JOURNAL OF BACTERIOLOGY, Feb. 1968, p. 376-384
Vol. 95, No. 2

Purification and Properties of Lysozyme Produced by *Staphylococcus aureus*

J. HAWIGER

Department of Bacteriology, State Institute of Hygiene, Chocimska Street, Warsaw, Poland

Received for publication 28 October 1967

A method based on cold ethyl alcohol fractionation at different *p*H levels and ionic strengths and on gel filtration on a Sephadex G-200 column was used to concentrate and purify lysozyme from the culture supernatant fluid of *Staphylococcus aureus* strain 524. The final, nondialyzable product exhibited a 163-fold rise in specific activity over that of the starting material. Staphylococcal lysozyme is a glycosidase which splits *N*-acetylamino sugars from the susceptible substrate. Staphylococcal lysozyme was shown to be similar to egg white lysozyme in its optimal temperature for reaction, optimal *p*H, activation by NaCl and Ca^{++} ions, inhibition by sodium citrate and ethylenediaminetetraacetate, and inactivation by Cu^{++} ions and sodium dodecyl sulfate. It differs from the egg white lysozyme in its temperature susceptibility range (staphylococcal lysozyme is inactivated at 56 C). It acts on whole cells and cell walls of *Micrococcus lysodeikticus*, murein from *S. aureus* 524, and cell walls of *S. epidermidis* Zak. The last substrate was not susceptible to the action of egg white lysozyme in the test system used. The mechanism of action of staphylococcal lysozyme seems to be analogous to that of egg white lysozyme; however, the biological specificity of the two enzymes may be different.

Staphylococcus aureus strain 524 excretes a lytic enzyme. The activity of the crude, nondialyzable product is similar to that of egg white lysozyme (E.C.3.2.1.17.—mucopeptide *N*-acetylmuramyl hydrolase). Both lyse viable cells and cell walls of *Micrococcus lysodeikticus* Fleming and liberate *N*-acetylamino sugars and reducing sugars (23). Lysozyme production is a frequent property of staphylococcal strains isolated from various sources; all 503 tested strains of *S. aureus* and 13 of 35 strains of *S. epidermidis* produced an enzyme which was shown (by a modified plate method) to lyse *M. lysodeikticus* (J. Hawiger, J. Clin. Pathol., *in press*).

Lack of purification methods has prevented research on staphylococcal lysozyme. In this report, a method of purification of staphylococcal lysozyme is presented which makes it possible to obtain this enzyme in a form suitable for studying its properties. Factors influencing the activity of staphylococcal lysozyme have been investigated, and its spectrum of action on bacterial substrates has been determined. These properties of staphylococcal lysozyme have been compared with those of egg white lysozyme.

MATERIALS AND METHODS

Bacterial strains. S. aureus strain 524 was obtained from H. J. Rogers, National Institute for Medical Research, London, England. This strain produces hyaluronidase (24), lytic enzyme (lysozyme; 23), coagulase, clumping factor, and staphylokinase, and it is resistant to $HgCl_2$. It was obtained and stored in a dry state. After reconstitution by inoculation into soft agar with glucose it was inoculated into stabbed agar cultures. The latter were stored at 4 C and used for further cultures.

M. lysodeikticus Fleming strain, obtained from the Strain Collection of the State Institute of Hygiene, was stored in stabbed agar cultures at 4 C.

S. epidermidis strain Zak was isolated in this laboratory from a case of conjuctivitis. Glucose is fermented by this strain under aerobic and anaerobic conditions in Hugh-Leifson medium (OF test), but mannitol is not fermented. Coagulase, clumping factor, staphylokinase, and Tween 80 lipase are not produced. The strain induces no hemolysis on sheep blood-agar, produces no lysozyme in solid (plate method) and liquid media, and is sensitive to $HgCl_2$. It was stored in stabbed agar cultures at 4 C.

Media. Brain Heart Infusion (Difco) and Smolelis and Hartsell medium (28) were used.

Enzymes and other reagents. Egg white lysozyme (three times crystallized, dialyzed, and lyophilized, batch No. 75B-8830, with activity of 35,000 Sigma units per mg), trypsin from bovine pancreas (type III, twice crystallized and lyophilized, with activity of 10,000 BAEE units per mg), and albumin (fraction V) were purchased from Sigma Chemical Co., St. Louis, Mo. Ribonuclease (five times crystallized) was obtained from Bios Laboratories, Inc., New York,

N.Y.; pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; tris(hydroxymethyl)aminomethane (Tris) and sodium lauryl sulfate were obtained from British Drug Houses Ltd., Poole, Dorset, Great Britain; *N*-acetyl-D-glucosamine was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., Great Britain; *p*-dimethylaminobenzaldehyde was obtained from FOCH, Gliwice, Poland; and Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden.

Instruments. Optical density was measured on a Havemann photocolorimeter (Berlin, Germany), and on a Coleman Junior colorimeter. A Titri-*p*H-meter (Orion, Budapest, Hungary) was used to determine *p*H. Mickle's apparatus for cell disintegration and a fraction collector (Unipan, Warsaw, Poland) were also used.

Preparation of the lysozyme substrates. Standard suspensions of *M. lysodeikticus* cells were prepared as described by Smolelis and Hartsell (28). Cells were suspended in 0.06 M phosphate buffer containing 0.1% NaCl, and the density of the suspension was adjusted in such a way that, after adding 2 ml of buffer solution to 2 ml of suspension, the optical density was 0.3 as measured at a wavelength of 550 mμ.

