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Extracellular Cell Wall Lytic Enzyme from *Staphylococcus aureus*: Purification and Partial Characterization

ESKIN HUFF, CAROL S. SILVERMAN, NANCY J. ADAMS, AND WOODRUFF S. AWKARD
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

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An autolysin obtained from culture fluid of *Staphylococcus aureus* strain 8507 was purified 3,000-fold. One milligram of this preparation (S-5DL) will solubilize 12 mg of cell wall in 1 hr. The major activity is *N*-acetylmuramyl-L-alanine amidase. Recovery of lytic activity in the purified preparation was repeatably only 20% of the starting level. This suggests that other cell wall lytic enzymes may be present in the starting material. The S-5DL enzyme has been compared to freeze-thaw extracted enzyme (AFZ). Both enzymes precipitate in 0.01 M KPO_4 (*p*H 6.0) and dissolve in 0.1 to 0.7 M NaCl. Fifty per cent of the AFZ activity and 66% of the S-5DL activity bind rapidly to cell walls of *S. aureus* at 0 C in the presence of magnesium ion. None of the AFZ activity and 66% of the S-5DL activity bind to cell walls at 0 C in the absence of magnesium ion. The cell walls of nine different strains of *S. aureus* were compared for level of native autolysin activity. These same walls after inactivation of the native autolysin were tested for susceptibility to the S-5DL enzyme.

Autolysins present in the cells and cell walls of *Staphylococcus aureus* were the subject of recent reviews (6, 19). Tipper showed that the major enzyme associated with isolated *S. aureus* cell walls is *N*-acetylmuramyl-L-alanine amidase (19). A small amount of endo-β-*N*-acetylglucosaminidase activity was also observed in *S. aureus* cell walls. The presence of *N*-acetylmuramyl-L-alanine amidase and glycylglycine endo-peptidase was demonstrated in a purified preparation from culture filtrates of *S. epidermidis* (16, 17). Partial purification of an apparently surface localized autolysin of *S. aureus* was reported (6). The mechanism of action of this enzyme and other isolated intracellular staphylococcal cell wall lytic enzymes has not been reported.

Characterization of an extracellular cell wall lytic enzyme of *S. aureus* was attempted to distinguish it from intracellular cell wall lytic enzymes. It is our hope that such studies will lead to the determination of the specific location of the activities in the cell and the function of the enzymes during cellular growth.

MATERIALS AND METHODS

Bacterial strains. *S. aureus*, our strain number 8507 (phage pattern 52/80), was obtained from Rose Lieberman; *S. aureus* 2476, 968, 1159, and 1200 from Ruth Korman; group H streptococci Challis and Wickey from Jon Ranhand; *Bacillus cereus* NRRL 569 from Arthur Saz; *S. aureus* ATCC 9144 (Oxford), *Micrococcus lysodeikticus* ATCC 4698, and *S. aureus* ATCC 13801 (H) from ATCC; *S. aureus* strain Morris (an encapsulated strain) and LS from Archibald Scott.

Purification of the culture supernatant enzyme. A strain of *S. aureus* (8507) produces an enzyme in the culture supernatant which is lytic toward cell walls, but not whole cells, of the parent organism (6). This strain was grown in Trypticase Soy Broth (Difco) with shaking on a New Brunswick shaker incubator. A 100-ml amount of an 18-hr shake culture 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 optical density at 660 nm (OD_{660}) increased from a value of 0.05 to a final value of 1.0, at which point the yield of cell wall lytic activity in the culture fluid was maximal. If the starting OD_{660} was 0.2 or greater, the yield of enzyme in the culture supernatant was low, presumably because exponential growth was never achieved. The cells from 20 flasks were removed by centrifugation at 3,020 × *g* in a Szent-Gyorgyi and Blum continuous-flow attachment to the Sorvall centrifuge. An aluminum coil in ice was used to cool the medium as it passed to the centrifuge. The culture supernatant was collected in a container in ice. Ammonium sulfate (5 kg) was added with stirring to each 10 liters of culture supernatant. After five days at 5 C, the precipitate settled to the bottom; thus, the bulk of the supernatant fluid could be siphoned off (S-1). The remaining suspension was centrifuged for 30 min at 3,600 × *g*. The precipitate (P-1) from 20 liters of culture fluid was suspended in

