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Purification and Characterization of Three Separate Bacteriolytic Enzymes Excreted by *Staphylococcus aureus*, *Staphylococcus simulans*, and *Staphylococcus saprophyticus*

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As a further development of previous investigations showing that different staphylococcal species display different bacteriolytic activity patterns (lyogroups), the bacteriolytic enzymes excreted by three different *Staphylococcus* species, *Staphylococcus aureus* (lyogroup I), *S. simulans* (lyogroup II), and *S. saprophyticus* (lyogroup IV), have been purified and characterized. A representative strain from each species was grown in a preselected medium made of fully dialyzable products. Culture supernatants were collected in the appropriate growth phase. Two different affinity adsorbents were used for enzyme purification. One was obtained by coupling lysozyme-digested pure peptidoglycan from *Micrococcus luteus* to cyanogen bromide-activated Sepharose 4B. The second affinity adsorbent used was chitin. The *S. aureus* bacteriolytic enzyme bound to the solubilized peptidoglycan but not to chitin, whereas the opposite was true for the *S. simulans* enzyme. The bacteriolytic enzyme from *S. saprophyticus* did not bind to either the Sepharose 4B-peptidoglycan resin or to chitin, and its purification was achieved by two ion-exchange chromatography steps combined with gel filtration. All three enzymes were purified to apparent homogeneity. Their subsequent characterization indicated that all acted as endo-β-*N*-acetylglucosaminidases. However, the three glucosaminidases differed significantly in their kinetics of activity and bacteriolytic spectrum against heat-killed cells of a variety of microorganisms. Very different values also resulted from molecular weight determinations: 80,000 for the *S. aureus* enzyme, 45,000 for the *S. simulans* enzyme, and 31,000 for the *S. saprophyticus* enzyme. Other important differences were observed in their stability, optimal pH and ionic strength for their activity, and their responses to temperature and divalent cations. These results confirmed the previous proposal that different staphylococcal species excrete different lytic enzymes.

We have previously shown that production of bacteriolytic enzymes is a general property of staphylococci (23, 24) and have reported that we were able to subdivide over 1,000 human *Staphylococcus* isolates into six groups (lyogroups) based on their bacteriolytic activity patterns analyzed through a simple in vivo assay (33). Further studies showed excellent correlation between the staphylococcal lyogroups and the individual species as recognized according to several physiological and biochemical properties (31, 32). Two different sets of coagulase-positive staphylococci, recognized using a similar in vivo assay of bacteriolytic activity, were found to exactly overlap with the two species acknowledged within such organisms (34).

These studies indicated that the analysis of bacteriolytic activity was the most efficient single test for *Staphylococcus* species separation and identification. The in vivo assay used, however, did not demonstrate whether the different bacteriolytic activities observed were actually due to different lytic enzymes excreted. This prompted us to start purification and characterization of the bacteriolytic enzymes from representative strains of each lyogroup.

This paper describes purification and distinctive properties of the bacteriolytic enzymes from three different *Staphylococcus* species: *Staphylococcus aureus* (lyogroup I), *S. simulans* (lyogroup II), and *S. saprophyticus* (lyogroup IV). It is shown that the enzymes have been purified to apparent homogeneity and that they clearly differ in many important properties, although they share the same specificity of action (endo-β-*N*-acetylglucosaminidase activity).

MATERIALS AND METHODS

Materials. Hen egg white lysozyme was supplied by Miles Laboratories, Inc. (Elkhart, Ind.) as a lyophilized powder and was used without further purification. Amberlite, analytical grade, was obtained from Rohm and Haas (Philadelphia, Pa.). Sephadex and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Chitin was from ICN Pharmaceuticals, Inc. (Plainview, N.Y.). Equipment for ultrafiltration (Diaflow) was purchased from Amicon N.V. (The Hague, Holland). Acrylamide, *N,N'*-methylene bisacrylamide, ammonium persulfate, and *N,N,N',N'*-tetramethylethylenediamine were from Bio-Rad Laboratories (Richmond, Calif.). $(NH_4)_2SO_4$, ultrapure grade, was from Carlo Erba, Divisione Chimica (Milan, Italy).