Cells of *S. aureus* 524 and *S. epidermidis* Zak were grown on Smolelis and Hartsell medium, then washed three times with water, heated at 60 C for 60 min to inactivate autolytic enzymes, and lyophilized.

Cell walls of *M. lysodeikticus*, *S. sureus* 524, and *S. epidermidis* Zak were prepared according to Morse (14). The purified walls were lyophilized.

Murein (mucopeptide) from cell walls of *S. aureus* 524 was prepared by extraction with 5% trichloroacetic acid for 30 min at 90 C; it was then washed three times with redistilled water and lyophilized (14).

Determination of lysozyme activity. Reduction of the optical density of *M. lysodeikticus* suspensions was measured in two ways. The first method, based on the procedure reported by Smolelis and Hartsell (28), was used for preliminary comparison of the activity of lysozyme preparations obtained with that of crystalline lysozyme from egg white. The second method, based on the procedure described by Kashiba et al. (11), was used for the determination of percentage reduction of the optical density of the standard suspension of *M. lysodeikticus* cells induced by lysozyme contained in the tested solution. The activity of the lysozyme was determined in the tested sample by adding 2 ml to 2 ml of the standard suspension of *M. lysodeikticus* cells. Immediately after mixing, optical density was measured on a photocolorimeter at a wavelength of 550 mμ. The mixture was then incubated for 30 min at 37 C on a water bath and the optical density was measured again. The results were used for calculating percentage reduction of the standard suspension density (index of lysis) according to the equation: percentage reduction of optical density = [optical density (0 min) − optical density (30 min)] /optical density (0 min) × 100. On the other hand, a staphylococcal lysozyme unit was arbitrarily referred to as the amount of enzyme which induced reduction of the optical density of standard *M. lysodeikticus* suspension by 0.001 during 30 min at 37 C.

Reduction of the density of murein (mucopeptide) and cell wall suspension was determined as follows: cell walls or murein was dissolved in 2 ml of 0.06 M sodium phospate buffer (*p*H 6.2) containing 0.1% NaCl. The concentration of cell walls was 10 mg/ml, and of murein 8 mg/ml. After addition of 2 ml of lysozyme solution and incubation at 37 C, the suspension density was determined at 660 mμ.

Determination of the amount of N-acetylamino sugars released (22). Samples of 0.5 ml were taken after various times of incubation of cell walls or murein with lysozyme solution. After adding 0.1 ml of potassium tetraborate, samples were heated for 35 min in a boiling-water bath (7). The prolonged incubation is a modification of the original reaction, because disaccharide liberated develops weak color after 3 min of incubation. The results were calculated as micromoles of *N*-acetylglucosamine, for which a standard curve was prepared.

Total nitrogen was assayed by the Kjeldahl micromethod (10).

Protein was determinated by Lowry's method (10), with albumin (fraction V) as standard.

Paper electrophoresis was performed by the method of Kabat and Mayer (10). Three strips of Whatman no. 1 filter paper were placed in the chamber, and 0.05-ml portions of the fractions to be tested were applied; 0.05 M Veronal (*p*H 8.0 and 9.0) and glycine (*p*H 10.0) buffers were used. The electrophoresis was run at room temperature for 18 hr at a current of 4.5 ma and 90 v. After drying, the strips were stained with amide black 10B. In some experiments Tris-ethylenediaminetetraacetate (EDTA) buffer, *p*H 8.9 (1), was used, and a 6.5 ma current; the electrophoresis was run for 16 hr.

Lysozyme dialysis. A 3-ml amount of lysozyme, containing 0.5 mg of protein per ml, was dialyzed in dialysis sacs (Visking Co., Chicago, Ill.), which were previously rinsed with redistilled water, against 300 ml of sodium acetate solution (0.1 M)-NaCl (0.1 M), *p*H 7.5, which was changed every hour. After 4 hr, the contents of the dialysis sack were tested for lysozyme activity.

Determination of the effect of temperature on the rate of the lysozyme reaction. Lysozyme samples [2 ml containing 0.5 mg of protein per ml of 0.06 M phosphate buffer (*p*H 6.2) with 0.1% NaCl added] were mixed with 2-ml portions of *M. lysodeikticus* suspension and incubated at 0, 10, 22, 31, and 37 C. Lysozyme activity was determined at 10-min intervals.

Determination of the effect of pH on lysozyme activity. Lyophilized *M. lysodeikticus* cells were washed three times with redistilled water, centrifuged, and suspended in a suitable buffer in a proportion such that 2 ml of suspension mixed with 2 ml of buffer showed a density of 0.3 at a wavelength of 550 mμ. A 2-ml portion of the suspension thus prepared at a given *p*H was mixed with 2 ml of lysozyme solution in the same buffer. Percentage reduction of the optical density was determined after 30 min of incubation at 37 C.

Determination of the effects of metal ions and the other compounds on lysozyme activity. M. lysodeikticus cells were washed three times with redistilled water and suspended in a solution of the compound to be

tested, in such a proportion that the suspension had a density of 0.3 when mixed with 2 ml of lysozyme solution. Percentage reduction of the optical density was determined after 30 min of incubation at 37 C.