70 ml of water and centrifuged for 3 hr at 81,000 × *g*. The precipitate was resuspended to 100 ml (P-2) for assay. The supernatant fraction (S-2) was rapidly decanted; the *p*H was adjusted to 6.5 with 2 N KOH and placed in two 20-inch lengths of ca. 1.38-inch dialysis tubing with a marble in each section. The tubing sections were placed in two plastic cylinders on a rocking platform (6 cycles per min) and dialyzed against 1,500 ml of 0.01 M KPO_4 (*p*H 6.5). The buffer was changed three times on the first day and then daily for 4 to 5 more days, until a total of 12 liters of buffer had been used. The dialyzed enzyme (S-2D) was centrifuged for 3 hr at 94,000 × *g*. The supernatant fraction (S-3) was decanted; the precipitate (P-3) was resuspended to a final volume of 10 ml with 0.01 M KPO_4 (*p*H 6.5) in a 10-ml polycarbonate tube and centrifuged for 3 hr at 81,000 × *g*. The supernatant (S-4) was discarded and the centrifuge tube was washed with 0.5 ml of 0.01 M KPO_4 (*p*H 6.5), care being taken not to lose any precipitate. The precipitate (P-4) was resuspended in 0.6 ml of 0.01 M KPO_4 (*p*H 6.5) by using a small glass Tenbrook homogenizer. A 0.6-ml amount of 0.01 M KPO_4-1.4 M NaCl (*p*H 7.0) was added to the resuspended P-4 to dissolve it, and the mixture was centrifuged for 30 min at 81,000 × *g*. A small-scale rotating dialysis device was prepared as follows. A 2-inch square of dialysis membrane was placed on the lower end of a 40 mm (length) by 29 mm (inner diameter) section of glass tubing which had been carefully fire-polished. An O-ring (inner diameter, about 2 mm smaller than the outer diameter of the glass tubing) was placed under the membrane and forced up over the glass tubing. This procedure pulls the membrane tightly over the lower end of the tubing. The supernatant (S-5) was removed and placed on the inner surface of the dialysis membrane. The device was lowered into 0.002 M ammonium acetate (*p*H 6.5) until the liquid level was even with the O-ring and then rotated at 600 rev/min around its vertical axis for 90 min. The buffer was changed three times. The dialyzed solution (S-5D) was lyophylized and stored at −20 C over anhydrous magnesium perchlorate under a vacuum. Lyophylized enzyme (S-5DL) stored in this manner has not lost activity in 6 months.

In an alternate procedure, S-5 was precipitated by the addition of 0.6 g of ammonium sulfate per ml of solution. Such ammonium sulfate-precipitated preparations (S-5A) lost activity at the rate of 50% per month when stored at 5 C. A flow sheet of the purification and alternate purification procedure is shown in Fig. 1.

Enzyme at the P-1, S-2, S-2D, P-3, and P-4 stages was quite stable and could be stored frozen or at 5 C. The *p*H at which S-2 is dialyzed is important. In a preliminary experiment, the supernatant fraction (S-2) was dialyzed for 2 hr against 0.01 M KPO_4 at *p*H values of 6.1, 6.5, and 6.9 followed by centrifugation for 15 hr at 34,000 rev/min in a Spinco SW 39 rotor. The overall enzyme recovery was 66, 82, and 36%, respectively. Recovery in the precipitate was 56, 71, and 23%, respectively.

Preparation of the ammonium sulfate precipitate of *S. aureus* 8507 whole cell freeze-thaw extract (AFZ) was as previously described (6).

Preparation of cell walls. Native cell walls (CWN) were prepared by sucrose gradient fractionation (6). Treatment with formaldehyde (6) completely inactivated cell wall-bound autolysins in all the cell wall preparations studied. Formaldehyde-treated cell walls were found to be inhibitory to the purified lytic enzyme even after several washes in buffer. Such inhibition could be prevented if the formaldehyde-treated cell walls were washed free of the sucrose in which they were stored, suspended in 0.1 mg of albumin (bovine serum albumin; Armour Pharmaceutical Co.) per ml in 0.001 M KPO_4 (*p*H 6.0), centrifuged, and resuspended in the same solution. These albumin-treated cell walls (CWB) were either used immediately or stored in 20% sucrose-1 M KCl.

Cell walls used to demonstrate the mode of action of the lytic enzyme were prepared as usual through the formaldehyde treatment. They were then incubated with 0.1 M sodium pyrophosphate buffer (*p*H 9.2) at 37 C for 6 hr (18). Longer treatment failed to remove more alanine. Such formaldehyde and pyrophosphate treated cell walls (CWO) were lysed by the purified enzyme S-5DL at 1.5 times the rate as CWB cell walls.

Lytic assay. The reaction mixture used for assay of the enzyme consisted of 40 μmoles of $MgCl_2$, 10 μmoles of KH_2PO_4 (*p*H 6.0), 28 μg (dry weight) of albumin-treated cell walls [25 nmoles of cell wall $^{32}PO_4$, prepared as described previously (6)], 100 μg of bovine plasma albumin, and 0.5 to 1.5 units of enzyme per ml of reaction mixture. Before addition of the cell walls and enzyme, the *p*H level of the mixture was adjusted to 6.0 with KOH. Enzyme was always added last to the reaction mixture. Incubation was at 37 C for 20 min. The reaction mixture was chilled; 15 ml of water at 0 C was added and then filtered through a membrane filter (0.45 μm pore size; Millipore Corp.). Radioactivity was measured as described previously (6). A unit of enzyme activity is defined as that amount of enzyme necessary to release 1 nmole of $^{32}PO_4$ per ml of standard reaction mixture in 1 hr. Bovine plasma albumin (0.1 mg/ml) was used to suspend all lyophylized enzyme preparations and for all enzyme dilutions.

