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Lysis of *Staphylococcus aureus* Cell Walls by a Soluble Staphylococcal Enzyme

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Enzyme preparations of *Staphylococcus aureus* were examined for their ability to solubilize ^{32}P-labeled cell walls of the parent organism. Enzymatic activity was observed in the growth medium, in soluble fractions, and associated with native cell walls. Enzyme associated with isolated cell walls could be inactivated with formaldehyde without reducing the susceptibility of the walls to the action of added enzyme. When cells are frozen and thawed, 50 to 75% of the intracellular enzyme is released along with 2% of the intracellular protein. This freeze-thaw extracted enzyme has little, if any, activity on intact *S. aureus* cells. It appears that the enzyme resides near the cell wall and acts on the cell-wall inner surface.

The production of lytic and autolytic enzymes by a variety of bacteria is well established (3, 4, 7, 8, 18, 21, 26, 27). The field of bacteriolysis was reviewed recently (22, 24). Culture filtrates of *Staphylococcus aureus* are lytic toward *Micrococcus lysodeikticus* cell walls (20) and whole and heated cells (14, 25). Autolysates of *S. aureus* cells lyse *M. lysodeikticus* cell walls (19) and acetone-extracted cells (19). Autolysates of *S. aureus* cell walls have been reported to be lytic toward *M. lysodeikticus* cells and cell walls (15). It is not known whether the staphylococcal enzyme which attacks *M. lysodeikticus* is the same one as that attacking *S. aureus* cell wall. There is, however, precedence for such activity in a single enzyme, since the *Chalaropsis* B enzyme (12) lyses both *S. aureus* and *M. lysodeikticus*, as does streptomyces "32" enzyme (9). In all of these studies, enzymatic lysis was detected by the reduction in turbitidy of cell or cell-wall preparations.

To study further the lytic enzymes present in *S. aureus*, a measure of enzyme activity more sensitive than turbidity reduction was needed. Phosphorus-32-labeled cell walls of *S. aureus* were prepared from cells grown for several generations in Trypticase Soy Broth (TSB, BBL) containing $^{32}PO_4$. The appearance of ^{32}P in Millipore filtrates (Millipore Corp., Bedford, Mass.) is shown to be a measure of cell-wall lysis and is sensitive to the release of 0.1% of the cell-wall phosphate.

This study confirmed that cell-wall lytic activity occurs in culture filtrates of *S. aureus* (20, 25) and is also associated with isolated cell walls (15). Lytic enzyme is localized in the cell in such a manner that much of the enzyme can be released by freezing and thawing without disruption of the cells. Although more than one lytic enzyme may be present in the crude freeze-thaw extract, purification of the lytic enzyme in this fraction by various means [chromatography on diethylaminoethyl (DEAE) cellulose, DEAE Sephadex, Sephadex-50, -100 and -200;, adsorption on $CaPO_4$-gel; fractional precipitation with $(NH_4)_2 \cdot SO_4$; centrifugation in a sucrose gradient; and disc electrophoresis] has not been possible. Therefore, the properties, conditions for optimal activity, and some kinetic studies of the crude lytic enzyme preparation are reported.

MATERIALS AND METHODS

Preparation of cell walls. Fifty ml of a 17-hr shake culture of *S. aureus* cells (Oeding strain 8507) were used to inoculate 500 ml of medium. Cells were grown at 37 C for 2.5 hr with shaking (120 rev/min in a New Brunswick shaker incubator) and harvested in a Servall centrifuge for 20 min at 5,800 × *g*. Unlabeled cells were grown in TSB. ^{32}P-labeled cells were grown in a medium containing (per liter): Trypticase (BBL), 17 g; Phytone (BBL), 3.0 g; NaCl, 5.0 g; glucose, 2.5 g; tris(hydroxymethyl)aminomethane, 2.9 g; and $^{32}PO_4$, 40 mc. All cells were suspended in water to an optical density (OD) at 660 mμ of 60 or less, centrifuged, suspended to the same OD_{660}, and frozen. For the preparation of cell walls, a 180- to 430-mg sample of ^{32}P-labeled cells (OD_{660} no greater than 25) in 36 ml was shaken with 44 g of 66-μ diameter glass beads in a Braun disintegrator with cooling, until there was a 10-fold or greater reduction in OD_{660} (5 to 7 min). During disruption and in subsequent steps, the sample was kept at 4 C. After centrifugation for 15 min at

