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Properties and Purification of *N*-Acetylmuramyl-L-Alanine Amidase from *Staphylococcus aureus* H

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The principal autolytic enzyme activity of the cell sap of *Staphylococcus aureus* H has been purified 400-fold. It is an *N*-acetylmuramyl-L-alanine amidase. This enzyme has a molecular weight of 8 to 10 $\times$ 10^5, a *p*H optimum of 7.3, an ionic strength optimum of 0.16 M and a K_m of 10^{-3} M murein repeating units.

Staphylococcus aureus lytic enzymes were first studied by Welsch and Salmon in 1950 (22) and by Mitchell and Moyle in 1957 (13). Recently, a variety of staphylococcal *extracellular* lytic enzymes have been partially purified. These include a glycosidase from strain 524 (6), an amidase from Oeding 8507 (11), a glucosaminidase from strain M19 (21) which also has amidase and peptidase activities in crude preparations, an amidase and glycine endopeptidase from strain EP-K1 (15), and an amidase, glucosaminidase, and glycine endopeptidase in strain K-6-W1 (1). The Oeding 8507 strain has also been shown to contain an amidase in both the cell wall and the soluble fraction (11). A wall-associated amidase has also been reported in strain Copenhagen (19). Our present report is the first study of a soluble *intracellular* autolytic enzyme in staphylococci.

We have previously shown (2, 17) that *S. aureus* H autolytic activity is present in both soluble (100,000 $\times$ *g* supernatant fluid) and wall fractions of broken cells. We report here that the soluble enzyme is an (*N*-acetylmuramyl)-L-alanine amidohydrolase and that a small activity of glycosidase is present in the wall but is not detectable in the soluble fraction. The main autolytic activity in the wall is the amidase previously reported (2).

A radioactive assay was developed for the soluble amidase based on the high sensitivity of relatively uncross-linked murein extracted from cells grown in the presence of penicillin, "penicillin murein." By using this assay, the enzyme has been purified 400-fold by ultrafiltration and by chromatography on Sepharose and diethylaminoethyl (DEAE)-Sephadex columns, and its properties have been studied. The high molecular weight of this amidase makes it unique among lytic enzymes so far studied.

MATERIALS AND METHODS

Cell strain and culture methods. *S. aureus* H (bacteriophage type 52A, 79, 80) was used throughout this study. Culture methods were as described previously (17).

Cell-free extract. Enzyme preparation was as previously described (17), except a Sorvall Omnimixer was used for cell breakage (14). This cell-free extract is a 100,000 $\times$ *g* supernatant fraction dialyzed against 0.03 M sodium phosphate, *p*H 6.8. This crude preparation is used extensively in the work reported here and is referred to as "dialyzed cell-free extract." Later work was done with a 400-fold partially purified preparation (see below) and this preparation is denoted "purified enzyme."

Substrate preparation. Normal murein and penicillin murein (murein extracted from cells grown in the presence of penicillin) were produced as previously described (17). The murein was *N*-acetylated to lower the background free amino groups when required. This acetylation was performed at *p*H 8.0 to 8.5, with 0.2 N NaOH being added to maintain this *p*H during the addition of acetic anhydride. There were two additions of acetic anhydride, each of 4 μmoles of acetic anhydride/mg of murein. This reaction mixture contained 10 mg of murein/ml. The reaction was performed at room temperature, and the reaction time for each addition was 5 min.

For the preparation of solubilized penicillin murein, 12 mg of penicillin murein was treated with 200 μg of Chalaropsis B enzyme (a gift of J. H. Hash) in 1.0 ml of 0.1 M sodium phosphate buffer, *p*H 7.3. The turbidity decreased from an optical density (OD) of 30.5 to 2.6 in 190 min. After boiling, the insoluble material was removed by centrifugation and the soluble material, denoted "solubilized penicillin murein," was tested as substrate for the soluble *S. aureus* H autolytic activity.

Soluble enzyme assay methods. Three different assay systems were used for crude, cell-free extract or purified enzyme. A very sensitive radioactive assay based on solubilization of tritiated murein was the standard method. A less sensitive but rapid turbidimetric assay and a free amino group assay found occasional use.