Bacterial strains and culture conditions. One typical representative strain from each of lyogroups I, II, and IV (33) was chosen. Lyogroup I, whose representative was strain AT-12, overlaps completely with *S. aureus*; lyogroup II, whose representative was strain O-12, overlaps completely with *S. simulans*; and lyogroup IV overlaps with a cluster of three very closely related species, *S. saprophyticus*, *S. cohnii*, and *S. xylosus* (31, 32). The representative of this last lyogroup (strain BO-3) could be identified by conventional methods as *S. saprophyticus* (27). The three strains were from our institute's collection. All had been originally obtained from clinical material processed in the Clinical Bacteriology Laboratory of our institute. The strains were grown in fully dialyzable media (see Results and Table 1) at 37°C in a gyrotory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.).

Ammonium sulfate precipitation and extensive dialysis. Ammonium sulfate precipitation was carried out according to Green and Hughes (8). The resuspended ammonium sulfate precipitate was transferred into dialysis tubing and dialyzed at 4°C against several changes of appropriate buffer (see Results), each one having a 200-fold-larger volume than that of the dissolved ammonium sulfate precipitate.

Preparation of peptidoglycan. Heat-killed cells of *Micrococcus luteus* ATCC 4698 (50 g, dry weight) were extracted with 1.6 liter of 10% (wt/vol) trichloroacetic acid for 24 h at 4°C. The mixture was then centrifuged at 8,000 × *g* for 15 min, and the pellet was suspended in 2 liters of distilled water. A 20% stock solution of sodium dodecylsulfate (SDS) was added dropwise to the suspension to a final concentration of 4%. The suspension was heated for 45 min at 100°C and incubated overnight with stirring at 37°C. The suspension was centrifuged at 8,000 × *g* for 15 min to remove unbroken cells, and the resulting supernatant was centrifuged at 78,000 × *g* for 30 min at 20°C. The pellet was washed five times with distilled water to remove the detergent, resuspended in 1 liter of 0.05 M phosphate buffer (pH 6.5), and digested first with DNase (50 μg/ml) and RNase (50 μg/ml) for 24 h at 37°C and then with pronase (30 μg/ml) for 2 h at 37°C. This treatment is more effective in removing the proteins covalently bound to peptidoglycan than the more frequently used trypsin treatment (5). The mixture was finally centrifuged at 78,000 × *g* for 30 min at 20°C, and the pellet was washed three times with distilled water. The pellet of the last washing, analyzed as described elsewhere (6), was found to be pure peptidoglycan.

Enzymatic digestion of the peptidoglycan. Hen egg white lysozyme (0.1 mg in 1 ml of 0.15 M sodium acetate buffer, pH 6.6) was added to a suspension of 1 g of pure peptidoglycan of *M. luteus* ATCC 4698 in 99 ml of the same buffer and preincubated at 37°C for 30 min. The reaction mixture was shaken at 37°C for 24 h, and the digested peptidoglycan was heated at 120°C for 1 h to inactivate the lysozyme. The insoluble material was removed by centrifugation (150,000 × *g* for 6 h), and the supernatant was lyophilized.

Preparation of the Sepharose 4B-peptidoglycan resin. The cyanogen bromide-activated Sepharose 4B (about 6 g, dry weight) was washed with cold water and mixed with a solution of 300 mg of lysozyme-digested peptidoglycan in 40 ml of 0.1 M borate buffer, pH 10.0. The mixture was gently shaken by a flask shaker for 20 h at 4°C. After coupling was complete, the excess lysozyme-digested peptidoglycan was washed away with coupling buffer solution on a glass filter, and any remaining active groups were blocked by treatment with 1 M ethanolamine, pH 8.0, for 2 h. The final product was washed five times alternately with acetate buffer solution (0.1 M, pH 4.0) and borate buffer solution (0.1 M, pH 8.0). The Sepharose 4B-peptidoglycan resin could be stored for a long time in a refrigerator.

Chromatography on Sepharose 4B-peptidoglycan resin. The sample obtained from extensive dialysis was applied to a column (0.9 by 12 cm) of Sepharose 4B-peptidoglycan resin preequilibrated with 0.078 M phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with 1 M phosphate buffer, pH 8.0, and then with 0.1 M glycine-NaOH, pH 9.7, containing 1.8 M NaCl at a flow rate of 20 ml/h and at room temperature.