Thin-layer chromatography. A total of 4 μmoles of unknown or standard dinitrophenyl (DNP) amino acid in acetone or acid-acetone [6 N HCl:acetone (2:23)] was spotted in four applications (0.2 μliters per application) onto Silica gel G thin-layer plates (250 μm thick) prepared by Analtech Inc. Solvent 1 was the upper layer of a mixture of toluene-pyridine-ethylenechlorohydrin-0.8 N ammonia in water (100:30:60:60) (reference 2). Solvent 2 was a mixture of chloroform-methanol-glacial acetic acid (95:5:1) (reference 2). Solvent 3 was the upper layer of a mixture of *n*-butanol-glacial acetic acid-water (4:1:5). Solvent 4 was a mixture of benzene-pyridine-glacial acetic acid (80:20:2) (reference 2).

Chemical and analytical methods. The appearance of free amino groups during lysis was measured by reaction with fluorodinitrobenzene (4); the appearance of reducing sugars by the ferricyanide method (4). Total phosphate was determined by the procedure of Chen, Toribara, and Warner (3) as modified by Ames and Dubin (1). Protein was determined by the procedure of Lowry et al. (7).

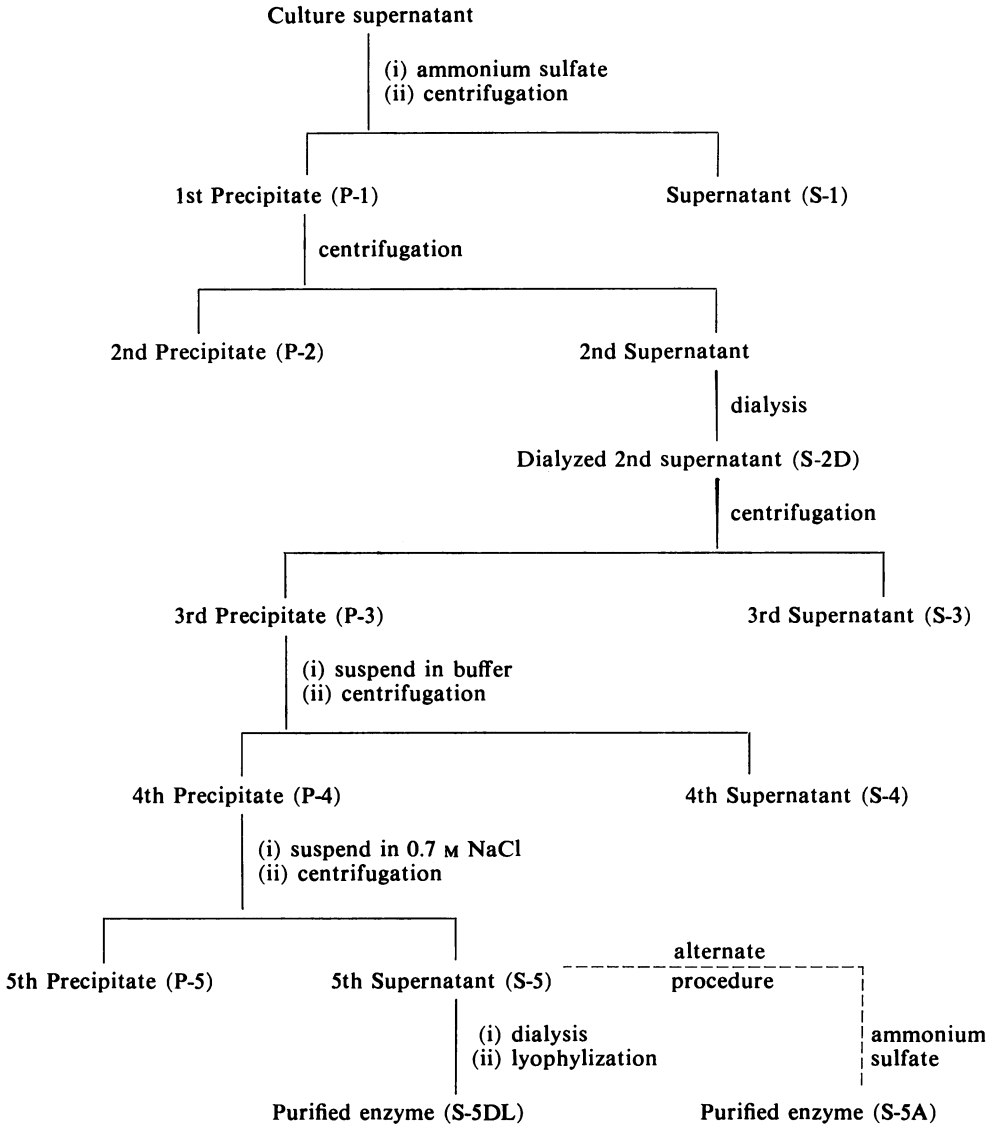

FIG. 1. *Purification of S. aureus 8507 culture supernatant cell wall lytic enzyme.*

Materials. Toluene, reagent-grade, was extracted three times with one-tenth volume of concentrated H_2SO_4, washed with water, 2 N Na_2CO_3, and again with water. After drying over calcium chloride, it was distilled. Pyridine, chloroform, and methanol were spectro-grade from Eastman Kodak. Ammonium sulfate (Mann Research Laboratories) was enzyme-grade. Other chemicals were reagent-grade.