The radioactive assay was performed as follows. A 0.025-ml sample of ^{3}H-L-lysine-labeled penicillin murein (4 mg/ml; reference 17) was added to a 4-ml polycarbonate test tube and prewarmed at 37 C. A 0.025-ml sample to be tested for enzyme activity was then added, and the tubes were incubated at 37 C. For the dialyzed, cell-free extract this contained 50 to 250 μg of protein. At various times, tubes were removed and boiled for 1 min. Distilled water (0.20 ml) was added, and the tubes were centrifuged at 35,000 $\times$ g for 10 min. Then 0.20 ml of the supernatant fraction was counted in scintillation counting equipment as previously described (17). The enzyme unit is defined as the amount of enzyme which will release 1 μmole of murein repeating unit in 1 min.

The turbidimetric assay was performed by incubating at 37 C, in a final volume of 2.7 ml, 18 mg of *N*-acetylated murein, 100,000 $\times$ g supernatant enzyme dialyzed against 0.03 M sodium phosphate buffer (*p*H 6.8), and 300 μmoles of sodium phosphate buffer. Final *p*H was 7.3. At intervals, 25-μliter samples were diluted into 0.5 ml of distilled water and absorbancy was measured at 582 nm.

The free amino group assay was as follows. The same incubation mixture as in the turbidimetric assay above was used. Samples were removed at various times, boiled for 1 min, and then assayed for fluorodinitrobenzene (FDNB) reacting material essentially as described by Ghuysen et al. (4). The soluble and insoluble dinitrophenol (DNP) compounds were separated by centrifugation, and the absorbancy of the soluble DNP compounds was determined in 2 N HCl at 420 nm. The insoluble pellet was washed, hydrolyzed in 4 N HCl for 4 hr at 95 C, diluted to a final concentration of 2 N HCl, and the absorbancy was determined at 420 nm.

Unless otherwise stated, penicillin murein was used as substrate for enzyme assay.

Enzyme purification. Ultrafiltration was used for concentrating and purifying autolytic activity from cell-free extract volumes up to 400 ml. This was performed in an Amicon Diaflo ultrafiltration apparatus model 402 using an XM-50 membrane which retains molecules with a molecular weight of about 50,000 or more. Nitrogen pressure up to 30 psi was used, and a flow rate of about 1.0 ml/min was obtained. For smaller volumes in later stages of purification, model 52 (65-ml capacity) and model 12 (10-ml capacity) were used for concentration.

Sepharose 4B in a Pharmacia K50/100 column (5.0 by 100 cm) with upward flow adaptors was used. The volume of Sepharose 4B in the column was 1,440 ml, and the column height was 74.5 cm. The column was packed using the Pharmacia Gel Reservoir apparatus. All products were obtained from Pharmacia Fine Chemicals, Inc. The ultrafiltered cell-free extract sample size applied to the column was 20 to 40 ml. The column was eluted with 0.03 M sodium phosphate, *p*H 6.8. Ten-milliliter fractions were collected at a flow rate of 60 ml/hr. Samples were pooled as shown in Fig. 7 and concentrated by ultrafiltration.

DEAE-Sephadex A-50 in a Pharmacia K 25/45 column (2.5 by 45 cm) was used. The volume of DEAE-Sephadex A-50 in the column was 100 ml, and the column height was 20.5 cm. For this size column no more than 8-ml samples containing about 17 mg of protein could be applied to the column. The sample was placed on the column in 0.03 M sodium phosphate, *p*H 6.8, and eluted by a step gradient of 80 ml of the same buffer and 120 ml of 0.1 M sodium phosphate, *p*H 7.3. The flow rate was about 50 ml/hr and 5-ml fractions were collected. In other experiments, smaller columns were used with a corresponding ratio of applied protein to column cross-sectional area. Elution buffer volume to column volume ratios were also the same for all columns.

Determination of the products of enzymic action. Five incubation tubes were used for enzymic product determination. Acetylated penicillin murein or acetylated normal murein was incubated with and without dialyzed cell-free extract, and dialyzed cell-free extract alone was incubated. The murein concentration was 6.7 mg/ml. The protein concentration of the dialyzed cell-free extract was 0.67 mg/ml, and the buffer was 0.11 M sodium phosphate, *p*H 7.3. Each tube was incubated at 37 C for 24 hr. Samples were removed at various times; the reaction was stopped by boiling, and the contents of the tubes were subjected to the following procedures. Determinations of *N*-acetylamino sugars, of *N*-terminal amino acids, and of the configuration of the *N*-terminal alanine by D- and L-alanine estimation were by modifications of the methods of Ghuysen et al. (4). Reducing sugar determination was performed using the method of Thompson and Shockman (18).