8,700 × *g*, the sediment of cell walls was suspended in 30 ml of 1 M KCl and centrifuged for 10 min at 35,000 × *g*. These KCl-washed ^{32}P-labeled cell walls were combined with the yield of cell walls from 790 mg of unlabeled, freshly disrupted *S. aureus* at the same stage of preparation, suspended in 30 ml of 1 M KCl, and centrifuged 10 min at 35,000 × *g*. The final sediment was suspended in 7.7 ml of 20% sucrose in 1 M KCl. At this stage, and at later stages of purification, cell walls can be stored at −25 C in sucrose-KCl without aggregation. Just prior to separation on a sucrose gradient, the cell walls in sucrose-KCl were thawed, diluted to 30 ml with 1 M KCl-0.02 M KPO_4 (*p*H 7.4), centrifuged for 10 min at 35,000 × *g*, and suspended in 6.5 ml of 1 M KCl-0.02 M KPO_4 (*p*H 7.4) or until the OD_{660} = 12. Two-ml samples of this solution were separated on a sucrose gradient as previously described (13). The cell-wall fractions in sucrose-1 M KCl were pooled and frozen. This preparation will be referred to as native cell walls.

For inactivation of cell wall-bound enzyme, a sample of 9 mg of ^{32}P-labeled cell walls was washed free of sucrose by centrifugation at 35,000 × *g* for 20 min, suspended in 100 ml of 4% formaldehyde in 0.10 M KPO_4 (*p*H 7.0), and maintained at 23 C for 3 days. Excess formaldehyde was removed by centrifugation and the cell walls were suspended in 20% sucrose containing 0.5 M KCl, 0.05 M KPO_4 (*p*H 7.0), and 1% formaldehyde. This suspension was frozen and stored at −25 C. This preparation will be referred to as HCHO-treated cell walls.

M. lysodeikticus cell walls were prepared in the same manner as *S. aureus* native cell walls from cells grown in TSB.

Preparation of the ammonium sulfate precipitate of the S. aureus whole cell freeze-thaw extract (AFZ enzyme fraction). *S. aureus* (Oeding strain 8507) was grown in TSB with shaking on a New Brunswick shaker incubator. A 100-ml amount of an 18-hr shake culture of *S. aureus* cells was used to inoculate 1,000 ml of medium in a 2-liter Erlenmeyer flask; cells were grown at 37 C for 2.5 hr with shaking. The OD_{660} increased from a value of 0.16 to a final value of 0.80. The cells from 12 flasks were harvested in a Szent-Gyorgyi & Blum continuous-flow attachment to the Servall centrifuge. All cells were suspended in water at 25 C to an OD_{660} of 60. The cells were centrifuged 10 min at 35,000 × *g* in a Spinco no. 30 rotor, suspended to the same OD_{660} in water, and frozen. After remaining frozen overnight or longer, the cells were thawed, centrifuged 10 min at 35,000 × *g*, and the supernatant solution was saved. Ammonium sulfate (0.6 g per ml of supernatant solution) was added with stirring in the cold for 20 min, the precipitate was centrifuged for 10 min at 35,000 × *g*, the supernatant solution was discarded, and the precipitate was suspended in a solution of ammonium sulfate (0.6 g/ml). This suspension is enzyme preparation AFZ.

Standard assay for cell-wall lysis. The standard reaction mixture used for assay of the enzyme consisted of 40 μmoles of $MgCl_2$, 0.20 μmole of $CoCl_2$, 0.1 μmole of ethylenediaminetetraacetic acid (EDTA), 10 μmoles of NaH_2PO_4 (*p*H 6.0), 28 μg (dry weight) of formaldehyde-treated cell walls (25 nanomoles of cell-wall $^{32}PO_4$), and 0.5 to 1.5 units of enzyme per ml of reaction mixture (1.3 to 3.9 μg of protein per ml). Prior to addition of the cell walls and enzyme, the *p*H of the mixture was adjusted to 6.0 with NaOH. Incubation was at 37 C for 20 min. The reaction mixture was chilled and filtered through a Millipore filter of 0.45-μ pore size. Radioactivity in the aqueous Millipore filtrates was determined by measurement of the Cerenkov radiation resulting from the passage of high-energy β-particles emitted by ^{32}P through water (10). Radioactivity of cell walls filtered onto Millipore filters was determined by immersing the filters directly in scintillation solution consisting of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene. Measurements of Cerenkov radiation and liquid scintillation were made on a Packard Tri-Carb liquid scintillation spectrometer. A unit of enzyme activity is defined as that amount of enzyme necessary to release 1 nanomole of $^{32}PO_4$ per ml of standard reaction mixture in 1 hr.

Total phosphate was determined by the procedure of Chen, Toribara, and Warner (5) as modified by Ames and Dubin (1).