RESULTS

Solubility of the culture supernatant enzyme. The culture supernatant enzyme is insoluble at low salt concentration (0.01 M KPO_4, *p*H 6.5) and soluble in the presence of 0.1 to 0.7 M sodium chloride. This property is readily observed by sucrose gradient fractionation (8) as shown in Fig. 2. In 0.1 M NaCl, the enzyme sediments with an apparent molecular weight of 30,000. In 0.01 M sodium or potassium phosphate (*p*H 6.5), the enzyme sediments to the bottom of the tube. Most of the lytic activity is in the precipitate, whereas the bulk of the protein is in the low-molecular-weight fraction (fractions 9, 10, and 11). Insoluble enzyme can be solubilized by suspension in 0.1 M to 0.7 M NaCl. This ability to solubilize and precipitate the enzyme with changes in the salt concentration has been used to provide the most important steps in the procedure used for purification of the enzyme. The

lytic activity obtained by freezing and thawing of the cells (6) solubilizes and precipitates in a similar manner (Fig. 2).

Purification of the culture supernatant enzyme. The purification procedure is outlined in Fig. 1. Recovery and specific activity of the enzyme at various stages of purification are presented in Table 1. The assay for lysis was satisfactory in the range of 0.1 to 5 units of enzyme, which gave linear function with respect to time (Fig. 3) and enzyme concentration (Fig. 4).

Mode of action. When the purified culture supernatant enzyme (S-5A) was incubated with *S. aureus* 8507 cell walls, N-terminal amino groups were released without the release of reducing groups (Fig. 5). In this experiment (curve A), 50 mg of alkaline-treated cell walls (CWO) was reacted with 4,900 units of S-5A for 8 hr (total volume, 42 ml). The mixture was reated with fluorodinitrobenzene. Nine micro-

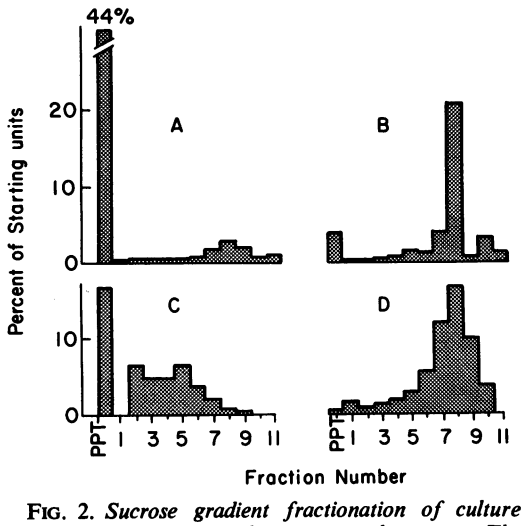

FIG. 2. *Sucrose gradient fractionation of culture supernatant and freeze-thaw extracted enzyme. The ammonium sulfate precipitate of freeze-thaw extracted enzyme (AFZ) previously described (6) or culture supernatant enzyme at the S-2 stage of purification was dialyzed for 30 min against the following buffers: A, 705 units of S-2 dialyzed against 0.01 M KPO_4 (pH 6.5); B, 705 units of S-2 dialyzed against 0.05 M KPO_4-0.1 M NaCl (pH 6.5); C, 1,080 units of AFZ dialyzed against 0.01 M KPO_4 (pH 7.0); D, 688 units of AFZ dialyzed against 0.05 M KPO_4-0.3 M NaCl (pH 7.0). The enzyme (0.2 ml) was then layered on a 5 to 20% sucrose gradient containing KPO_4 and NaCl at the same concentration and pH as used for dialysis. After centrifugation for 16 hr at 99,000 × g, 10 or 11 equal volume fractions plus the precipitate were collected and assayed for lytic activity. Enzyme recoveries for A, B, C, and D were 55, 39, 46, and 57%, respectively.*

TABLE 1. *Summary of the purification of cell wall lytic enzyme from Staphylococcus aureus 8507 culture supernatant*

Step	Fraction	Vol	Enzyme	Specific activity[a]	Activity recovered
		ml	*units/ml*		%
1	CS	20,000	54.5	5.45	100
	S-1	25,000	14.1	1.95	32
2	P-1	102	12,050	35	112
3	S-2	95	11,100	970	96
	P-2	100	258	6,800	2
4	S-2D	340	2,250	758	70
	S-3	318	2,200	778	64[b]
5	S-5DL	10.1[c]		14,800	13.6[d]

[a] Expressed as units per milligram of protein, except for last value which is expressed as units per milligram (dry weight).

[b] In other enzyme preparations, recovery was 15, 13, and 5%, making the present value of 64 unusually high.

[c] Milligrams.