Molecular weight determination. Sucrose gradient centrifugation of the dialyzed cell-free extract or of the purified enzyme was performed by the procedure of Martin and Ames (12). Five-milliliter linear gradients of 5 to 20% sucrose (in 0.03 M sodium phosphate, *p*H 7.3) were centrifuged in an SW65 rotor in the Beckman model L centrifuge at 38,000 rev/min for 3 hr. Eight-drop fractions were collected resulting in 22 to 23 fractions. The protein standards used were beef liver catalase (Sigma Co.), molecular weight 250,000 (16), and purified ferritin (a gift of B. D. Stollar), molecular weight 750,000 (9). The catalase was assayed by measuring the time for 0.05 ml of H_2O_2 (1 to 800 dilution of a 30% solution) to decrease from an OD of 0.45 to 0.40 at 240 nm with 0.010 ml of cell-free extract. The reciprocal of this time is the catalase activity in arbitrary units. This assay is based on a method given in Sigma Co. literature. Ferritin was determined by measuring the absorption at 400 nm. The amidase activity was determined by using the radioactive assay given above.

RESULTS AND DISCUSSION

Autolytic activity at different growth stages. Cells were harvested at various times during the growth of the culture, and autolytic

activity in the soluble fraction of broken cells was determined by using the radioactive assay. There were indications of increased activity in the exponential phase of growth, but exponential cells never yielded more than two times the specific activity of stationary cells. At no time could lytic activity be detected in the culture supernatant fraction.

A number of other *N*-acetylmuramyl-L-alanine amidase-producing bacteria have been shown to have this enzymic activity in maximal amounts during exponential growth. These include *S. aureus* Copenhagen (19), *S. aureus* Oeding 8507 (10, 11), *Bacillus subtilis* 168 (23), and *B. cereus* T (H. J. Singer and B. D. Church, Bacteriol. Proc., p. 32, 1964). That cells that are actively dividing have more autolytic enzyme correlates well with a postulated function for lytic enzyme in cell division.

Determination of the sites of action of the autolytic activity. In the following experiments, acetylated murein, containing no free amino groups, was used as substrate to differentiate enzymically released free amino groups from endogenous free amino groups. The soluble material resulting from enzymic attack by dialyzed cell-free extract on acetylated *S. aureus* murein was treated with FDNB (see Materials and Methods). When acid hydrolysates of this material were analyzed by thin layer chromatography, only DNP-alanine was detected. Only *N*-terminal L-alanine was found to be released by the enzyme. These data are summarized in Table 1. About three times as many *N*-terminal L-alanine groups are released from penicillin murein as from normal murein. This is because about three times as much murein is solubilized from penicillin murein as from normal murein. The number of *N*-terminal amino groups released per repeating unit of murein solubilized is the same for either substrate.

Cell walls of *S. aureus* H were allowed to autolyze and reducing groups were measured. The results are shown in Fig. 1. Eighty nanomoles of reducing groups per milligram of murein were found to be present initially. If all these reducing groups represent ends of glycan chains, this would suggest an average glycan chain length of 7.5 disaccharide units. This agrees well with previously reported data using a borohydride reduction method (D. Mirelman, A. N. Chatterjee, H. J. Singer, D. R. D. Shaw, and J. T. Park, Bacteriol. Proc., p. 47, 1969). About 80 nmoles more of reducing groups were produced by autolysis after a 24-hr incubation period. Thus, there is apparently a small amount of glycanase present in the cell wall. The 100,000 $\times$ *g* supernatant fraction, i.e., the cell-free extract, on the other hand, contained no detectable glycanase activity.