RESULTS

Endogenous release of ^{32}P from native cell walls. The content of lytic enzyme attached to isolated cell walls caused the release of 4% of the cell-wall phosphate per hour (1,050 picomoles per ml per hr out of a total of 25,000 picomoles of cell-wall phosphate; Table 1) under the standard conditions for assay of the enzyme. This endogenous release made it difficult to determine whether added enzyme fractions had lytic activity or were stimulating endogenous release of ^{32}P. Formaldehyde treatment of cell walls reduced the level of endogenous release of ^{32}P from isolated cell walls from 4% down to 0.2% per hr (from 1,050 down to 30 picomoles per ml per hr; Table 1). During the treatment of cell walls with formaldehyde, less than 1% of the total cell-wall phosphate was released. Heat inactivation of native cell walls was not as successful for inactivation. When native walls were heated for 30 min at 100 C and a *p*H of 6.0, there was only a threefold reduction in the endogenous rate. During the heat treatment, 10% of the total cell-wall phosphate was released.

The endogenous activity of native walls was approximately equal to the activity of formaldehyde-treated cell walls with 2 μg of added AFZ enzyme fraction (Table 2). The further addition of 2 μg of enzyme caused an equal increase in release in both the formaldehyde-treated and native cell walls. This was taken as evidence that formaldehyde treatment of cell walls does not alter their susceptibility to the action of the enzyme.

Lytic enzyme activity in various fractions of S. aureus. With the release of ^{32}P from formaldehyde-treated cell walls used as an assay of lysis, a

TABLE 1. *Effect of magnesium, cobalt, ethylenediaminetetraacetic acid (EDTA) and lytic enzyme on the ^{32}P release from the Staphylococcus aureus cell walls*[a]

Addition	$^{32}PO_4$ Release (pmoles per ml per hr)			
	Native walls	HCHO walls	HCHO-walls + added enyme	HCHO-walls with absorbed enzyme
None	680	85	115	1,155
+Co	885	45	95	1,310
+Mg	855	35	550	1,615
+Co+Mg	945	30	525	1,680
+EDTA	530	105	115	695
+EDTA+Co	905	75	125	1,310
+EDTA+Mg	725	50	435	1,765
+EDTA+Mg+Co (complete)	1,050	30	620	1,980

[a] Complete reaction mixture contained 0.04 M $MgCl_2$, 2×10^{-4} M $CoCl_2$, 10^{-4} M EDTA, 0.01 M NaH_2PO_4, and 28 μg/ml of the indicated type of cell wall (25 nmoles of $^{32}PO_4$/ml), final *p*H 6.0. Native walls were incubated in the absence of added enzyme (native walls). Formaldehyde-treated walls were incubated in the absence of added enzyme (HCHO-walls) and in the presence of 0.61 units per ml of AFZ enzyme fraction (HCHO-walls + added enzyme). Formaldehyde-treated walls with absorbed AFZ were prepared by incubating 27 μg of HCHO-cell walls and 310 μg (93 units) of AFZ enzyme fraction at 0 C for 10 min in the presence of 0.028 M $MgCl_2$ and 0.01 M NaH_2PO_4, *p*H 6.0. Walls were centrifuged and resuspended in 0.01 M NaH_2PO_4, *p*H 6.0; recentrifuged and resuspended in 0.01 M NaH_2PO_4, *p*H 6.0; and recentrifuged and suspended in 0.001 M NaH_2PO_4, *p*H 6.0. These cell walls were then incubated in the reaction mixture in the absence of added enzyme (HCHO-walls with absorbed enzyme).

TABLE 2. *Comparison of native and formaldehyde-treated cell walls as substrates for lytic enzyme of Staphylococcus aureus*[a]

Tube no.	Cell-wall type	Enzyme (μg/ml)	^{32}P release (nmoles per ml per hr)	Increase in ^{32}P release (nmoles per ml per hr)
1	Native	—	0.61	
2	Native	2.02	0.89	0.28
3	HCHO-treated	2.02	0.55	
4	HCHO-treated	4.04	0.80	0.25

[a] ^{32}P-labeled native and HCHO-treated cell walls were tested for the release of ^{32}P under the conditions of the standard assay in the presence of the indicated amounts of AFZ enzyme fraction.