RESULTS

S. aureus strain 524 was grown for 6 hr in 10 ml of Brain Heart Infusion medium at 37 C. The culture was then transferred to 100 ml of the same medium in an Erlenmeyer flask. After 7 hr of incubation at 37 C on a shaking machine (150 rev/min), the culture was transferred into a 6-liter flask with 1 liter of warmed Brain Heart Infusion medium. Rotation of the shaker was reduced to 90 rev/min, and the culture was incubated for 12 hr at 37 C. The supernatant fluid was then centrifuged at 4 C and 2,200 rev/min, for 60 min.

Fractionation of the supernatant fluid is presented in Fig. 1. The modified Cohn method (4) of cold ethyl alcohol fractionation at variable *p*H and ionic strength was used for the purification of the lysozyme. The method was previously applied to the purification of staphylococcal coagulase (31). The conditions under which staphylococcal lysozyme was obtained and fractionated were a little different from the procedure applied for preparing coagulase. The precipitate was dissolved in 0.1 M sodium acetate solution, with 0.1 M NaCl added. The yield of fractionation of fraction LIIIS was much higher in 0.1 M phosphate buffer at *p*H 7.5 than in buffer at *p*H 6.1 or 6.5. Fractions after Sephadex G-200 gel filtration containing the highest amount of lysozyme ("peak") were combined and designated fraction LIV. The course of the filtration in the Sephadex G-200 column is illustrated in Fig. 2.

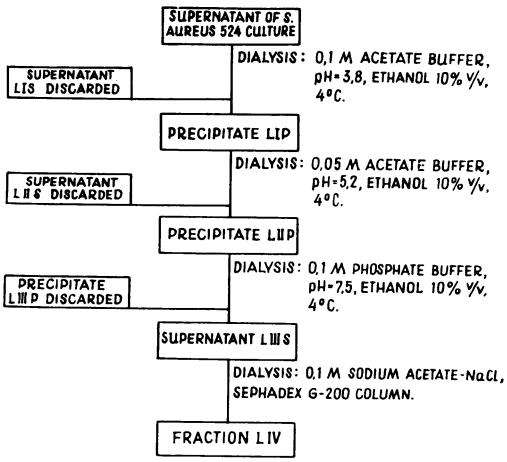

FIG. 1. *Purification of staphylococcal lysozyme.*

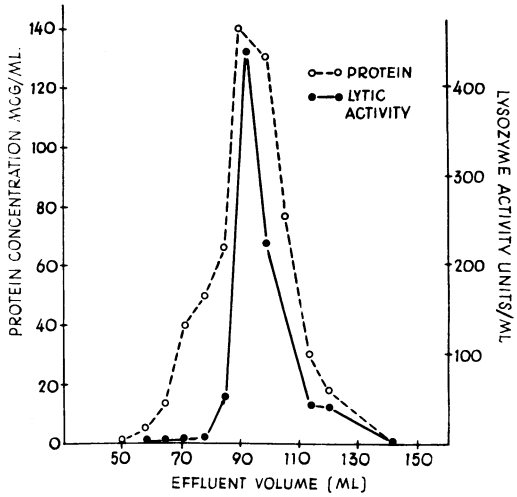

FIG. 2. *Sephadex G-200 column filtration of fraction LIIIS.*

The results obtained in the different steps of fractionation are illustrated in Table 1.

The product contained 0.14 mg of protein per ml; its activity, as measured with *M. lysodeikticus* cells supension, corresponded to the activity of 0.0036 mg of crystalline lysozyme from egg white. Nitrogen content amounted to 15%, as calculated in relation to protein. Fraction LIIIS and LIV showed no coagulase activity.

Fractions LIIIS and LIV were analyzed by paper electrophoresis to determine their homogeneity. The results were as follows: in 0.05 M Veronal buffer (*p*H 8.0), fraction LIIIS showed the presence of one strongly stained band which corresponded to the mobility of globulins when compared with electrophoretic pattern of serum proteins tested under identical conditions. However, in a more sensitive system of Tris-EDTA buffer (*p*H 8.9), the same preparation showed the presence of at least three fractions. It should be emphasized that under these conditions partition of serum proteins is also higher (1). The final product (fraction LIV) obtained by Sephadex G-200 filtration, under the same conditions, showed the presence of only one fraction (Fig. 3).

The product lost a part of its activity upon lyophilization, but at 4 C it could be stored for as long as 4 weeks without any significant loss of activity. It did not dialyze through a Visking membrane at 4 C.

Effect of lysozyme concentration (Fig. 4) on the course of the lysozyme reaction. When added in various concentrations to standard *M. lysodeikticus* suspension, lysozyme induced various decreases in the suspension density under standard conditions (37 C, 30 min). The decrease was

TABLE 1. *Characteristics of fractions obtained during preparation of staphylococcal lysozyme*

Fraction	Conditions of fractionation			Protein (mg/ml)	Activity[a]	Total activity	Purification index	Per cent recovery
	*p*H	Ionic strength	Ethyl alcohol (%)					
Supernatant fluid.....	—	—	—	15.8	19	300,000	—	—
LIP.....	3.8	0.1	10	0.76	631	120,000	33	40
LIIP.....	5.2	0.05	10	4.1	116	90,000	6	30
LIIIS.....	7.5	0.1	10	1.72	1,500	75,000	78	25
LIV.....	7.5	0.1	—	0.14	3,100	37,000[b]	163	12

[a] Activity in units per milligram of protein.
[b] Calculated as total activity of fractions obtained by the subsequent filtration of the whole LIIIS fraction.