Chromatography on chitin. The dialysis product was

TABLE 1. Composition of the dialyzable media used to grow the staphylococcal strains for bacteriolytic activity production

Component	Medium composition (g/liter) for:		
	S. aureus	*S. simulans*	*S. saprophyticus*
Casamino Acids[a]	20	30	15
Yeast extract[a]	25	18	30
Brain heart infusion broth[a]	200	300	300
Sodium chloride[b]	2.5	2.2	5.0

[a] The concentrations reported refer to the initial solutions which were dialyzed against three changes of water. At each change, the volume of water was equal to that of the initial solution. The dialyzable portions of the three dialysis steps were pooled. Subsequently, equal volumes of the final dialyzable solutions of Casamino Acids, yeast extract, and brain heart infusion broth were pooled.

[b] The concentrations reported refer to the final ones, i.e., those obtained by adding NaCl directly to the pool of the dialyzable solutions of Casamino Acids, yeast extract, and brain heart infusion broth.

applied to a column (1.8 by 30 cm) of chitin prepared as described by Jensen and Kleppe (11) and preequilibrated with 0.05 M phosphate buffer, pH 6.3. The column was washed with the same buffer until the absorbance at 280 nm of the effluent equalled that of the buffer, and then the enzyme was eluted by a linear gradient of phosphate buffer (pH 8.0) ranging from 0.05 to 0.8 M. The elution was carried out at a flow rate of 20 ml/h at room temperature.

First column chromatography on Amberlite IRC-50. A 200-g (dry weight) portion of Amberlite IRC-50 equilibrated in 0.05 M phosphate buffer, pH 6.3, was added to 2 liters of the sample obtained from extensive dialysis against the same buffer, and the mixture was shaken overnight and then allowed to sediment for 1 h at 4°C (the rest of this purification step was carried out at room temperature). The supernatant, devoid of lytic activity, was discarded. The resin, with the bacteriolytic enzyme bound to it, was washed with 0.05 M phosphate buffer, pH 6.3, and then a column (1.8 by 60 cm) was packed with it. The bacteriolytic activity was eluted with a linear gradient established with 200 ml of 0.1 M phosphate buffer, pH 7.5 (first chamber), and 200 ml of the same buffer containing 0.8 M NaCl (second chamber). Elution was performed at a flow rate of 500 ml/h, and 4-ml fractions were collected.

Gel filtration through Sephadex G-50. Sephadex G-50 was packed in a column (3.5 by 80 cm) and equilibrated with 0.05 M phosphate buffer, pH 6.5. The active fractions obtained from the preceding Amberlite chromatography step were pooled, dialyzed at 4°C for 6 h against 0.05 M phosphate buffer, pH 6.5, and then applied to the Sephadex G-50 column. Elution was performed with 0.2 M phosphate buffer, pH 6.5, at a flow rate of 20 ml/h; 6-ml fractions were collected.

Second column chromatography on Amberlite IRC-50. The active fractions from the Sephadex column step were pooled and diluted four times with water. The pH was adjusted to 6.3 with HCl. The pooled fractions were applied to a column (1.8 by 45 cm) of Amberlite IRC-50 in the same manner as described above, except that 5-ml fractions were collected.

Enzyme substrates. Heat-killed cells of *M. luteus* ATCC 4698 were used as standard lytic enzyme substrate. Heat-killed cells of a variety of other microorganisms were also tested as substrates to assess the bacteriolytic spectrum of the purified enzymes. Cell walls were purified from *M. luteus* ATCC 4698 according to the standard procedures referred to above for peptidoglycan preparation (6).

Assay of lytic activity. Bacteriolytic activity was assayed on a suspension of heat-killed cells of *M. luteus* ATCC 4698 as described by Parry et al. (20). One unit of enzyme activity was arbitrarily defined as the amount of test lytic activity required to give a linear decrease of absorbance at 640 nm of 1%/min at 37°C.

Molecular weight determination. Both SDS-polyacrylamide gel electrophoresis and gel filtration were used for molecular weight determination. Rabbit muscle aldolase (molecular weight, 150,000), β-galactosidase monomer from *Escherichia coli* (molecular weight, 130,000), bovine serum albumin (molecular weight, 68,000), ovalbumin (molecular weight, 43,000), and α-chymotrypsinogen A from bovine pancreas (molecular weight, 25,000) were used as known protein standards. For the electrophoretic procedure, 10% slab gels were prepared according to Laemmli (14), and molecular weights were calculated according to Shapiro et al. (28). For gel filtration, Sephadex G-200 was equilibrated with 0.2 M phosphate buffer, pH 7.0, packed in a column (2 by 100 cm) and calibrated with reference proteins; molecular weights were calculated according to Andrews (2).