number of preparations of *S. aureus* were examined for lytic activity. Activity was observed in culture filtrates, in supernatant fluids of washed whole cells after freezing and thawing, and in supernatant fluids of disrupted cells. Activity was also found attached to the isolated cell walls as mentioned above. The appearance of lytic activity in these fractions with growth time is demonstrated in Fig. 1. Maximum enzyme activity was present in the culture medium and the freeze-thaw extract of the cells in 3 to 4 hr after inoculation with a 10% inoculum. After 4 hr of growth, the culture medium had four times as much enzyme activity as remained in the cell. The enzyme activity remaining in the cell was evenly divided between that which was removed in the freeze-thaw extraction and that released by disruption of the remaining cells in the Braun disintegrator. The activity in the freeze-thaw extract was not a

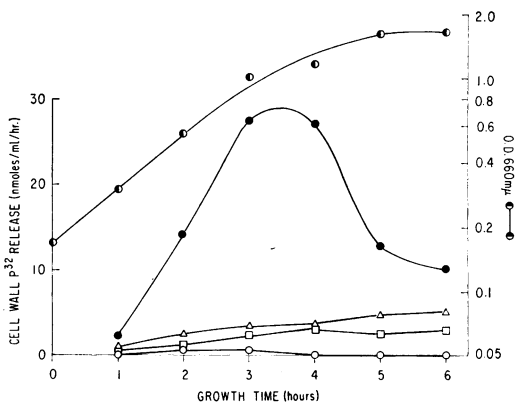

FIG. 1. *Time course of appearance of cell-wall lytic activity in various cell fractions during growth of Staphylococcus aureus. An inoculum of 100 ml of an overnight shake culture of S. aureus in Trypticase Soy Broth was placed in 1,000 ml of fresh medium in a 2-liter flask. Shaking was continued and, at hourly intervals, a 250-ml sample of medium was removed. Optical density was measured at 660 mμ (◐). The cells were centrifuged for 20 min at 5,800 × g. The supernatant fraction = "culture medium." The cells were resuspended in 25 ml of water and centrifuged for 10 min at 35,000 × g. The supernatant fraction = "wash of cells." The cells were resuspended to 25 ml with water and frozen overnight. Cells were thawed and centrifuged for 10 min at 35,000 × g. The supernatant fraction = "freeze-thaw extract." The cells were resuspended to 36 ml and disrupted for 5 min with the Braun disintegrator in the presence of 44 g of glass beads. The cell walls were centrifuged for 10 min at 35,000 × g. The supernatant fraction = "Braun super." Enzyme activity was measured under standard assay conditions. Activity in the culture medium (●); wash of cells (○); freeze-thaw extract (□); Braun super (△) were corrected to the total activity in the original 250-ml sample.*

carry-over of enzyme from the culture medium, since the wash of the cells before freezing had very little enzymatic activity (Table 3). Because less than 2% of the protein in the cell was released into the freeze-thaw extract, this fraction had the highest specific activity of any examined (Table 3). Compared to the unextracted disrupted cells, the freeze-thaw extract represented a 25-fold purification of the enzyme. The amount of enzyme extracted by freezing and thawing was relatively unaffected by the volume of water extract. When 5.5-hr grown cells (OD_{660} = 0.66 at harvest time) were washed, suspended, and frozen at a cell concentration of OD_{660} = 55, 27.5, and 13.2, the yield of enzyme in the extract after thawing was 3.3, 3.8, and 3.8 units per mg of cells (dry weight), respectively. Cells were routinely suspended in water to an OD_{660} = 60 in order to obtain a concentrated enzyme in the freeze-thaw extract. The freeze-thaw extract was further concentrated by precipitation with ammonium sulfate. This precipitate was stored as a suspension in ammonium sulfate (0.6 g/ml) at 2 C. This preparation is AFZ enzyme fraction.

When chromatographed on DEAE cellulose, the AFZ enzyme fraction yielded several protein peaks (Fig. 2). The majority of the activity did not elute from the column; that which did elute came off prior to the main protein peak. The presence of more than one peak of activity on DEAE-cellulose suggests that more than one lytic enzyme is present in the AFZ enzyme fraction.

Properties of the freeze-thaw enzyme fraction. A large amount of the ammonium sulfate precipitate of the freeze-thaw extract was suspended in ammonium sulfate (0.6 g/ml) and stored at 2 C. When stored in this manner, the enzyme proved to be stable for 7 months.

TABLE 3. *Specific activity of enzyme fractions during growth of Staphylococcus aureus*[a]

Time	Release of cell wall ^{32}P (nmoles per hr per mg of protein)			
	Culture medium	Wash of cells	Freeze-thaw extract	Braun supernatant fraction
hr				
1	0.3	6.1	248	12
2	2.2	21.8	360	19.2
3	4.1	18.2	445	16.1
4	4.0	2.6	628	12.7
5	2.1	1.3	457	16.0
6	1.6	0.6	555	14.7

[a] Growth conditions and preparation of various fractions were as in Fig. 1.