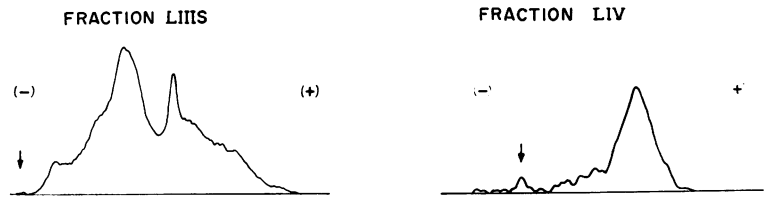

FIG. 3. *Electrophoresis of fractions LIIIS and LIV in Tris-EDTA buffer, pH 8.9.*

proportional to the amount of lysozyme added; i.e., it was correspondingly higher when lysozyme concentration was higher. It was possible, thereore, to accept arbitrarily a unit of the staphylococcal lysozyme activity which was applied for comparison of activities of the individual fractions obtained during the purification procedure.

Effect of temperature. The reaction depended on the temperature at which lysis of *M. lysodeikticus* suspensions occurs. The rate of the reaction was highest at 37 C, and the lowest at 0 C.

The effect of temperature on the activity of the staphylococcal lysozyme and the egg white lysozyme is illustrated in Fig. 5. It was found that at 56 C activity of staphylococcal lysozyme decreased to 15% of the initial value, and temperatures of 70 and 100 C inactivated it almost completely. On the other hand, lysozyme from the egg white retained 80% of its activity after heating at 56 C, 72% after heating at 70 C, and 56% after heating at 100 C. Activity at 37 C was accepted as the "initial" level.

Effect of pH on the reaction. Staphylococcal lysozyme was active over a wide *p*H range. Lytic activity, expressed in terms of percentage reduction of the suspension density, was highest in the *p*H range from 6.0 to 9.4 (Fig. 6).

For comparison, activity of egg white lysozyme was determined in the same buffers. The results were almost identical. The curves of activity at various *p*H values are intentionally discontinued at the point corresponding to *p*H 6.8 for phosphate buffer, because starting from this value lysozyme activity in phosphate buffer decreased as hydrogen ion concentration decreased. For example, at *p*H 7.4 and 8.0, the reduction of optical density amounted to 40 and 20%, respectively. Alkaline shift of the *p*H of phosphate buffer had a similar effect on the activity of crystalline lysozyme from egg white. As can be seen in Fig. 6, activity of both staphylococcal lysozyme and egg white lysozyme did not decrease in Tris buffer at the same *p*H.

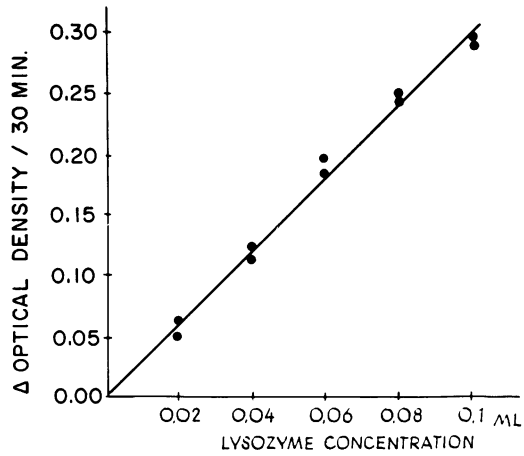

FIG. 4. *Staphylococcal lysozyme activity in relation to its concentration.*

Effect of monovalent and divalent ions, chelating agents, and other compounds on lysozyme activity. NaCl in various concentrations was added to the mixture, and lysozyme activity was determined.

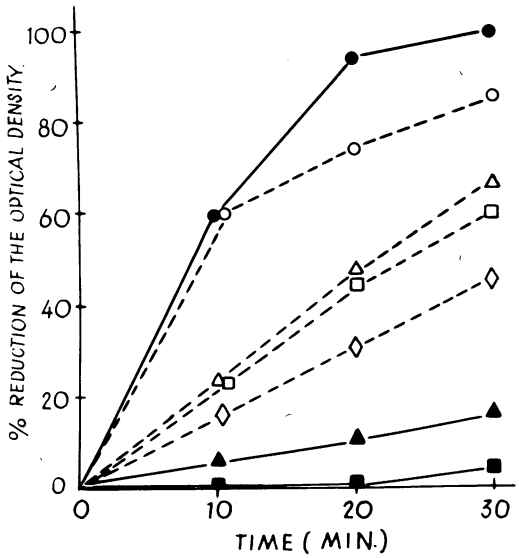

FIG. 5. *Thermal sensitivity of staphylococcal lysozyme (140 μg of protein/ml) in comparison with crystalline egg white lysozyme (2 μg ml). Samples diluted in 0.06 M phosphate buffer (pH 6.2), with 0.1% NaCl added, were heated for 15 min at various temperatures and cooled in a water bath at 37 C. Then lytic activity was tested with Micrococcus lysodeikticus cell suspension. Solid lines indicate staphylococcal lysozyme and broken lines indicate egg white lysozyme. Symbols: (●, ○) 37 C; (▲, △) 56 C; (□) 70 C; (◇) 100 C; (■) 70 C and 100 C.*

Unfortunately, the determinations might be influenced by NaCl that remained in the lysozyme solution after its fractionation; the NaCl could not be removed by dialysis against water because of loss of activity under such conditions. Nevertheless, an activating effect of NaCl on staphylococcal lysozyme activity was evident. The activity was highest in a solution containing 10^{-1} M NaCl. Similar results were obtained with egg white lysozyme.