Reducing power and free amino group determination. The appearance of reducing substances, *N*-acetylamino sugars (Morgan-Elson reaction), and free amino groups in soluble fragments during enzymatic hydrolysis of *M. luteus* cell walls was determined as described by Ghuysen et al. (7). To measure the rate of hydrolysis of glycosidic linkages, the total amino sugar content was determined after acid hydrolysis of cell walls (2 M HCl at 100°C for 2 h) followed by evaporation of acid in vacuo in the presence of KOH and P_2O_5. As standards, glucose was used for reducing power determination, glycine was used for free amino group determination, and glucosamine hydrochloride was used for the amino sugar reaction.

Isolation and characterization of disaccharide from lysis products of *M. luteus* peptidoglycan. Disaccharide was purified from staphylococcal enzyme digests of *M. luteus* peptidoglycan according to the scheme of Wadström and Hisatsune (36) by ion-exchange chromatography with Amberlite CG-120 followed by Sephadex G-10 gel filtration. The sugar components and the reducing end of the purified disaccharide were identified by paper chromatography according to Mirelman and Sharon (18). Quantitative amino sugar analysis was performed with a Unichrom (Beckman) amino acid analyzer (6, 15).

Inhibition of bacteriolytic enzymes by α- and β-derivatives of 2-acetamido-2-deoxy-D-glucose. The turbidimetric method of Prasad and Litwack modified by Neuberger and Wilson (19) was used. The alkylglycosides were prepared by the method of Zilliken et al. (39), and α- and β-anomers were separated on a Dowex 1-X2 (200 to 400 mesh) resin column as described by Austin et al. (3).

Determination of exo-β-*N*-acetylglucosaminidase activity. The purified staphylococcal bacteriolytic enzymes (200 U/ml) were assayed at pH 4.4 and 6.5 according to the method described by Levvy and Conchie (16), by using *p*-nitrophenyl-β-*N*-acetylglucosaminide as a substrate and measuring the *p*-nitrophenol released. Control experiments were performed with exo-β-*N*-acetylglucosaminidase from bovine kidney (Serva).

Other procedures. Spectrophotometry and pH determinations were performed in a Beckman DB spectrophotometer and Corning 119 ph meter, respectively. Protein concentration was estimated by the method of Lowry et al. (17) or from spectrophotometric measurement of the absorbance at 280 nm. Immunodiffusion tests were performed as described by Stollar and Lawrence (29). Testing of biologically active extracellular staphylococcal substances other than bacteriolytic enzymes was carried out according to the standard methods (1). For SDS-polyacrylamide gel electrophoresis, 10 to 15% slab gels were prepared by the method of Laemmli (14). Three different buffers (acetate, phosphate, and Veronal), covering an overall 4.0 to 9.5 pH range, were used to test the effect of pH on enzyme activity.

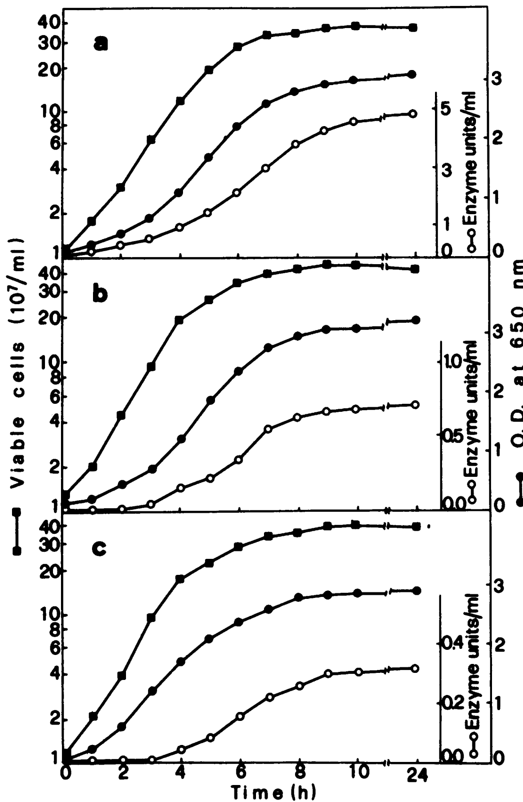


FIG. 1. Bacterial growth and enzyme production of *S. aureus* strain AT-12 (a), *S. simulans* strain O-12 (b), and *S. saprophyticus* strain BO-3 (c). At intervals, samples were removed and assayed for viable cells (■), OD (●), and bacteriolytic activity in the supernatant (O).