Radioactive assay of autolytic activity. When the solubilization of radioactive penicillin murein was plotted as a function of time for 4 hr, a sigmoid curve resulted (Fig. 2). Presumably, as the structure of the murein is opened during initial attack by the soluble autolysin, the rate of solubilization increases since more groups are made sensitive to the enzyme. In addition, it may take more than one "hit" to cleave off a piece of even only partially uncross-linked murein. The 0- to 20-min time interval was chosen as the standard assay period. As shown in Fig. 3, the 20-min assay is linear as a function of increasing protein in the range of 50 to 250 μg of protein. The *p*H optimum was previously shown to be about 7.3, and the ionic strength optimum was about 0.16 M (17). When 5 μmoles of ammonium sulfate was added to the reaction mixture, a stimulation of up to 65% was obtained for the enzyme. Ammonium sulfate was added for all assays except when noted.

Comparison of assays. As seen in Fig. 4, turbidity measurement shows that acetylated penicillin murein is solubilized by cell-free extract to a greater extent than acetylated normal murein, as was indicated previously (17). Presumably, the autolysin-sensitive bonds present less steric hindrance in the less cross-linked murein prepared from cell walls synthesized in the presence of penicillin. FDNB-reacting material also rises with time at a more rapid rate and to a greater extent with the acetylated penicillin murein than with acetylated normal murein. However, a strict inverse proportionality is not seen between the turbidity curves and the DNP curves, presumably because continued attack by the enzyme on already solubilized murein releases additional amino groups. A comparison of solubilization of radioactive wall and turbidity decrease did give strict inverse proportionality (17) as would be expected since these two methods measure solubilization of substrate rather than the absolute number of chemical bonds broken.

Other substrates for the autolysin. Acetylated penicillin murein was solubilized by treatment with Chalaropsis B enzyme, an *N*-acetylmuramidase (5). When the Chalaropsis B-treated substrate was then incubated at 37 C with *S. aureus* H-dialyzed, cell-free extract, 55% of the amide bonds were hydrolyzed in 4 hr. With substrate not treated with Chalaropsis enzyme, the same amount of extract re-

TABLE 1. *Estimation of N-terminal* D- *and* L-*alanine released by dialyzed cell-free extract from penicillin murein and normal murein*[a]

Murein	Moles of *N*-terminal L-alanine released/mole of repeating unit murein incubated	Moles of *N*-terminal L-alanine released/mole of repeating unit murein solubilized	Moles of *N*-terminal D-alanine released/mole of repeating unit murein incubated or solubilized
Penicillin murein + enzyme	0.61	0.78	0
Normal murein + enzyme	0.20	0.76	0
Penicillin murein − enzyme	0	0	0
Normal murein − enzyme	0	0	0

[a] Samples of the soluble fraction, shown in Fig. 5, were removed at 24 hr, and part of each sample was treated with FDNB. The FDNB-treated material and also untreated material was hydrolyzed and, after purification, D- and L-alanine were estimated. The value of 0 means less than 0.01 mole/mole. For details of procedures see Materials and Methods.

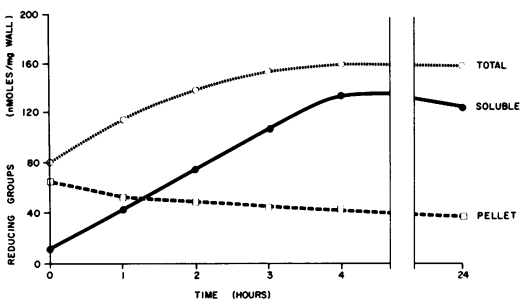

FIG. 1. *Release of reducing groups during autolysis of S. aureus H cell walls. Cells were grown and walls were prepared as described in Materials and Methods. S. aureus H walls were suspended at a final concentration of 14.8 mg/ml in 0.1 M sodium phosphate, pH 7.3, and incubated at 37 C. Samples were removed at the time intervals shown and the reaction was stopped by boiling. Samples were assayed for reducing sugars by the method of Thompson and Shockman (18) and this represented the total reducing sugar. Another sample was centrifuged, and the soluble fraction and the washed pellet were assayed in the same manner.*

leased only 14% of the amide bonds in 4 hr. Thus, solubilizing the murein increased fourfold the rate at which the soluble autolysin reacts.