Staphylococcal lysozyme was activated by monovalent Ag^{+} ions at a concentration of 10^{-5} to 10^{-3} M $AgNO_3$.

The chelating agents (substances binding Ca^{++} ions) were tested in concentrations of 10^{-6} to 10^{-1} M. Sodium citrate in concentrations of 10^{-3} and 10^{-2} M was found to increase staphylococcal lysozyme activity, whereas in higher concentrations (10^{-1} M) it inhibited lysozyme effect on *M. lysodeikticus* cells. The effect of EDTA was uniform; in concentrations of 10^{-4} M or higher, it inhibited staphylococcal lysozyme activity (Fig. 7).

The data discussed above suggest that staphylococcal lysozyme activity is probably associated with the presence of bivalent metal ions. Therefore, lysozyme activity was determined in a system to which Ca^{++} or Cu^{++} was added. Lysozyme was activated by Ca^{++} in concentrations of 10^{-5} to 10^{-3} M $CaCl_2$. On the contrary, Cu^{++} inhibited lysozyme action on *M. lysodeikticus* cell suspensions. $CuSO_4$ concentrations of 10^{-2} to 10^{-3} M made measurement impossible, as a result of precipitation of the suspension. The concentration of 10^{-4} M totally inhibited lysozyme activity,

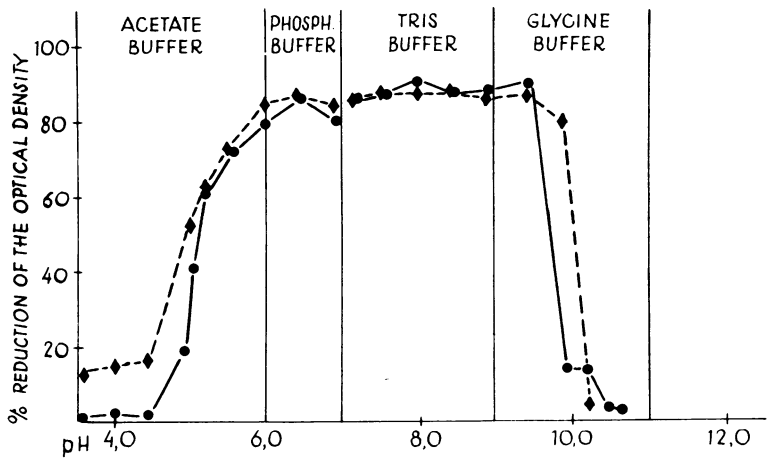

FIG. 6. *Effect of pH on the activity of staphylococcal lysozyme (solid line) in comparison with egg white lysozyme (broken line). The activity was tested in the following buffers: 0.05 M sodium acetate, pH 3.6 to 5.6; 0.05 M sodium phosphate, pH 6.0 to 8.0; 0.05 M Tris-chlorides, pH 7.2 to 9.0; and 0.05 M glycine-NaOH, pH 8.6 to 10.6.*

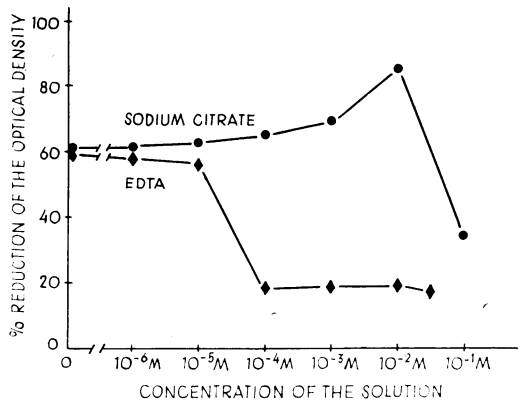

FIG. 7. *Effect of sodium citrate and EDTA on staphylococcal lysozyme activity.*

and lower $CuSO_4$ concentrations had no effect on the activity.

Staphylococcal lysozyme activity, as well as the activity of egg white lysozyme, was totally inhibited by all the concentrations of sodium dodecyl sulfate used (1.4×10^{-2}, 10^{-2}, and 10^{-3} M).

Effect of staphylococcal lysozyme on cells and cell walls of M. lysodeikticus, S. aureus 524, and S. epidermidis Zak. Lyophilized bacterial cells were suspended in 0.06 M phosphate buffer of *p*H 6.2 containing 0.1% NaCl, in such a proportion that 2 ml of the suspension mixed with 2 ml of buffer had a density equal to 0.3, as in case of *M. lysodeikticus*. Staphylococcal lysozyme, added in a concentration of 0.17 mg of protein per ml, induced no visible lysis of *S. aurues* 524 and *S. epidermidis* Zak.

Staphylococcal lysozyme in a concentration of 0.085 mg of protein per ml was added to cell wall suspension. Final concentration of the cell walls in the reacting mixture was 5 mg/ml. The effect of lysozyme, as measured by reduction of density of cell wall suspension (curve A) and by the amount of *N*-acetylamino sugars released, calculated as micromoles of *N*-acetylglucosamine (curve B), is depicted in Fig. 8–10.

Reduction of the density of *M. lysodeikticus* cell wall suspension occurred rather rapidly; more than 80% of walls were digested after 1 hr (Fig. 8). The decrease in density was accompanied by an increase in *N*-acetylamino sugars released, the amount of which in 1 ml of the mixture after 3 hr was equal to 0.1 μmole, as calculated per *N*-acetylglucosamine.