RESULTS

Preparation and choice of growth media made of fully dialyzable products. Several media were prepared by mixing, in various proportions, sodium chloride and the dialyzable portions of solutions of Casamino Acids (Difco Laboratories, Detroit, Mich.), yeast extract (Difco), and brain heart infusion broth (Difco). Media were inoculated with the three strains to select the medium composition yielding the highest bacteriolytic activity in the supernatant.

The compositions of the growth media selected for the three strains used are reported in Table 1.

Evaluation of lytic enzyme production during bacterial growth. Many experiments were performed to evaluate the growth phase during which the lytic enzyme present in the culture medium was most abundant, and autolysis of growing cells did not occur or was negligible. Avoiding autolysis was important to prevent the liberation of cellular cytoplasmic proteins into the media and possible contamination of the extracellular lytic enzymes with intracellular autolysins. This was probably the main reason why no signs of more than one bacteriolytic enzyme in each strain tested emerged in the course of this study.

Bacteriolytic enzyme production during bacterial growth of the *S. aureus*, *S. simulans*, and *S. saprophyticus* strains is shown in Fig. 1.

Lytic activity could be found in *S. aureus* supernatants beginning at an early exponential growth phase, and activity increased with time proportionally to the rise in optical density (OD) and viable count. With the strain used, 3 U of lytic enzyme could be obtained by collecting the cultures at an OD of 2.6, while the cells were still exponentially growing and autolysis was negligible.

In the *S. simulans* cultures, extracellular lytic activity was detectable only after the cultures had reached an OD of 0.2. With further incubation, the detectable lytic activity increased proportionally to the OD and viable count, reaching a maximum of 0.5 U at the end of exponential growth. The supernatants of this strain were collected at an OD of 2.7, when they contained approximately 0.6 U/ml and the bacteria were growing exponentially. Also in this instance, a reasonably good enzyme yield could be obtained under conditions which did not permit autolysis.

With *S. saprophyticus*, extracellular lytic activity was less consistently detectable than with the previous two strains, and it was evident and easily detectable only at the end of the exponential phase. Cultures of this strain were collected at an OD of 2.8. In this case, avoiding the effects of autolysis was probably less certain than with the previous two strains.

Purification of the *S. aureus* bacteriolytic enzyme. The recovery and specific activity at each purification step are reported in Table 2.

Ammonium sulfate at 40% saturation caused precipitation of 36% of the total protein content of the supernatant, but precipitated only 2% of the lytic activity. This precipitate was therefore discarded. When the supernatant was 50% saturated with ammonium sulfate, >50% of the total protein and <10% of the lytic activity remained in solution. However, <50% of the lytic activity could be recovered in the precipitate. Altogether this step gave a fivefold purification with a recovery of activity of 46.1%.

The resuspended precipitate was then dialyzed for 24 h against four changes of 0.078 M phosphate buffer, pH 7.0. This step was a very important one in the procedure adopted for purification of all three enzymes because it led to the elimination of all medium components plus all dialyzable bacterial products. By this

TABLE 2. Purification of the bacteriolytic enzymes from *S. aureus*, *S. simulans*, and *S. saprophyticus*