[d] In other enzyme preparations, the recovery in this fraction varied from 13 to 22%.

moles of N-terminal amino acid (as alanine) was released in this time period. The dinitrophenylamino derivatives were released from the cell wall by hydrolysis in 2.9 N HCl for 16 hr at 105 C. The HCl was removed in vacuo over soda lime. The residue was extracted with ether and acid-acetone (2 ml of 12 N HCl diluted to 25 ml with acetone). The acid-acetone extract was dried and extracted with ether. The combined ether extracts were applied to a Silica gel G thin-layer plate and developed in solvent 2 for 1 hr. The bands of color were scraped off the plate; the silica gel was then acidified with 6 N HCl and repeatedly extracted with acetone to remove yellow color The acetone was treated with $NaHCO_3$ and centrifuged at 200 × *g*; the supernatant fluid was filtered through Gelman Alpha Metricel filter paper, evaporated to less than 1 ml, and then diluted to 1.00 ml with acetone. The OD_{420} of each of the extracted bands was measured in 1% sodium borate (Table 2). The major DNP material (band 3) traveled identically to DNP-alanine in thin-layer chromatographic solvents 1, 2, 3, and 4 and accounted for 54% of the color present. A small amount (4.6%) of material (unknown 2) migrated in the same manner as DNP-glycine. Two faint compounds (unknowns 1 and 3) accounted for another 4%

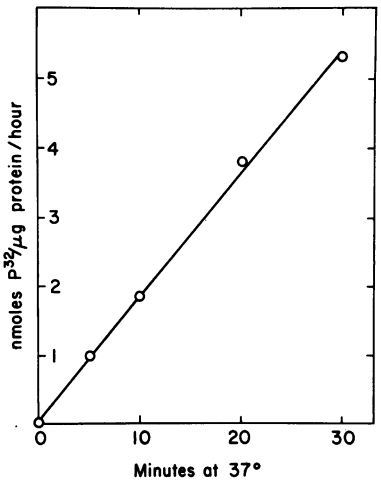

FIG. 3. *Kinetics of the release of ^{32}P from S. aureus cell walls by purified culture supernatant enzyme. Conditions were the same as indicated in the standard assay except that the reaction was run for variable times as indicated.*

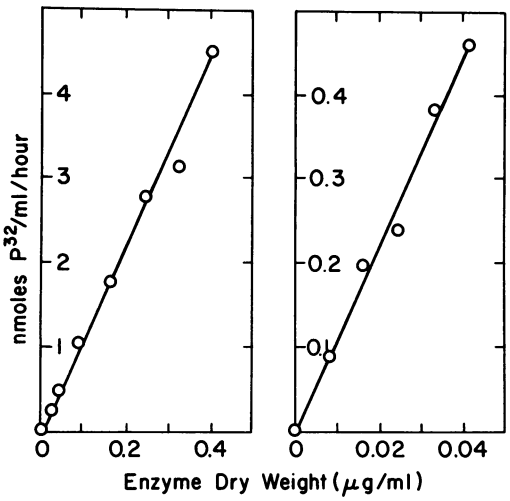

FIG. 4. *Variation of the rate of lysis of S. aureus cell walls with concentration of purified culture supernatant enzyme. Conditions were the same as the standard assay for lytic enzyme except that the amount of S-5DL enzyme was varied as indicated.*

of the total. The balance of the color (37.5%) was from dinitrophenol (DNP-OH) and dinitrophenylamine (DNP-NH_2). The rotation of the isolated DNP-alanine was compared to authentic DNP-D- and DNP-L-alanine by using a Perkin Elmer automatic polarimeter (model 141). The amount of material limited the accuracy of the determination. The observed rotation indicated the isolated material to be at least 75% DNP-L-alanine.

Properties of the purified lytic enzyme. The *p*H optimum of the enzyme is 6.1 in phosphate buffer. Near optimal activities are obtained in acetate, phosphate, and phosphite buffers from *p*H 5 to 7 (Fig. 6). Potassium chloride inhibits activity when present at concentrations of 0.05 M or

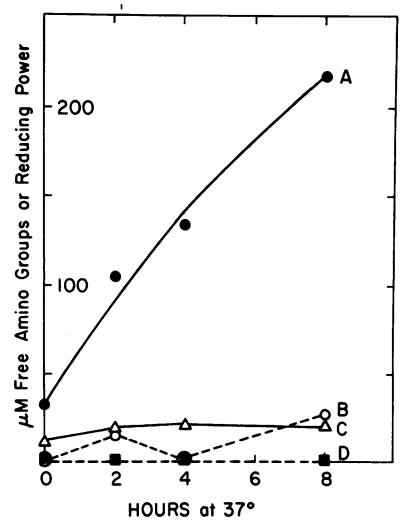

FIG. 5. *Release of free amino groups from S. aureus cell walls by purified culture supernatant enzyme. In curves A and C, the reaction mixture was 40 µmoles of $MgCl_2$, 10 µmoles of KH_2PO_4 (pH 6.0), 1.2 mg of cell walls (CWO), and 117 units of S-5A (final pH, 6.0) per ml of reaction mixture. The reaction mixture for curve B was the same as that for curve A, except that it was without cell walls; the reaction mixture for curve D was the same as that for curve A, without enzyme. Release of free amino groups was measured in curves A, B, and D. Free reducing sugar was measured in curve C.*