For comparison, crystalline lysozyme from egg white was added in a concentration of 24 μg/ml to cell wall suspension. With *M. lysodeikticus* cell walls, density reduction was 89% after 1 hr, and the increase in *N*-acetylamino sugars was 0.08 μmole after 3 hr.

Cell walls of *S. aureus* 524 were not lysed by staphylococcal or egg white lysozyme, whereas murein of *S. aureus* 524 was sensitive to staphylococcal lysozyme (Fig. 9). However, the sensitivity differed from that observed in *M. lysodeikticus* cell walls. The decrease in density of murein was much slower and amounted to about 30% after 1 hr. The rate of the reaction was markedly slower during further incubation. The amount of *N*-acetylamino sugars increased most rapidly during the first hour of incubation (0.04 μmole), after which the increase was slight. The reaction with egg white lysozyme followed a similar course.

Staphylococcal lysozyme reacted in a different way with purified cell walls of *S. epidermidis* Zak (Fig. 10). Cell walls of this strain were sensitive to staphylococcal lysozyme and resistant to egg white lysozyme in the concentration used. With staphylococcal lysozyme, 66% reduction of

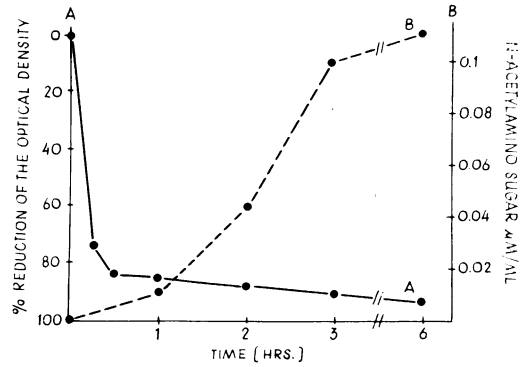

FIG. 8. *Effect of staphylococcal lysozyme on Micrococcus lysodeikticus cell walls.*

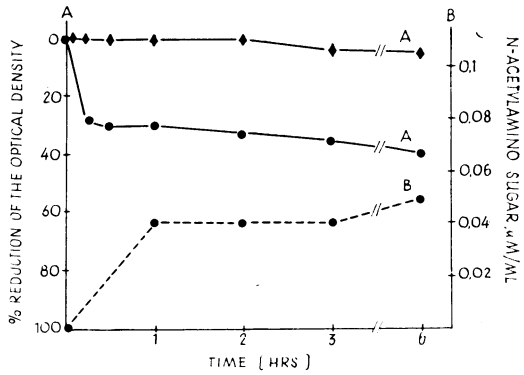

FIG. 9. *Effect of staphylococcal lysozyme on cell walls and murein of Staphylococcus aureus 524. Symbols: ◆, cell walls of S. aureus 524; ●, murein of S. aureus 524.*

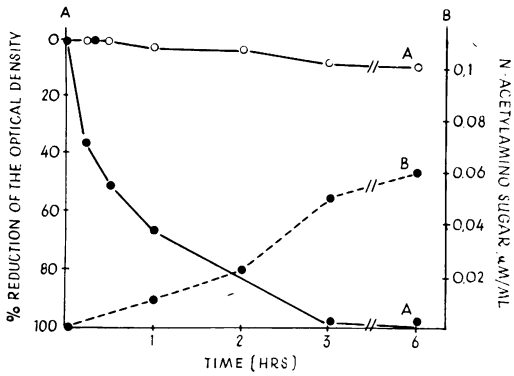

FIG. 10. *Effect of staphylococcal lysozyme and egg white lysozyme on Staphylococcus epidermidis Zak cell walls. Symbols:* ●, *staphylococcal lysozyme;* ○, *egg white lysozyme.*

density was observed during the first hour; then the reaction proceeded, reaching almost 100% reduction after 3 hr. The amount of *N*-acetylamino sugars increased to 0.05 μmole after 3 hr of incubation.

DISCUSSION

Partial purification of staphylococcal lysozyme was possible by means of ethyl alcohol fractionation at variable *p*H and ionic strength. The decrease in activity, observed in fraction LIIP in spite of high protein concentration, might indicate the presence of an inhibitor, which could be concentrated at this stage (thus inhibiting lysozyme activity) and could be removed at later stages of fractionation.

The final product obtained was 40 times less active than crystalline lysozyme from egg white, as can be inferred from the amount of lysozyme obtained per milligram of protein. The activity of these enzymes was measured by reduction of density of the standard suspension of *M. lysodeikticus* cells. On the other hand, activity of staphylococcal lysozyme was only about seven times less than activity of crystalline egg white lysozyme when purified cell walls of *M. lysodeikticus* were used as substrate. Thus, it should be mentioned that, according to criteria accepted by International Union of Biochemistry for standard enzyme unit (9), the determination of absolute specific activity of staphylococcal lysozyme will be possible only when a synthetic, chemically defined lysozyme substrate is available.

The possible effect of ethyl alcohol on lysozyme during fractionation remains to be explained. It seems not to play any role, for its effect on egg white lysozyme structure was observed at a concentration higher than 60% (8). Therefore, the fractionation method used gives a product which may be subjected to further purification and testing.

The staphylococcal lysozyme obtained shows some similarities to egg white lysozyme and staphylococcal autolysin (16–21). The two lysozymes differ in sensitivity to temperature. Staphylococcal lysozyme is heat-labile and is inactivated at 56 C. Kashiba et al. (11) reported that staphylococcal lysozyme was inactivated by heating at 100 C for 15 min, but it seems likely that these authors worked with an unpurified preparation, which may have affected the determinations. Thermal sensitivity of egg white lysozyme is different. Its activity is not lost even at 100 C.