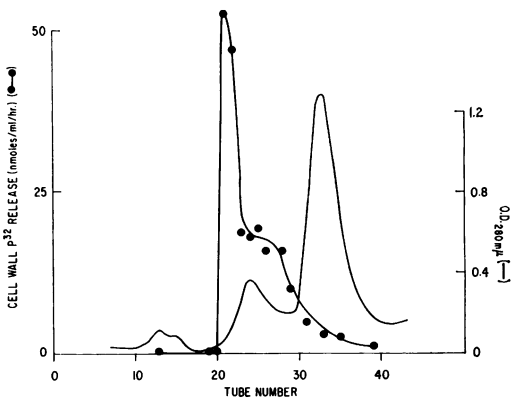

FIG. 2. *Diethylaminoethyl (DEAE) cellulose chromatography of Staphylococcus aureus cell-wall lytic enzyme. A sample of AFZ enzyme (6,430 units) in 0.005 M NaH_2PO_4 (pH 7.0) was placed on a 2- × 22-cm column of DEAE-cellulose and eluted with 100 ml of 0.01 M NaH_2PO_4 (pH 7.0)—0.02 M NaCl—10^{-5} M ethylenediaminetetraacetate (EDTA), followed by a linear gradient generated with 150 ml of the same buffer and 150 ml of 0.10 M NaH_2PO_4 (pH 7.0)—1.0 M NaCl—10^{-5} M EDTA. Fractions of 10 ml were collected. Enzyme was assayed in the standard manner and activity expressed as the release in nmoles of ^{32}P per hr per ml of column eluate. Recovery of the enzyme was 2,300 units.*

When the enzyme was incubated with formaldehyde-treated cell walls in the absence of magnesium, there was little release of cell-wall phosphate (Table 1). Magnesium stimulates the activity of enzyme acting on formaldehyde-treated walls. The addition of cobalt and EDTA further increases the activity of the enzyme. The highest activity was observed in the presence of cobalt, EDTA, and magnesium. Variation of the $MgCl_2$ concentration is illustrated in Fig. 3. The *p*H optimum for the enzyme is 5.9 (*see* Fig. 4). Under the standard assay conditions, the formaldehyde-treated walls released negligible ^{32}P in the absence of added enzyme within the *p*H range 4 to 7. The release of ^{32}P was proportional to the added protein in the range of 0 to 2.5 units of enzyme activity (Fig. 5, inset). As the amount of added protein was increased, the cell walls appeared to become saturated with enzyme. Further addition of enzyme above a protein concentration of 340 μg per ml caused no increase in rate of release of ^{32}P above the value of 33% released during a 20-min assay period. At the highest level of protein in Fig. 5, ammonium sulfate (from the AFZ suspension in ammonium sulfate) was present at a concentration of 0.06 M. At this concentration of ammonium sulfate, there was a 25% inhibition of the enzyme in a standard assay mixture at a low enzyme concentration (8.1 μg of protein per ml).

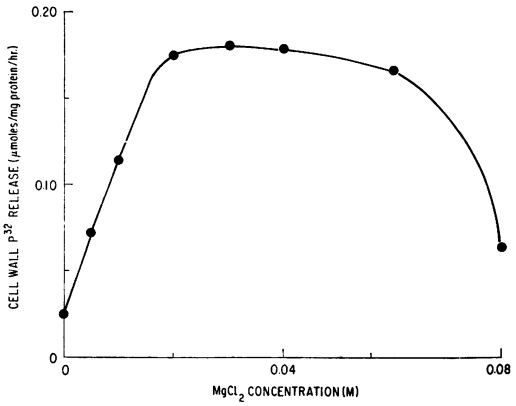

FIG. 3. *Effect of magnesium concentration on the cell-wall lytic activity of the AFZ enzyme fraction. Assay conditions were as indicated in the standard assay, with the exception of the magnesium concentration. The pH was adjusted to 6.0 at each level of magnesium to avoid the effect of magnesium on pH in the presence of phosphate.*

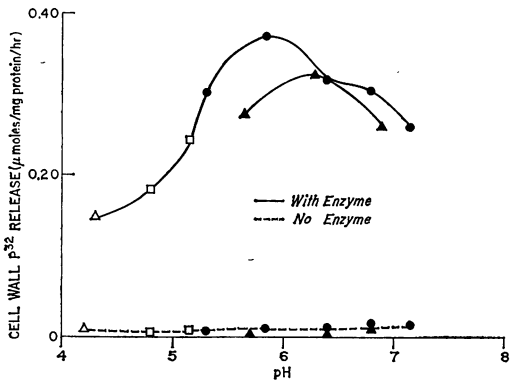

FIG. 4. *Dependence of cell-wall lytic activity of the AFZ enzyme fraction on pH. Assay conditions were as indicated in the standard assay, with the exception that formate (△); acetate (□); phosphate (●) and N-tris-(hydroxymethyl) methyl-2-amino-ethanesulfonic acid (▲) buffers were used as indicated. All pH values are the measured final pH of the reaction mixture.*