The uridine pyrophosphate-bound precursor of *S. aureus* murein, uridine diphosphate-*N*-acetylmuramyl-L-alanyl- D -isoglutamyl-L-lysyl-D-alanyl-D-alanine, and the *N*-acetylmuramyl pentapeptide derived from the uridine compound were not hydrolyzed at all by the cell-free extract under the optimal conditions for hydrolysis of known substrates.

Van Heijenoort and Van Heijenoort (20) obtained different results by using *Escherichia coli N*-acetylmuramyl L-alanine amidase and purified substrates. These authors found amidase activity by using as substrate *E. coli N*-acetylmuramyl tri- or pentapeptide, but obtained little or no activity on β (1-4)*N*-acetyl-

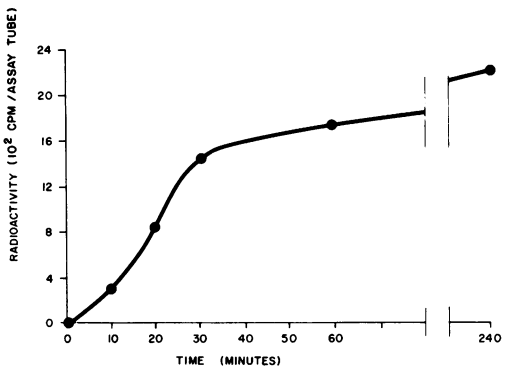

FIG. 2. *Time course of solubilization of radioactive penicillin murein by dialyzed, cell-free extract. The procedure for this experiment is described in Materials and Methods.*

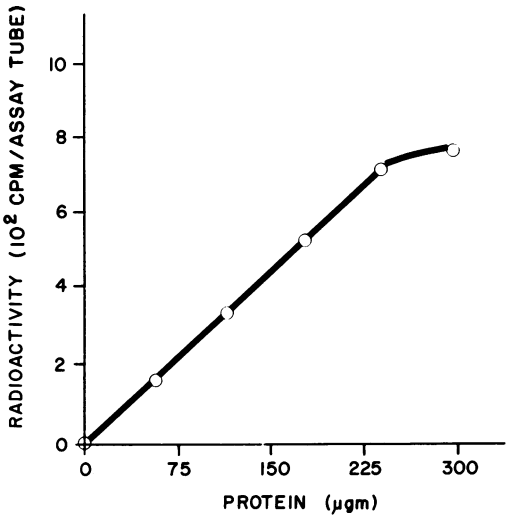

FIG. 3. *Solubilization of radioactive penicillin murein by varying amounts of dialyzed, cell-free extract. The procedure for this experiment is described in Materials and Methods.*

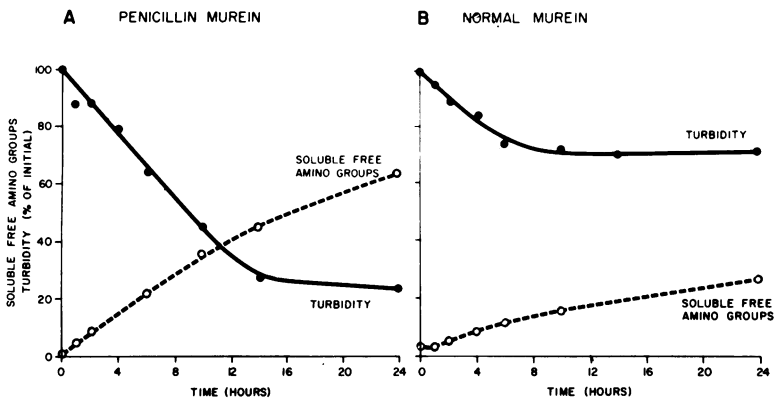

FIG. 4. *Correlation of turbidity reduction and free amino group solubilization by dialyzed, cell-free extract using acetylated penicillin murein (A) and acetylated normal murein (B) as substrates. Samples were removed from an incubation mixture (see Materials and Methods) at the times shown, and the reaction was stopped by boiling. Parts of the samples were treated with FDNB as described in Materials and Methods, and the quantities of soluble DNP derivatives are shown in the figure. The units of soluble free amino groups are nanomoles per 100 nmoles of repeating unit of murein incubated, so that this plot represents the percent of possible amide bonds released. Part of the sample (0.025 ml) was diluted with 0.5 ml of distilled water, and the turbidity was determined at 582 nm. A, Contained acetylated penicillin murein; B, contained acetylated normal murein. Controls without enzyme showed no soluble free amino groups and less than 10% turbidity reduction.*

glucosaminyl-*N*-acetylmuramyl tri- or tetrapeptide.

