration of 0.01mM were Cu^{2+}, Ag^{+}, Fe^{2+} and Pb^{2+}. Several ions had a lower inhibitory effect and none had a stimulatory effect on the activity. The counterions, e.g. Cl^{-}, SO_4^{2-} and NO_3^{-}, did not influence the results of these experiments.

Effect of thiol compounds and chelating agents. Attempts were made to reactivate enzyme inhibited by Ag^{+} and Hg^{2+} ions. Dialysis against 0.1M-sodium phosphate buffer, pH 6.5, with the addition of EDTA, dithioerythritol or cysteine in the concentration range 10–0.1mM was not successful. After preincubation (200 units/ml; 4°C, 15min) of enzyme and chelating agent or thiol compounds cell suspension was added. The results are given in Table 2. EDTA and sodium citrate in a high concentration (0.01M) as well as cysteine after pH adjustment were found partially to inhibit the activity after preincubation for 15min at 20°C, but cysteine in a lower concentration (1mM) showed a slightly stimulatory effect. The same situation was found for dithioerythritol (1–0.1mM). Higher concentrations inhibited the enzymic activity slightly, but inactivated the enzyme on storage.

Attempts were made to stabilize the semi-purified and the purified enzyme by thiol compounds and chelating agents (Table 2). EDTA (0.1mM) had a certain stabilizing effect, but storage in a 1mM solution overnight destroyed most of the activity. When dithioerythritol (0.1 or 0.01mM) was added the half-life increased more than three times. British Anti-Lewisite (1,2-dithioglycerol), penicillamine, β-mercaptoethylamine and mercaptoethanol (0.01 and 1mM) were also tried for storage and in the test system, and gave similar results to those obtained for cysteine.

Effect of other reducing agents. Enzymes with a pronounced instability due to labile thiol groups are common. Reducing agents, such as glutathione, ascorbate and thioglycollate often protect these groups from oxidation and inactivation. These reducing agents in 1mM final concentration and with 0.1mM-dithioerythritol were found partly to prevent inactivation of crude and purified preparations of the glucosaminidase. However, on storage in solutions with a low redox potential no regain of activity was found. Bubbling of oxygen through a sample, or addition of hydrogen peroxide (0.1–1 μM) drastically inactivated the enzymic activity.

Effect of inhibitors. The glucosaminidase is probably a thiol enzyme. Degradation of whole cells of *M. lysodeikticus* was affected to a certain extent by the addition of thiol compounds (Table 2). The enzyme was preincubated at 4°C for 30min with

Table 2. *Effect of chelating agents and thiol compounds on bacteriolytic activity of purified glucosaminidase on whole cells of* M. lysodeikticus

Agent tested	Concentration (mM)	Bacteriolytic activity after storage for 5 min at 4°C	Bacteriolytic activity after storage for 24 h at 4°C
None (control)	—	230	150
EDTA (sodium salt)	10	100	10
	1	140	40
	0.1	200	110
	0.01	190	170
Diethylenetriamine penta-acetic acid (sodium salt)	10	150	50
	1	180	90
	0.1	190	130
Cupferron	1	200	130
	0.1	240	130
2,2′-Bipyridyl	1	160	120
	0.1	200	130
1,10-Phenanthroline	1	160	90
	0.1	210	140
Sodium citrate	100	150	80
	50	190	90
Cysteine	10	150	100
	1	270	180
	0.1	230	160
Dithioerythritol	10	160	70
	1	230	110
	0.1	290	210
	0.01	230	190

1 mM-*N*-ethylmaleimide, 0.1 mM-*p*-hydroxymercuribenzoate or 0.1 mM-iodoacetate. This resulted in a loss of about half of the lytic activity when compared with a control experiment. Sodium dodecyl sulphate (0.1%, w/v) inactivated more than half of the activity. A complete loss was noticed at a concentration of 1%. Butan-1-ol (1–2%, v/v) did not influence the stability. Chelating agents, such as EDTA, diethylenetriamine penta-acetate, phenanthroline, cupferron and bipyridyl were also tested. Storage in a 1 mM solution of these compounds in 0.1 M-sodium phosphate buffer, pH 6.5, resulted in a partial loss of activity (Table 2).