Bacteriolytic enzyme from:	Purification stage	Enzyme activity		Sp act (U/mg)
		Total (U)	Recovery (%)	
S. aureus (lyogroup I)	Culture supernatant	92,000	100	2
	$(NH_4)_2SO_4$ precipitation	42,000	46.1	10.5
	Extensive dialysis	42,490	46.1	31.5
	Chromatography on Sepharose 4B-peptidoglycan	10,580	11.5	327.6
	Diaflow ultrafiltration	10,520	11.4	329.4
S. simulans (lyogroup II)	Culture supernatant	12,000	100	0.27
	Extensive dialysis	11,850	98.7	0.8
	Chromatography on chitin	9,960	83.0	27.7
	$(NH_4)_2SO_4$ precipitation	3,240	27.0	165
S. saprophyticus (lyogroup IV)	Culture supernatant	10,000	100	0.12
	$(NH_4)_2SO_4$ precipitation	8,970	89.7	2.88
	Extensive dialysis	8,780	87.8	11.52
	First chromatography on Amberlite IRC-50	6,290	62.9	48.85
	Sephadex G-50 chromatography	5,330	53.3	148
	Second chromatography on Amberlite IRC-50	4,680	46.8	156

step a threefold purification was obtained with virtually no loss in the lytic activity.

As described in Materials and Methods, two different affinity adsorbents were prepared: Sepharose-bound lysozyme-digested peptidoglycan and chitin. Reversible and complete binding of *S. aureus* lytic enzyme to the peptidoglycan was achieved. On the contrary, its efficient binding to chitin was not obtained. The Sepharose 4B-peptidoglycan resin was therefore used for purification of the *S. aureus* lytic enzyme. A column having a total binding capacity of 5 mg of protein was prepared, equilibrated with 0.078 M phosphate buffer (pH 7.0), and loaded with the dialysate from 10 liters of *S. aureus* supernatant. The column was then washed with the same phosphate buffer to remove the unbound proteins and with 1 M phosphate buffer, pH 8.0, to remove the loosely bound proteins. The first wash removed a large amount of protein but no lytic activity. The second wash removed a smaller amount of protein and, again, no lytic activity. To remove the lytic enzyme, the column was washed with 0.1 M glycine-NaOH buffer, pH 9.7, supplemented with 1.8 M NaCl. With this buffer, a sharp protein peak was removed from the column which exactly coincided with an equally sharp peak of lytic activity. The pattern of elution is shown in Fig. 2. The lytic activity-containing material was immediately added with phosphate buffer, pH 6.0, to bring the pH to 6.5. The affinity chromatography step allowed a 10.4-fold purification and caused a loss of activity of 34.6%.

The enzyme was then filtered on Amicon calibrated-pore membranes (XM50) with an exclusion size of 50,000 molecular weight to remove salts without diluting the enzyme and to improve purification by removing possible contaminants with a molecular weight lower than 50,000.

Purification of the *S. simulans* bacteriolytic enzyme. The recovery and specific activity at each purification step are reported in Table 2.

As a first step for enzyme purification, the supernatant was dialyzed for 24 h against five changes of 0.05 M phosphate buffer, pH 6.3. This procedure did not cause evident loss of activity but it allowed a greater than threefold purification of the enzyme and made the preparation more suitable for the following step.

The *S. simulans* lytic enzyme did not efficiently bind to the Sepharose 4B-peptidoglycan resin under any of the conditions tested, whereas it bound rather efficiently to chitin. Chitin columns were prepared, loaded with the sample obtained from the previous step, and eluted as described in Materials and Methods. The elution profile is shown in Fig. 3. It is evident that all lytic activity was eluted with a phosphate buffer concentration of 0.5 M, whereas most of the proteins did not bind to the column and a few were eluted with lower concentrations of the buffer. This step caused 15.7% loss of activity and a 34-fold purification.

The active fractions were pooled and submitted to fractionated precipitation with ammonium sulfate. A concentration of 50% saturation