The ammonium sulfate carried over into the assay from the AFZ enzyme fraction was less than 0.03 M and not inhibitory at all other levels of protein tested. The rate of release of ^{32}P was constant during the first 4 hr of incubation with 0.35 units of enzyme per ml (Fig. 6). The AFZ enzyme fraction showed Michaelis kinetics (Fig. 7). Although the exact substrate for the enzyme is not known, the K_m for the enzyme in terms of the added amount of cell-wall total phosphate is 1.2×10^{-5} M. The enzyme was not saturated with substrate under the standard assay conditions and, therefore, care was taken to add exactly 25 nanomoles of cell-wall $^{32}PO_4$ when assaying.

When high levels of cell walls and enzyme were incubated under standard conditions, there was a parallel release of ^{32}P from ^{32}P-labeled cell walls and a decrease in OD_{660} (Fig. 8). In an experiment identical to that shown in Fig. 8, except that *M. lysodeikticus* cell walls were used instead of *S. aureus* cell walls, the rate of decrease in turbidity was 20-fold greater with the *M. lysodeikticus* cell walls than with the *S. aureus* cell walls. When *S. aureus* cells grown for 3.5 hr (final OD_{660} of 0.2)

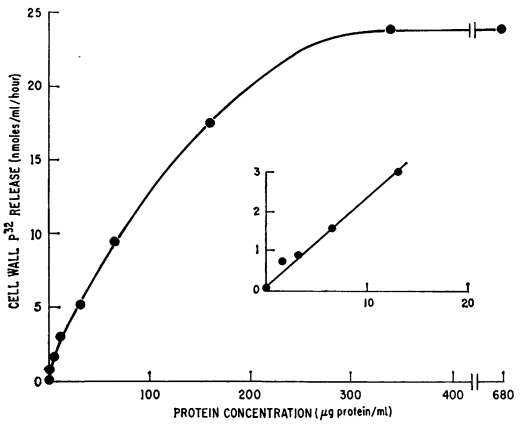

FIG. 5. *Variation of rate of cell-wall lysis with concentration of AFZ enzyme fraction. Conditions were as indicated for standard assay mixture, except for the amount of added enzyme which varied from 1.6 to 680 μg of protein per ml. Inset shows the activity at low enzyme levels.*

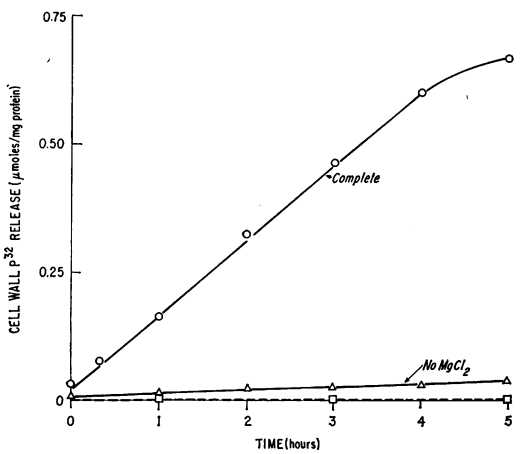

FIG. 6. *Time course of release of ^{32}P from Staphylococcus aureus cell walls in the presence of AFZ enzyme. The complete system (○) consisted of the standard assay system incubated for the indicated time. The controls were less magnesium (△) and less enzyme (□).*

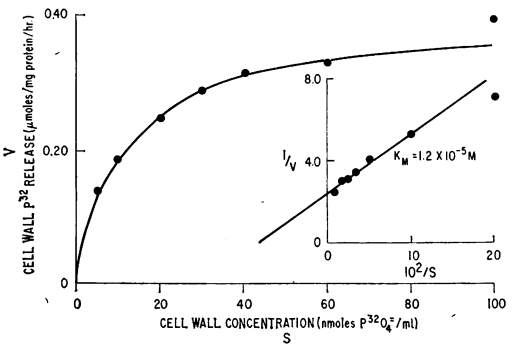

FIG. 7. *Michaelis constants for cell-wall lytic enzyme from Staphylococcus aureus. Under conditions of standard assay for cell wall lytic enzyme, 5.5 to 110 μg/ml of cell wall (5 to 100 nmoles of cell-wall $^{32}PO_4$/ml) were added. The Lineweaver Burk plot of this data is shown in the inset. The K_m of 1.2 × 10^{-5} M cell-wall phosphate corresponds to 13.8 μg of cell-wall (dry weight) per ml.*