Some basic proteins, such as lysozyme, are inhibited in a nonspecific way by a wide range of acidic substances, e.g. nucleic acids, acidic polysaccharides and acidic proteins (Salton, 1957; Cattan & Bourgoin, 1968; Caputo, 1955; Sela & Steiner, 1963; Pryme, Joner & Jensen, 1969). Samples of purified glucosaminidase (100 units/ml) were incubated with 0.4 mg and 4 mg of RNA, DNA, hyaluronic acid or teichoic acid for 15 min at 20°C, and for 24 h at 4°C. They were then assayed under standard conditions. No inhibitory or stimulating effect was noticed for any of these compounds.

A crude extracellular material from strain M18 was separated by isoelectric focusing. Fractions of isoelectric point 2–5 inhibited the activity and caused an increase in the initial lag phase in the ordinary assay system. Digestion with purified staphylococcal nuclease (isoelectric point 10.1; Wadström, 1967), deoxyribonuclease, ribonuclease or trypsin did not destroy the inhibitory effect.

Sensitivity to proteolytic enzymes. Half of the activity remained after 10 min of incubation with trypsin and after 30 min it was completely destroyed (see the Materials and Methods section). The sensitivity to other proteases such as subtilisin, pronase and chymotrypsin was also pronounced.

Heat-stability. Lysozymes are alkaline proteins, heat-stable in acidic and neutral buffers, but unstable when heated in an alkaline environment (Jollés, 1964). The heat-stability for staphylococcal glucosaminidase at different pH values and temperatures was investigated. Samples of crude supernatant and of a purified enzyme were heated for different time-intervals after dialysis against the following buffers (all 0.1 M): glycine–hydrochloric acid, sodium acetate, sodium phosphate, tris–hydrochloric acid, glycine–sodium hydroxide. At pH values below 6 the purified enzyme was found to be resistant to boiling for 15 min (Fig. 5*a*). A drastic loss of activity was found on heating for 5 or 15 min at pH 6 and 7 and in alkaline buffers. The stability on heating at different temperatures and pH values was also studied. Approximately half

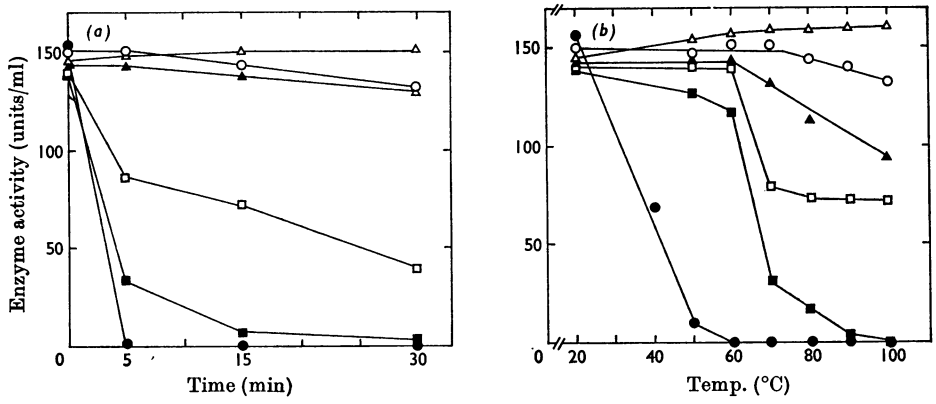

Fig. 5. (*a*) Heat-stability of purified staphylococcal glucosaminidase heated to 100°C at different pH values. Freeze-dried enzyme was dissolved in and dialysed against the following buffers: glycine–HCl, pH 3.2 (○); sodium acetate, pH 4.5 (△) and pH 5.6 (▲); sodium phosphate, pH 6.1 (□) and pH 7.0 (■); glycine–NaOH, pH 9.5 (●). The final pH was in each case measured before and after the heating procedure at 4°C. Samples were taken at 5, 15 and 30 min, chilled instantly to 0°C and immediately assayed under standard conditions. (*b*) Stability of purified staphylococcal glucosaminidase heated at different temperatures and pH values for 5 min. For symbols and description of the experimental condition see (*a*), except for sodium acetate, pH 6.1 (▲) and sodium phosphate, pH 6.2 (□).

of the lytic activity remained after heating at 60°C for 5 min at pH 6.2 (Fig. 5*b*). Addition of bovine serum albumin (1 mg/ml) had a stabilizing effect on the enzymic activity and increased the half-life 2–3 times at this temperature. A crude preparation of culture supernatant, not concentrated or concentrated ten times (protein concentration about 1 and 10 mg/ml), was dialysed and investigated under similar conditions. A higher heat-resistance was observed; half of the activity remained after heating at 60°C for 15 min at pH 7.5.