TABLE 2. *Dinitrophenyl (DNP) compounds obtained from Staphylococcus aureus cell walls after lysis with purified S-5DL enzyme followed by treatment with fluorodinitrobenzene and acid hydrolysis*

Band no.	R_F	Compound	OD_{420} in 1-ml sample	Percentage of total OD_{420}
1	0.07	Unknown 1	0.31	1.4
2	0.19	Unknown 2	1.01	4.6
3	0.31	DNP-alanine	11.70	54.0
4	0.47	DNP-OH	1.2	5.5
5	0.51	DNP-NH_2	6.85	31.6
6	0.89	Unknown 3	0.61	2.8

greater (Fig. 7). The significance of the diphasic nature demonstrated in Fig. 7 is not known. Magnesium chloride stimulates the reaction, reaching an optimal concentration at 0.03 to 0.04 M. Higher concentrations of magnesium inhibit the activity (Fig. 8).

Magnesium-specific binding. When the S-5DL enzyme was incubated with cell walls at 0 C, there was an immediate uptake of 65 to 75% of the activity by the cell walls (Table 3). This uptake was not dependent on the presence of $MgCl_2$ (0.04 M) as in the case of the AFZ enzyme (*see* Table 3 and reference 6). AFZ absorption to cell walls was 44% in the presence of $MgCl_2$ (0.04 M) and 1% in the absence of magnesium. AFZ does

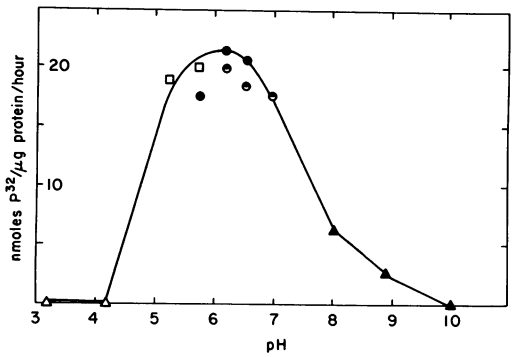

FIG. 6. *Rate of lysis of S. aureus cell walls versus pH. Conditions were the same as indicated in the standard assay with the exception that the potassium salts of formic acid (△); acetic acid (□); and phosphoric (●), phosphorus (◐), and N-tris-(hydroxymethyl) methyl 2-amino ethane sulfonic acids (▲) were used at a final concentration of 0.01 M. All pH values are the measured, final pH level of the reaction mixture after 20 min of incubation at 37 C.*

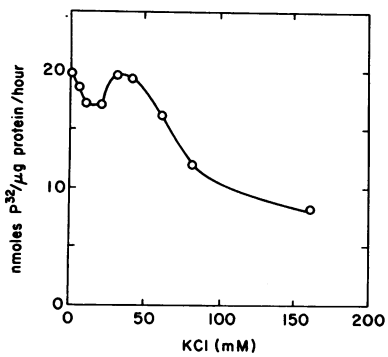

FIG. 7. *Rate of lysis of S. aureus cell walls versus KCl concentration. Conditions were the same as indicated in the standard assay with the exception that KCl was added as indicated. The pH level was adjusted to 6.0 in all cases.*

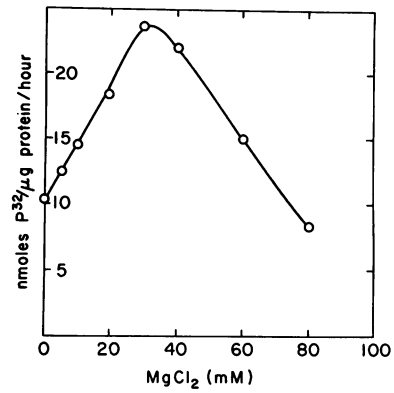

FIG. 8. *Rate of lysis of S. aureus cell walls versus magnesium ion concentration. Conditions were the same as for the standard lytic assay except that the $MgCl_2$ concentration was varied and the pH level was adjusted to 6.0 after the addition of magnesium; this was necessary since the $MgCl_2$ tended to make the reaction mixture acidic.*

TABLE 3. *Effect of magnesium ion on binding of the AFZ and S-5DL enzyme preparations to cell walls of Staphylococcus aureus 8507*

Expt	Enzyme added	Absorption time	Percentage of enzyme absorbed onto the cell walls[a]	
			With Mg	Without Mg
		min		
1[b]	1.64 units of S-5DL	0	59	62
		20	56	59
2[c]	1.56 units of AFZ	5	44	1.3
		19	52	0
3[c]	1.75 units of S-5DL	20	66	74
4[c]	2.32 units of S-5DL	20	70	64

[a] The reaction mixture was the same as that used for the standard lytic assay. After the indicated absorption period at 0 C, each mixture was centrifuged for 10 min at 20,000 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 (S-1) was assayed for unabsorbed enzyme by using the standard assay for lytic enzyme. The enzyme absorbed onto the ^{32}P-labeled cell wall precipitate (P-1) was assayed by incubating the resuspended cell wall precipitate (28 µg/ml) under the conditions used for the standard assay for lytic enzyme. The percentage absorption was (S-1 units)/(S-1 units + P-1 units) × 100.