Determination of K_m. In Fig. 5 the substrate concentration was varied and the initial rate of reaction using dialyzed, cell-free extract was measured with a modification of the radioactive assay. The Lineweaver-Burk plot shows a K_m of 1.0 mg of penicillin murein/ml. This corresponds to 9×10^{-4} M repeating units of murein. For comparison with in vivo substrate, the concentration of repeating unit in wall itself is about 1 M. A final concentration of 2 mg/ml was used for later assays.

Molecular weight. Sucrose gradient centrifugation of the dialyzed, cell-free extract was performed according to the procedure of Martin and Ames (12). Beef liver catalase and ferritin were used as standards (see Materials and Methods). The results for the cell-free extract are shown in Fig. 6. By using the distance traveled by the enzyme compared to the standard, an approximate molecular weight can be calculated assuming the enzyme to be spherical. With catalase as standard, the molecular weight of the autolytic activity is calculated to be 920,000. By using ferritin as standard, it is 800,000, giving an average of 860,000. These values are for the peak in Fig. 6. It is obvious from Fig. 6 that the peak is broad, presumably indicating aggregation. This figure also shows a small amount of activity under the catalase peak (molecular weight 250,000), further complicating exact determination and

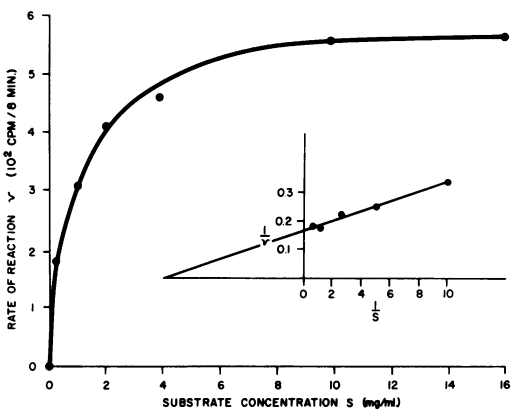

FIG. 5. *Determination of the K_m of dialyzed, cell-free extract autolytic activity with penicillin murein as substrate. The procedure for this experiment is described in the text.*

interpretation of molecular weight in terms of subunit size.

Sepharose 4B column chromatography (see below), by application of molecular weight determination methods described in Pharmacia Co. literature, yields a molecular weight for the autolytic activity of 950,000 to 1,050,000 by using cell-free extract or purified enzyme. Thus the molecular weight of the enzyme is in the range of 800,000 to 1,000,000.

Previously reported *N*-acetylmuramyl-L-alanine amidases and other autolytic enzymes

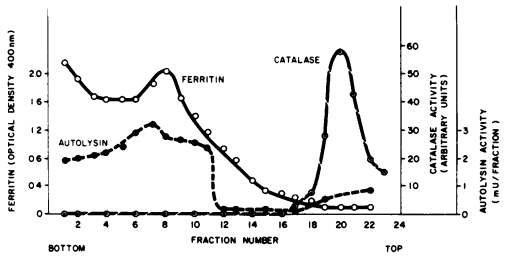

FIG. 6. *Sucrose gradient centrifugation of the dialyzed, cell-free extract autolytic activity. The procedure for this experiment is described in Materials and Methods.*

from various species have molecular weights of 30,000 or less (H. J. Singer, thesis, Tufts University, 1970). A recent report (21) indicates *S. aureus* M18 has an endo-β-*N*-acetyl-glucosaminidase with a molecular weight of 70,000, the highest molecular weight previously reported for any lytic enzyme.

Purification. The Amicon Diaflo ultrafiltration cell with XM-50 membrane retained the autolytic activity, yielding a 10-fold concentration of activity with 100% recovery of total activity and 1.3- to 5-fold increase in specific activity. Other methods for initially concentrating the autolytic activity by using ammonium sulfate or high-speed centrifugation were less effective than ultrafiltration.