The other two lytic enzymes found in staphylococci, autolysin and virolysin, although tested under different conditions, are heat-labile and are inactivated at a temperature as low as 45 C (19). Lysostaphin is also heat-labile, although it is inactivated at a little higher temperature of 70 C (26). In contrast to egg white lysozyme, therefore, all the hitherto mentioned lytic enzymes produced by staphylococci are sensitive to temperatures above 45 C.

Staphylococcal lysozyme is active in a wide *p*H range, which is similar to the range for egg white lysozyme. On the other hand, autolysin and virolysin show optimal activity within a similar but much more limited *p*H range when tested with other substrates (19). It seems that determination of a lytic activity in a *p*H range should be performed not only with commonly used phosphate buffer, but also with other buffers.

The effect of lysozyme on the sensitive substrate is much stronger in the presence of NaCl. Its role in the activity of egg white lysozyme and lysostaphin is distinct and consists of dissociation of complexes with acid polymers (12, 27) or with substrate (25). The role of NaCl in activation of staphylococcal lysozyme may be similar. Full activity of staphylococcal lysozyme seems to be related to the presence of bivalent cations, particularly Ca^{++}, in addition to being related to the presence of NaCl. This is suggested by the results of experiments with chelating agents, as well as by data indicating the activating effect of $CaCl_2$ on lysozyme action on *M. lysodeikticus* cells. The effect was also observed with egg white lysozyme, and with autolysin and virolysin (19). Activity of the autolytic system present in *S. aureus* cell walls is inhibited by EDTA (13).

The lysozyme-substrate reaction is not activated by all bivalent ions. The effect of Cu^{++} is opposite: in a concentration of 10^{-3} M it inhibits egg white lysozyme and staphylococcal lysozyme, autolysin, and virolysin. The inactivation of egg white lysozyme by Cu^{++} ions is irreversible; not only biological activity is destroyed, but also

changes in physical properties of lysozyme are produced (6).

The effect of sodium dodecyl sulfate was tested for practical purposes. This salt, in a concentration of 1.4×10^{-2} M was used for inactivating the lytic enzymes to protect cell walls of *Escherichia coli*, a microorganism in which the presence of at least three lytic enzymes has been found (29, 30). The activity of staphylococcal lysozyme, autolysin, and virolysin is also inhibited by this compound. It can thus be used for preparing "native" murein from *S. aureus*.

The range of biological activity of lysozyme is related to "accessibility" of the substrate on which it acts. Staphylococcal lysozyme, which has no lytic effect on living and heat-killed *S. aureus* 524 cells and their cell walls, acts on the substrate prepared from them in which phosphodiester teichoic acid-murein linkage was disrupted. Therefore, staphylococcal lysozyme is probably inhibited by the presence in the cell wall of teichoic acid linked by an ester bond.

The effect of staphylococcal lysozyme on the *S. epidermidis* Zak strain is of interest. Living cells of this strain were not lysed by either staphylococcal or egg white lysozyme in the concentrations used. On the other hand, purified cell walls were sensitive to staphylococcal lysozyme only. This finding cannot be explained unequivocally. Both cell wall preparation and the conditions were identical for the two enzymes. They must differ, therefore, in affinity to the same substrate. During purification, *S. epidermidis* cell walls are probably modified in such a way that they become sensitive to staphylococcal lysozyme. Cell walls of *S. epidermidis* contain glycerol teichoic acid (2), which presumably exerts no effect on the activity of staphylococcal lysozyme. This may be due to specificity of action of staphylococcal lysozyme. Other lytic agents are also characterized by specific biological activity, for example, streptococcal muralysin (3), endopeptidases SA, MR, and ML (15), and staphylococcal virolysin [which acts on *S. aureus*, but does not affect *M. lysodeikticus* cells (19)].

As far as staphylococci are concerned, it is only certain that *S. aureus* 524 cells are well protected against the action of lysozyme they produce. The sensitive substrate is coated by a number of radicals which "block" lysozyme action. The effect of staphylococcal lysozyme on the parental cells seems to be possible only when the substrate is "uncoated" by autolytic processes or by other factors.

ACKNOWLEDGMENTS

The author is greatly indebted to Bogumila Kluz and Maria Swiderska for technical assistance, and to Sigma Chemical Co., St. Louis, Mo., for supplying chemicals used in this study.

This investigation was supported by grant CDC-LP-3 from Communicable Disease Center, U.S. Public Health Service.