[b] Bovine plasma albumin (BSA) concentration, 0.012 mg/ml.

[c] BSA concentration, 0.102 mg/ml.

not contain an inhibitor for the absorption of S-5DL to cell walls in the absence of magnesium, since S-5DL still absorbed when premixed with AFZ before addition to the cell walls (Table 4, experiment 4). The portion of S-5DL which did not initially absorb to cell walls was not the result of the establishment of an equilibrium between absorbed and unabsorbed enzyme, since a second addition of cell walls did not further absorb out any enzyme (Table 4, experiment 3; compare S-1 with S-2 and P-2).

Specificity of the enzyme toward other cell walls. The relative rate of turbidity reduction of various species of cell walls by the S-5DL enzyme is compared in Table 5. It is of interest that the parent organism (8507) had a lysis rate with the S-5DL enzyme which was less than 50% that of any other strain. This suggests that the enzyme acts in a more controlled manner when lysing homologous cell walls. It would be interesting to see whether enzyme from the culture fluids of other *S. aureus* strains also discriminates against the parent cell walls. The differences in the rate of turbidity reduction probably reflect differences in the affinity of the S-5DL enzyme for the various cell walls as well as differences in the ratio of turbidity to content of L-alanine-muramic acid bond.

TABLE 4. *Effect of the AFZ enzyme on the magnesium-independent binding of the S-5DL enzyme to cell walls of Staphylococcus aureus*

Fraction assayed	Units[a]			
	With S-5DL		With S-5DL + AFZ	
	With Mg	Without Mg	With Mg	Without Mg
Expt 3[b]				
S-1	0.69	0.43	1.14	1.75
P-1	1.41	1.24	1.93	1.09
S-2	0.62			
P-2	0.02			
Sum S-1 + P-1	2.10	1.67	3.07	2.84
Expected units	1.75	1.75	3.12	3.12
Expt 4[c]				
S-1	0.66	0.82	5.88	8.72
P-1	1.57	1.47	3.92	1.29
Sum S-1 + P-1	2.23	2.29	9.80	10.01
Expected units	2.32	2.32	11.02	11.02

[a] Expressed as nanomoles of ^{32}P cell wall released per milliliter per hour; enzyme activities determined as in Table 3.

[b] S-5DL (1.75 units) was incubated with or without 1.37 units of AFZ, with the AFZ added last. In the tube with S-5DL + Mg, a portion of the supernatant S-1 was reabsorbed with 28 μg of cell wall per ml. The activity in S-2 and P-2 was determined in the same manner as S-1 and P-1, respectively.

[c] S-5DL (2.3 units) was incubated alone or premixed with 8.70 units of AFZ before addition to the reaction mixture containing cell walls.

TABLE 5. *Specificity of purified culture supernatant enzyme (S-5DL) toward various cell walls*

Organism	Decrease in OD_{660}/min × 1,000[a]			
	Autolysis CWN	Autolysis CWB	Lysis CWB[b]	Lysis CWN[b]
Staphylococcus aureus				
2476	10.8	0.00	19.3	
968	45.5	0.00	30.0	
8507	1.3	0.00	5.4	
H	2.0	0.00	14.1	
Oxford	0.82	0.00	17.8	
1159	3.00	0.00	18.5	13.5
1200	1.30	0.00	13.0	16.0
Morris	1.35	0.00	10.5	13.7
LS	3.00	0.00	22.6	14.1
Micrococcus lysodeikticus	11.5	0.00	25.9	
Bacillus cereus	0.72	0.00	0.00	
Streptococcus group H Wickey	0.00	0.00	1.74	1.65
Streptococcus group H Challis	0.00	0.00	1.00	1.20

[a] Cell walls were added to give a starting OD_{660} as near 0.400 as possible. Abbreviations: CWN, native cell walls; CWB, native cell walls after treatment with formaldehyde and albumin.

[b] S-5DL enzyme was added to a final concentration of 740 units/ml (50 μg/ml). When the starting OD_{660} varied from 0.4, the value, 1,000 × (decrease in OD_{660}/min), was multiplied by the factor, starting OD_{660}/0.4, to correct to the expected rate at 0.40 OD_{660}.

DISCUSSION

An autolysin obtained from culture supernatants of *S. aureus* has been purified to the level of 11,000 units per mg of protein. This represents a 1,000- to 3,000-fold purification, depending on the batch of culture supernatant. The major activity detected in the S-5A enzyme preparation was *N*-acetylmuramyl-L-alanine amidase. Suginaka et al. (16) have purified a lytic enzyme (ALE) from *S. epidermidis* culture supernatants to an equal potency as S-5DL amidase. This ALE enzyme contained equal amounts of *N*-

acetylmuramyl-L-alanine amidase and a glycyl-glycine endopeptidase (17). Less than 10% of the activity in S-5DL could have been due to splitting of the pentaglycine cross-bridge. An endo-acetylglucosaminidase was reported by Tipper (19) to be associated with cell walls. There was no evidence for the presence of this enzyme in S-5DL. The glycyl-glycine endopeptidase and the endo-acetylglucosaminidase activities may be present in the crude culture fluid and account for the fact that never more than 20% of the original lytic activity can be isolated in the final 11,000 units per mg of protein preparation. Wadstrom and Hisatsune (*personal communication*) partially purified an endo-β-*N*-acetyl-glucosaminidase from the culture supernatants of *S. aureus* M18.