Sepharose 4B chromatography of the above ultrafiltrate gave the results shown in Fig. 7. Total purification of 30- to 100-fold in peak tubes was obtained with 5- to 10-fold purification of all the tubes pooled. Recoveries as high as 90% were obtained. The pooled enzyme fractions were again concentrated by ultrafiltration prior to the next column step.

Chromatography of the ultrafiltered, pooled Sepharose 4B enzyme preparation on DEAE-Sephadex (Fig. 8) gave an average total purification of about 400-fold. A summary of one total purification procedure is given in Table 2. In other experiments, purification was greater with the initial ultrafiltration step, and purification and yield were greater after the Sepharose 4B chromatography.

Stability of the purified enzyme. The purified enzyme was not stable. Samples stored overnight in glass tubes at 4 C or −20 C resulted in complete loss of activity. Storage in polycarbonate tubes resulted in 25 to 50% loss of activity when stored overnight at −20 C. Essentially all activity was lost within 1 week when the enzyme was stored at −20 C in polycarbonate tubes. Other methods tested for stabilization included lyophilization, ultrafiltration, addition of bovine plasma albumin at 1 mg/ml, pancreatic ribonuclease at 1 mg/ml, glycerol at 20%, dimethyl sulfoxide at 20%, saturated ammonium sulfate, and phosphatidylethanolamine at 0.5 mg/ml. Substrate protection was not given by *S. aureus* H or *S. aureus* strain 52A5 (2) cell walls solubilized with cell-free extract or Chalaropsis B enzyme. No methods to give long term stabilization were found. This instability is unusual for an autolytic enzyme but it may be associated with the uncommonly large size of this enzyme.

The purified enzyme was easily heat inactivated. Heating for 10 min at 25 C resulted in 65% inactivation. The enzyme preparation was completely inactivated in 60 min at 37 C, in 30 min at 42 C, and in 10 min at 50 C. The crude, cell-free extract is stable at −20 C for at least 2 years (20% loss in activity) and is also much more stable to heat than the purified enzyme.

Activators and inhibitors of the purified enzyme. Ammonium sulfate produced a 20% stimulation of autolytic activity (Table 3). Ammonium acetate and ammonium chloride stimulate enzymic activity, whereas potassium sulfate and sodium sulfate inhibit the activity. Thus, it is apparent that ammonium sulfate stimulation is caused by the ammonium ion.

Cupric and plumbic ions and parachloromercuriphenylsulfonic acid at 10^{-4} M (each tested singly) inhibited the enzyme only 37, 25, and 12%, respectively. At 10^{-5} M, inhibition was 10% or less for all of the above. Mer-

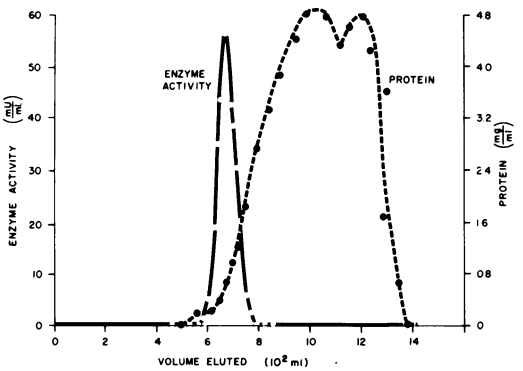

FIG. 7. *Sepharose 4B column chromatography. A Sepharose 4B filled column was run by using the procedures described in Materials and Methods. Sixteen liters of S. aureus H culture (OD of 3.0) were used to prepare 150 ml of 100,000 × g supernatant in 0.03 M sodium phosphate buffer, pH 6.8. This was concentrated to 20 ml with an Amicon ultrafiltration apparatus and an XM-50 membrane, and the concentrate was applied to the column. Fractions were assayed by using the radioactive method.*