Inhibition by antisera. Antiserum CPP 76/63 and CPP 68/36 (diluted 1:10), staphylococcal O 11 antiserum (diluted 1:50), rabbit antiserum prepared against strain M18 exoproteins (diluted 1:5) and EX 1480 antiserum (diluted 1:2) were found to inhibit the enzymic activity to about 50% under standard assay conditions. Control experiments of enzyme plus normal sera from rabbit and man were incubated under the same conditions. No inhibition or stimulation was noticed in any of these experiments.

DISCUSSION

About 20 different lysozymes isolated from a variety of sources, e.g. mammalian cells (Jollés, 1964), plants (Howard & Glazer, 1969; Glazer, Barel, Howard & Brown, 1969), bacteriophages (Tsugita & Inouye, 1968) and bacterial cells (Richmond, 1959*a*,*b*; Kingan & Ensign, 1968; Krulwich & Ensign, 1968) all seem to be basic low-molecular weight proteins that lyse specific bacteria. Staphylococcal glucosaminidase from strain M18 is also a basic protein with an isoelectric point at 9.5. An enzyme with the same isoelectric point is also produced by other strains of different bacteriophage types of *S. aureus*: Wood 46, 524, Duncan, Foggie, R1 and V8 (Wadström & Hisatsune, 1968). Only the enzyme from strain M18 has been characterized and found to have several properties similar to hen's-egg-white lysozyme. The production of the enzyme (isoelectric point 9.5) is assumed to be a general characteristic of *S. aureus* and not phage-induced as in the case of streptococcal glucosaminidase or muralysin (Barkulis *et al.* 1964).

Several properties of a bacteriolytic endo-β-*N*-acetylglucosaminidase from *S. aureus* strain M18 (Wadström & Hisatsune, 1970*a*,*b*) have been investigated. A hexosaminidase with the same isoelectric point was shown to be produced by other strains of *S. aureus*. Staphylococcal 'lysozyme' partially purified by Hawiger (1968) from strain 524 is probably identical with the glucosaminidase. Additional proof is given by the fact that both enzymes are inhibited by all the polyvalent staphylococcal antisera investigated and give reaction of identity in diffusion in gel (T. Wadström, unpublished work). The appearance of antibodies inhibiting the glucosaminidase activity in a variety of staphylococcal antisera also indicates that it is common for different strains of *S. aureus* to produce this enzyme.

The glucosaminidase exhibited a single pH optimum at pH 6.5–7.0 for *M. lysodeikticus* whole

cells, which agreed favourably with that obtained for the staphylococcal 'lysozyme' (Hawiger, 1968). The bacteriolytic muramidases and amidases from *Streptomyces albus* showed great variation in the pH maximum when the single enzymes were assayed on different bacterial species (Muñoz *et al.* 1966; Ghuysen *et al.* 1965*a*; Ghuysen, Tipper, Birge & Strominger, 1965*b*; Petit, Muñoz & Ghuysen, 1966). Significant differences of the pH profiles for purified staphylococcal glucosaminidase on whole cells of *M. lysodeikticus*, *B. megaterium* and *S. aureus* strain M18 and strain 3528 peptidoglycan were found. Whereas lysis of *M. lysodeikticus* was at a maximum in tris buffer, lysis of *B. megaterium* was more effective in phosphate buffer at the same pH.

Many mammalian muramidases are reported to be more rapidly inactivated by heat at an alkaline than at a neutral or acidic pH and show a high degree of resistance to digestion with trypsin (Jollés, 1964). This was also true for this staphylococcal glucosaminidase, e.g. heating to 100°C for 15min of the purified enzyme at a pH below 6.1 resulted in almost no inactivation. Staphylococcal 'lysozyme' from strain 524 was found to be heat-sensitive and was rapidly destroyed at temperatures above 56°C at pH 6.2 (Hawiger, 1968). The crude and purified glucosaminidase from strain M18 was found to be heat-sensitive at a pH above 5.6 and resistant to heat at lower pH values. Lysozymes from many species were also shown to be extremely temperature-stable at acidic pH values (Jollés, 1964; Parry, Chandan & Shahani, 1965). The stability of the purified enzyme also increased on storage in acidic buffer systems, as was reported for the hexosaminidase in lysostaphin (H. P. Browder & J. R. Young, personal communication).