It is clear from the studies of enzyme binding to walls that the majority of the S-5DL enzyme which rapidly absorbs to cell walls at 0 C in the absence of magnesium is different from the AFZ enzyme, since the latter does not absorb or inhibit the absorption of S-5DL to cell walls in the absence of magnesium. Magnesium ion was required for the absorption of the AFZ enzyme to cell walls but had no effect on absorption of the S-5DL enzyme. In the presence of magnesium, only 66% of the S-5DL enzyme activity absorbed to the cell wall. A second treatment with cell wall did not further absorb any enzyme. This suggests that either the enzyme which absorbs is different from that which does not absorb or the binding properties of a portion of the enzyme are altered by the first treatment with cell walls. Shockman and Cheney (14) have shown that the muramidase from *S. faecalis* cell walls is incompletely (87%) absorbed to cell walls.

It is reasonable to suppose that if either the S-5DL enzyme, which binds to cell walls, or the AFZ enzyme, which binds in the presence of magnesium, is present in the cells at the time of disruption, it might be absorbed to the cell wall and be indistinguishable from a lytic enzyme already attached. It is therefore difficult to be certain that the mechanism of autolysis of isolated cell walls of *S. aureus* (19) represents the true picture in vivo. In a careful study of the autolysin of *S. faecalis*, Pooley and Shockman (10) were able to determine the amount of unbound latent autolysin present in cells. They mixed unlabeled cell walls (treated with sodium decyl sulfate to inactivate autolysin bound to the cell walls) with ^{14}C-labeled cells in various proportions during disruption. The cell walls were isolated and the rate of release of ^{14}C was determined. Latent autolysin content was plotted against the per cent of ^{14}C in the isolated cell wall. Extrapolation to zero per cent ^{14}C showed that the amount of latent autolysin originally bound to the cell wall was only 15% of maximal at 100% ^{14}C. It was previously demonstrated that all the measurable latent autolysin was in the cell wall fraction. Thus, 85% of the soluble latent autolysin rapidly binds to the cell wall during disruption. In *S. aureus* 8507, the majority of the lytic enzyme attached to isolated cell walls is unaffected by the presence of inactivated walls during disruption and therefore must be attached to the cell walls in vivo (Huff, *in preparation*).

It was demonstrated that the level of autolysin present in *S. faecalis* is dependent on growth medium and temperature (12) as well as phase of growth (13). The rates of autolysis of several strains of *S. aureus* cells were compared (9). The presence of autolytic enzymes in *S. aureus* was reported in several laboratories (9, 6, 19, 20). The possible manner in which autolysins are linked to growth and cell division has been discussed (9, 13). Hydrolysis by amidase could act coordinately with polymerization of peptidoglycan (19) to form the head-to-tail type of bond present in *Micrococcus lysodeikticus* (5, 11). As yet there is no evidence for the presence of such a linkage in *S. aureus*. An amidase could (i) make a cell wall more flexible by decreasing the number of cross-linkages, (ii) open gaps in the murein network to allow absorption and excretion of large-molecular-weight compounds, (iii) lyse bridging wall material between daughter cells, thus allowing cell separation.

Autolysins were reported to occur in a wide variety of bacteria (15). However, we are not aware of any study within any bacterial species which determines whether autolysis is a general characteristic found in all strains. Both the general rule and the exceptions would be important points to establish in this study. It has been generally observed that most strains of streptococcus do not autolyze. The group H streptococcus studied here did not autolyze, and the cell walls obtained from these cells showed no evidence for autolysis. It may be that some organisms or groups of organisms do not need autolysins for normal growth. Such autolysins may be under strong control or are highly unstable so that they are difficult to demonstrate. In the present study, nine different strains of *S. aureus* showed measurable autolytic enzyme present in the cell walls, with values occurring in a 50-fold range. Since the susceptibility of H, 2476, Oxford, and 1159 strains to lysis by the S-5DL amidase was about the same but the autolysin content varied from 0.8 to 10.8, it would appear that some of the variation in autolysis may be due to differences in content of autolytic enzyme. Some of the observed differences in autolysis rate may

be due to strain differences in cell wall structure or degree of cross-linking, since strain 968 which showed a high autolysis rate was also much more quickly lysed by the S-5DL amidase.

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ADDENDUM IN PROOF

Jon H. Ranhand (*personal communication*) has recently demonstrated autolytic activity in cells of group H *Streptococcus* (strains Wickey and Challis) capable of undergoing DNA-mediated transformation. Cells incapable of undergoing transformation did not show autolysis.

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