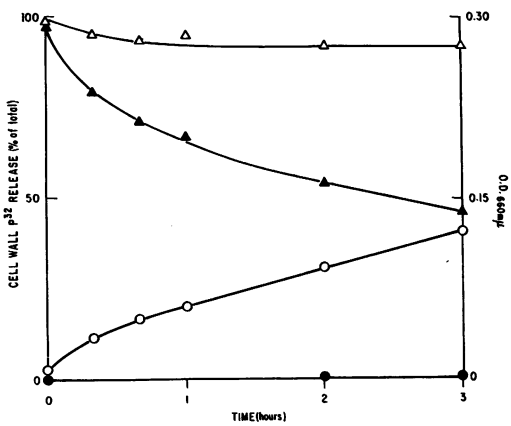

FIG. 8. *Effect of high levels of AFZ enzyme fraction on the release of ^{32}P from cell walls of Staphylococcus aureus and correlated changes in turbidity (OD_{660}). An incubation mixture containing 920 nmoles of formaldehyde-treated cell-wall $^{32}PO_4$/ml [1.06 mg of cell wall (dry weight) per ml] and 217 units of AFZ enzyme fraction/ml was incubated under conditions of standard assay. The OD_{660} (▲) and ^{32}P-release (○) were measured at the indicated times. OD_{660} (△) and ^{32}P-release (●) for the control without enzyme are indicated.*

were treated with 128 units of AFZ enzyme fraction for a 3-hr period, there was a decrease in OD_{660} of 0.08. The control without enzyme showed a decrease of 0.04 during the same time period. The decrease in turbidity in the absence of enzyme was on the same order of magnitude as observed by Mitchell and Moyle for autolysis of cells (15). Whether the greater rate of change in turbidity in the presence of enzyme represented lysis of the cells from without, superimposed on autolysis, or whether this was an enhancement of the autolysis of the whole cells in the presence of the added protein, is not clear. However, if the net rate of 0.04 decrease in OD_{660} per 3 hr with cells is compared to the rate of OD_{660} decrease with cell walls over a 3-hr period, and if correction is made for the fact that the turbidity of cell walls is 6.3% that of the cells, then the rate of lysis of cells per unit of enzyme is only 5% of the rate on cell walls.

Role of magnesium. When cell walls were incubated with the AFZ enzyme fraction, there was a rapid adsorption of the enzyme onto the cell wall; this occurred at 0 C, as shown in Table 4. Adsorption was dependent upon the presence of magnesium. Adsorption of the enzyme activity to the cell wall was incomplete (Table 4). This may have resulted from the fact that there were two different enzymes in the AFZ preparation, or may have occurred because the cell-wall binding sites were limiting. In a separate experiment similar to that of Table 4, 16 times as much cell

TABLE 4. *Absorption of cell-wall lytic enzyme to Staphylococcus aureus cell walls at 0 C in the presence and absence of $MgCl_2$*[a]

Time of absorption	Units of lytic enzyme activity (nmoles of $^{32}PO_4$ per ml per hr)			
	With $MgCl_2$		Without $MgCl_2$	
	Unabsorbed enzyme	Absorbed enzyme	Unabsorbed enzyme	Absorbed enzyme
min				
0	1.18	1.59	2.40	0.15
1	1.17	1.74	2.44	0.23
5	1.08	1.68	2.42	0.17
10	1.02	1.67	2.38	0.13
20	1.01	1.78	2.45	0.13

[a] The reaction mixture contained 2.45 units per ml of AFZ enzyme fraction (10 μg/ml of protein), 0.01 M NaH_2PO_4 (pH 6.0) with or without 0.04 M $MgCl_2$ as indicated. Formaldehyde-treated cell walls were added to each tube at a final concentration of 28 μg/ml. After the indicated absorption period at 0 C, each mixture was centrifuged for 10 min at 30,000 rev/min in a Spinco SW 39 swinging bucket rotor at 0 C. The final volume of the reaction mixture was 4.0 ml. The supernatant fluid was assayed for unabsorbed enzyme by using the standard assay for lytic enzyme. The enzyme absorbed onto the cell-wall precipitate (absorbed enzyme) was assayed by incubating the resuspended cell-wall precipitate (28 μg/ml) under the conditions used for the standard assay for lytic enzyme.

wall (450 μg) was used to adsorb the AFZ enzyme preparation (3 units per ml). In this case, there was still 38% of the enzymatic activity that did not adsorb. This unadsorbed enzyme was either a different enzyme or a different physical state of the adsorbed enzyme.