The hydrolysis of *M. lysodeikticus* cell walls by hen's-egg-white lysozyme is stimulated by sodium chloride (Davies, Neuberger & Wilson, 1969). However, several other bacteriolytic enzymes are reported to be inhibited at high ionic strengths, e.g. an *N*-acetylhexosaminidase from *Chalaropsis* (Hash, 1963), a staphylolytic enzyme from *Pseudomonas* (Zyskind, Pattee & Lache, 1965; Burke & Pattee, 1967), staphylolytic enzymes from *Streptomyces griseus* (Ward & Perkins, 1968) and an amidase from *S. albus* (Ghuysen & Strominger, 1963). Staphylococcal glucosaminidase is also inhibited by a high ionic strength, and no activation was obtained by addition of low concentrations of sodium chloride as reported for the hexosaminidase from strain 524 by Hawiger (1968). As reported by this author, no activation was found by any divalent cation investigated, which is peculiar, since both enzymes are inhibited by 0.1mM-EDTA.

Hawiger (1968) found that staphylococcal 'lysozyme' was activated by low concentrations of Ag^+ and Ca^{2+} ions, but EDTA and Cu^{2+} ions inhibited the activity. Tipper (1969) reported that autolysis of cell walls of strain Copenhagen was mainly caused by an endo-β-*N*-acetylglucosaminidase. Autolysis was inhibited by 0.01M-EDTA, but addition of an appropriate amount of Mg^{2+} reversed the inhibition without an enhanced rate of lysis being observed on addition of higher concentration of Mg^{2+}, Ca^{2+}, Mn^{2+} or Co^{2+}. Lysis was also found to decrease on addition of sodium chloride to the phosphate buffer of low ionic strength. These results are in general compatible with the results obtained for the extracellular glucosaminidase from strain M18, except for the effect of Ag^+ and EDTA on the staphylococcal 'lysozyme' of Hawiger (1968). This gives further evidence for the proposition that the staphylococcal 'lysozyme', the cell-bound autolytic enzyme and the extracellular glucosaminidase are the same, or enzymes with very similar properties.

Crude and purified enzyme preparations showed pronounced instability, especially on storage at 4°C. The addition of bovine serum albumin, glycine, carrier ampholytes and thiol compounds had a stabilizing effect. Dithioerythritol and dithiothreitol in final concentrations of 0.1mM proved superior to other thiol compounds studied. Cysteine stabilized the enzyme at a low concentration (1mM), but gave rise to inactivation at high concentrations on storage at 4°C. Chelating agents, such as EDTA, diethylenetriaminepenta-acetic acid and others, all had an inactivating effect and the heavy metal ions investigated all seemed to inactivate even at low concentrations (Table 1).

Several bacteriolytic enzymes are very sensitive to heavy metal ions and are inactivated at very low concentrations. Staphylococcal and streptococcal glucosaminidase (Barkulis *et al.* 1964), a staphylolytic enzyme from *S. epidermidis* (Suginaka, Kotani, Kato, Kashiba & Amano, 1968) and one from *Pseudomonas* (Burke & Pattee, 1967) as well as bacteriolytic protease from *Myxobacter* (Ensign & Wolfe, 1966) were found to be partly inactivated at a concentration of 1μM of several heavy metal ions.

The phage-induced streptococcal muralysin is believed to be an endo-β-*N*-acetylglucosaminidase (Barkulis *et al.* 1964). The presence of thiol compounds was also important for storage of this enzyme, which was otherwise inactivated by several metal cations and thiol-reacting compounds. In contrast, hen's-egg-white lysozyme does not possess free thiol groups (Jollés, 1964) but is irreversibly inhibited by some heavy-metal ions, although others have no significant effect (Feeney, MacDonnell & Ducay, 1956). Calcium and magnesium salts are reported to activate lysozyme (Smolelis & Hartsell, 1952), although much less effectively than monovalent cations. When compared, both streptococcal and staphylococcal

glucosaminidase are inhibited by Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Cu^{2+}; the inhibition can be reversed by EDTA in the case of the streptococcal enzyme. It is remarkable that the staphylococcal glucosaminidase is only inhibited by EDTA at 1 mM or higher concentrations after preincubation before assay, but no divalent cation seems to be important for the enzymic activity as reported for staphylococcal 'lysozyme' (Hawiger, 1968).

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