LITERATURE CITED

1. ARONSSON, T., AND A. GRÖNWALL. 1957. Improved separation of serum proteins in paper electrophoresis—a new electrophoresis buffer. Scand. J. Clin. Lab. Invest. **9**:338–341.
2. BADDILEY, J. 1959. Nucleotides and bacterial cell wall components. Proc. Chem. Soc. **117**: 177–182.
3. BARKULIS, S. S., C. SMITH, J. J. BOTRALIK, AND H. HEYMAN. 1964. Structure of streptococcal cell walls. IV. Purification and properties of streptococcal phage muralysin. J. Biol. Chem. **239**:4027–4033.
4. COHN, E. J., L. E. STRONG, W. L. HUGHES, D. J. MULFORD, J. N. ASHWORTH, M. MELIN, AND H. L. TAYLOR. 1946. Preparation and properties of serum plasma proteins. J. Am. Chem. Soc. **68**:459–464.
5. COLOWICK, S. P., AND N. O. KAPLAN [ED.]. 1955. Methods in enzymology, vol. 1. Academic Press, Inc., New York.
6. FEENEY, R. E., L. R. MCDONNELL, AND E. D. DUCAY. 1956. Irreversible inactivation of lysozyme by copper. Arch. Biochem. Biophys. **61**:72–83.
7. GHUYSEN, J. M., AND M. R. J. SALTON. 1960. Acetylhexosamine compounds enzymically released from Micrococcus lysodeikticus cell walls. I. Isolation and composition of acetylhexosamine-peptide complexes. Biochim. Biophys. Acta **40**:462–472.
8. HAMAGUCHI, K., AND A. KURONO. 1963. Structure of muramidase (lysozyme). III. Effect of 2-chloroethanol, ethanol and dioxane on the stability of muramidase. J. Biochem. (Tokyo) **54**:497–505.
9. INTERNATIONAL UNION OF BIOCHEMISTRY. 1965. Enzyme nomenclature. Recommendations, 1964 Elsevier Publishing Co., Amsterdam.
10. KABAT, E. A., AND M. M. MAYER. 1964. Experimental immunochemistry. Charles C Thomas, Publisher, Springfield, Ill.
11. KASHIBA, S., K. NIIZU, S. TANAKA, H. NOZU, AND T. AMANO. 1959. Lysozyme, an index of pathogenic staphylococci. Biken's J. **2**:50–55.
12. KERBY, S. P., AND G. S. EADIE. 1953. Inhibition of lysozyme by heparin. Proc. Soc. Exptl. Biol. Med. **83**:111–113.
13. MITCHELL, P., AND J. MOYLE. 1957. Autolytic release and osmotic properties of "protoplasts" from Staphylococcus aureus. J. Gen. Microbiol. **16**:184–194.
14. MORSE, S. I. 1962. Studies on the chemistry and immunochemistry of cell walls of Staphylococcus aureus. J. Exptl. Med. **116**:229–245.
15. PETIT, J. F., E. MUNOZ, AND J. M. GHUYSEN. 1966. Peptide cross-links in bacterial cell wall peptidoglycans studied with specific endopeptidases from Streptomyces albus G. Biochemistry. **5**:2764–2776.
16. RALSTON, D. J. 1963. Staphylococcal sensitiza-

tion: specific biological effects of phage K on the bacterial cell in lysis-from-without. J. Bacteriol. **85**:1185–1193.

17. RALSTON, D. J., B. S. BAER, M. LIEBERMAN, AND A. P. KRUEGER. 1955. Virolysin, a virus induced lysin from staphylococcal phage lysates. Proc. Soc. Exptl. Biol. Med. **89**:502–507.
18. RALSTON, D. J., B. S. BAER, M. LIEBERMAN, AND A. P. KRUEGER. 1957. Lysis-from-without of S. aureus K_1 by the combined action of phage and virolysin. J. Gen. Physiol. **41**:343–358.
19. RALSTON, D. J., M. LIEBERMAN, B. S. BAER, AND A. P. KRUEGER. 1957. Staphylococcal virolysin, a phage induced lysin: its differentiation from autolysin of normal cells. J. Gen. Physiol. **40**: 791–807.
20. RALSTON, D. J., AND M. MCIVOR. 1964. Cell-wall lysins of *Staphylococcus aureus* strains induced by specific typing phages. J. Bacteriol. **88**:667–675.
21. RALSTON, D. J., AND M. MCIVOR. 1964. Lysis-from-without of *Staphylococcus aureus* strains by combinations of specific phages and phage-induced lytic enzymes. J. Bacteriol. **88**:676–681.
22. REISSIG, J. J., J. L. STROMINGER, AND L. F. LELOIR. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. **217**:959–966.
23. RICHMOND, M. H. 1959. Lytic enzymes of Staphylococcus aureus 524. Biochim. Biophys. Acta **31**:564–565.
24. ROGERS, H. J. 1953. Variant populations within a hyaluronidase-producing culture of Staphylococcus aureus. J. Pathol. Bacteriol. **66**:545–551.
25. SCHINDLER, C. A. 1965. The role of NaCl in the lysis of Staphylococcus aureus by lysostaphin. J. Gen. Microbiol. **40**:199–205.
26. SCHINDLER, C. A., AND V. T. SCHUHARDT. 1965. Purification and properties of lysostaphin—a lytic agent for Staphylococcus aureus. Biochim. Biophys. Acta **97**:242.
27. SKARNES, R. C., AND D. W. WATSON. 1955. The inhibition of lysozyme by acidic polymers from pathogenic bacteria. J. Bacteriol. **70**:110–112.
28. SMOLELIS, A. N., AND S. E. HARTSELL. 1949. The determination of lysozyme. J. Bacteriol. **58**: 731–736.
29. WEIDEL, W., H. FRANK, AND H. H. MARTIN. 1960. The rigid layer of the cell wall of Escherichia coli strain. B. J. Gen. Microbiol. **22**:158–166.
30. WEIDEL, W., AND H. PELZER. 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Advan. Enzymol. **26**:193–232.
31. ZOLLI, Z., JR., AND C. L. SAN CLEMENTE. 1963. Purification and characterization of staphylocoagulase. J. Bacteriol. **86**:527–535.