When AFZ enzyme was adsorbed onto cell walls and tested for the requirement of magnesium, cobalt, and EDTA, the requirement for magnesium was much reduced (Table 1). Cell walls with adsorbed enzyme were 50% inhibited in the presence of EDTA. This inhibition with EDTA was overcome with cobalt and magnesium. Native cell walls showed the same pattern of activation in the presence of EDTA, Mg, and Co, as the formaldehyde-treated cell walls with adsorbed enzyme (Table 1). Since AFZ lytic enzyme rapidly attached to formaldehyde-treated cell walls in the presence of magnesium, giving rise to a cell wall-lytic enzyme complex, which behaved similarly to native cell walls, one might question whether the cell walls have attached enzyme before disruption of the cell. An alternative possibility is that the enzyme attached to the cell wall under the conditions of disruption of the bacteria, as in the case of cytoplasmic polyphosphate attachment to *Neurospora* cell walls during fractionation (11).

DISCUSSION

The release of 50% of the soluble intracellular autolytic enzyme activity from *S. aureus* upon freezing and thawing, with only 2% release of the intracellular protein, as reported here, is an example of the specific release of a degradative enzyme from a gram-positive organism. A number of degradative enzymes are released specifically from *E. coli* and some other gram-negative organisms by osmotic shock treatment (16). Osmotic shock has not yet been shown to cause such a specific release from any gram-positive organisms (17). It would be of interest to see whether osmotic shock would also release the intracellular cell-wall lytic enzyme of *S. aureus*. It is possible that the freezing and thawing technique might be of use for the specific release of other enzymes of staphylococci and related organisms.

The fact that cell-wall lytic enzyme is specifically removed from the cell suggests a surface localization. All *S. aureus* cell-wall preparations which have been tested have attached lytic activity. The demonstrated rapid magnesium-dependent binding of lytic enzyme to cell walls raises the question as to whether this enzyme is attached in vivo, or whether this attachment occurs after disruption. The slow autolysis of washed *S. aureus* and the insensitivity of the whole cells to lysis from without in the presence of the lytic enzyme suggest that this enzyme is associated with the inner surface of the cell wall. The presence of enzyme extracellularly could be a result of leakage of the enzyme through the cell wall or a specific excretion of enzyme associated with cell division.

The incomplete adsorption of enzyme by cell walls even in an excess of cell walls and the two peaks of enzyme activity observed on DEAE cellulose may be due to the presence of more than one lytic enzyme in the AFZ fraction.

The rate of autolysis in *S. aureus* (15), as well as in *B. subtilis* (26) and *S. faecalis* (22), is greatest during logarithmic growth. The rate of autolysis could be a function of a changing susceptibility of the cell wall during growth in the presence of a constant level of autolytic enzyme. The present studies, in which enzyme fractions were tested with cell walls from a single stage of growth, demonstrate that the enzyme activity found extracellularly and in the first wash of the cells is greatest during logarithmic growth. The intracellular content of enzyme remained relatively constant throughout the growth period. It would be of interest to see whether the rate of autolysis in the present strain of *S. aureus* varies with growth, as observed by Mitchell and Moyle, or whether the susceptibility of the cell wall to lysis changes during growth.

The occurrence of maximal autolytic enzyme activity in various bacteria during logarithmic growth suggests that the enzyme has some function associated with growth and cell division (15, 23, 26). Possibly such an enzyme acts specifically in the local areas of cell-wall growth (6, 22, 23) to hydrolyze the cross-linked matrix and to allow the insertion of cell wall subunits transported through the cell membrane via a lipid carrier (2, 26). The concept of localized action of autolytic enzyme on the cell wall is further supported by the observation of Mitchell and Moyle (15) that osmotic shocking of cells of *S. aureus* allowed to autolyze in the presence of 1.2 M NaCl gives rise to hemispherical halves of cell wall. The more rapid release of ^{32}P from pulse-labeled cell walls, as compared to uniformly labeled cell walls in the presence of the AFZ enzyme fraction, which will be reported subsequently, further supports a specific localized action of the enzyme. The presence of extracellular lytic enzyme could be a result of the fact that this enzyme is synthesized locally near the region of cross wall formation and then specifically released from the cell, perhaps at the time of cell division. Studies with synchronously dividing cells could test this theory.

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