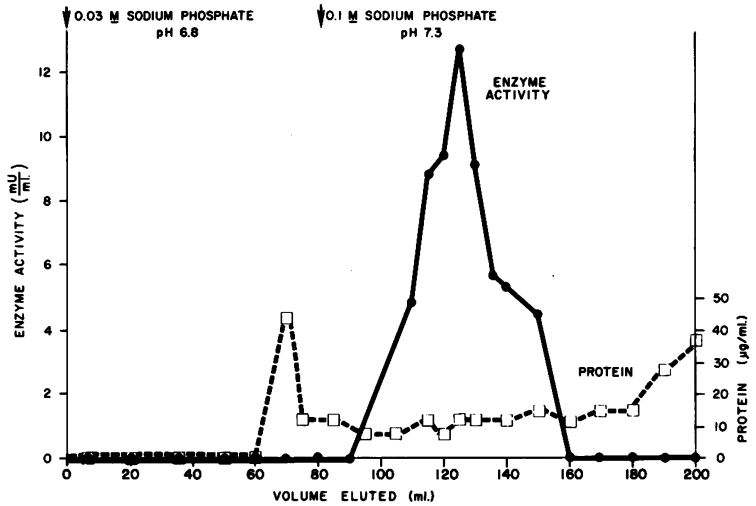

FIG. 8. *DEAE-Sephadex A-50 column chromatography. A DEAE-Sephadex A-50 filled column was run by using the procedure described in Materials and Methods. Eight milliliters of the concentrated Sepharose 4B enzyme fraction was placed on the column in 0.03 M sodium phosphate, pH 6.8.*

TABLE 2. *Summary of enzyme purification*[a]

Step	Vol (ml)	Enzyme activity (mU/ml)[b]	Total activity (mU)	Protein (mg/ml)	Specific activity (mU/mg)	Purification (-fold)	Yield (%)
Cell-free extract	150	41	6150	14	2.9	1	100
Ultrafiltration	20	337	6670	88	3.9	1.3	110
Sepharose 4B column	28	94	2630	2.2	43	6.7	43
DEAE-Sephadex column	25	13	325	0.011	1180	407	5

[a] The procedures are described in Materials and Methods. Enzyme was assayed by the radioactive assay. Protein was determined by the Lowry method.

[b] mU, Milliunits.

captoethanol at 10^{-3} M did not affect the enzyme. Ethylenediaminetetraacetic acid (EDTA) at 10^{-3} M inhibited the enzyme 75%. Magnesium ions neither stimulated the enzyme nor reversed the effect of EDTA.

Other properties of the purified enzyme. The *p*H optimum (7.3), ionic strength optimum (0.16 M), and K_m (1.3×10^{-3} M) were similar to the values obtained from the crude, cell-free extract autolytic activity. Accurate molecular weight determination by sucrose gradient centrifugation was not possible because of the instability of the purified enzyme, but the behavior of the purified enzyme on Sephadex and Sepharose columns suggested little or no change in the 900,000 molecular weight determined prior to purification.

This first study of a soluble intracellular autolytic activity in *S. aureus* has shown a uniquely high molecular weight L-alanine amidase. A wall-bound amidase also occurs in this strain as shown in previous work from this laboratory (2), but its molecular weight was not determined. The wall-bound autolytic enzymes of other *S. aureus* strains and of other bacterial strains are almost all of considerably smaller molecular weight than the 900,000 molecular weight autolytic activity obtained from the cell sap. Since it is hard to envision an intracellular function for an enzyme whose substrate is mostly if not completely extracellular, it encourages one to speculate that the large intracellular amidase may be a precursor of a (smaller) functional wall enzyme. Proof for such a hypothesis does not exist at present, nor, of course, does proof exist of postulated physiological modes of action of the wall amidase in cell growth and division.

TABLE 3. *Comparison of activators and inhibitors of the purified enzyme*

Addition[a]	Molarity	Activity (% of control)
Ammonium sulfate	0.15	120
Ammonium acetate	0.15	118
Ammonium chloride	0.15	108
Potassium sulfate	0.15	71
Sodium sulfate	0.15	64
Cupric sulfate	10^{-4}	63
Lead acetate	10^{-4}	75
PCMPS[b]	10^{-4}	88
Mercaptoethanol	10^{-3}	100
EDTA	10^{-2}	25
EDTA	10^{-3}	25
EDTA	10^{-4}	87
Magnesium chloride	10^{-3}	100
Magnesium chloride + EDTA	10^{-3}[c]	25

[a] The listed compounds were added to the reaction tubes in the radioactive assay with purified enzyme.

[b] PCMPS, Parachloromercuriphenylsulfonate.

[c] Magnesium chloride and EDTA each at 10^{-3} M.

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