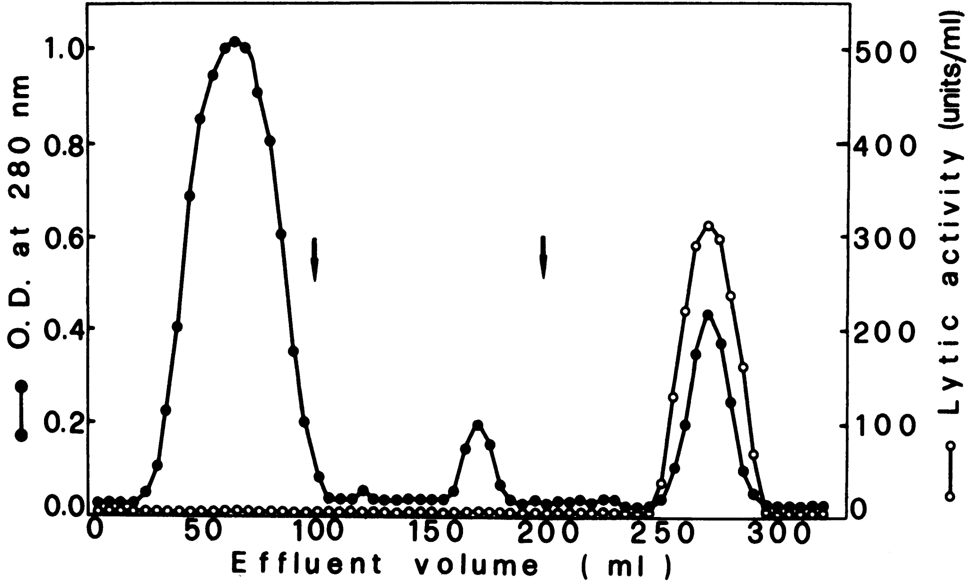


FIG. 2. Affinity chromatogram of *S. aureus* bacteriolytic enzyme. Symbols: OD at 280 nm (●); bacteriolytic activity (○). Arrows indicate addition of the elution buffers.

caused precipitation of other proteins, but not of the lytic enzyme, which was precipitated at 60% saturation. This step gave a sixfold purification and caused a reduction of 56% of the enzyme activity.

Purification of the *S. saprophyticus* bacteriolytic enzyme. The recovery and specific activity at each purification step are reported in Table 2.

The ammonium sulfate-fractionated precipitation proved to be very effective with this lytic enzyme. The crude supernatant was first brought to a concentration of 55% saturation,

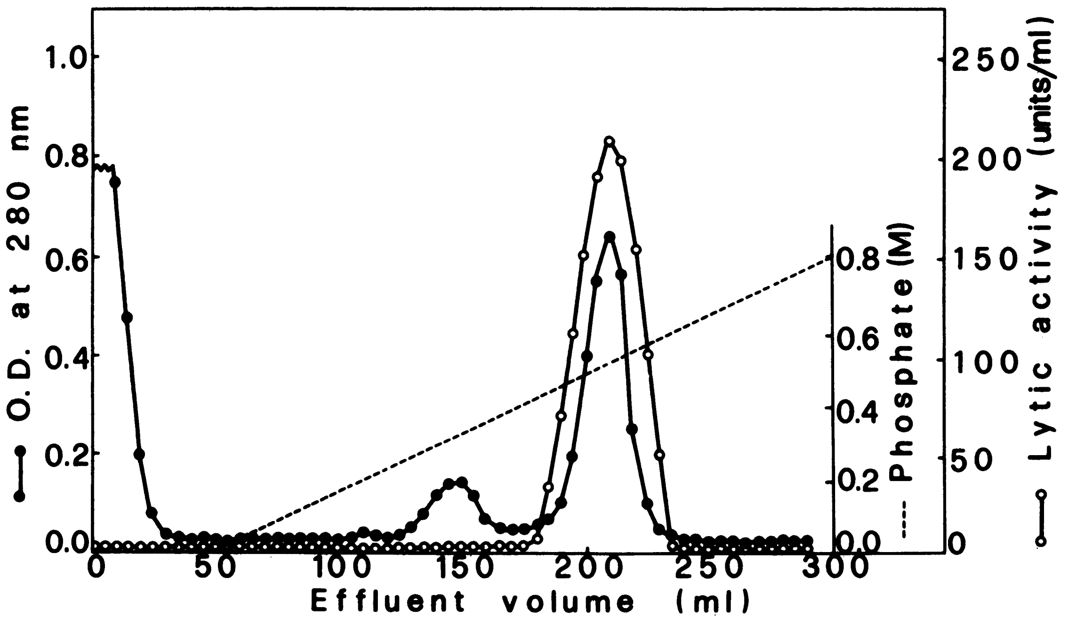


FIG. 3. Pattern of elution of *S. simulans* bacteriolytic enzyme from chitin. Elution was performed by a linear gradient of phosphate buffer (pH 8.0) from 0.05 to 0.8 M, and 5-ml fractions were collected. Symbols: OD at 280 nm (●); bacteriolytic activity (○).

and the sediment containing no more than 1% of the lytic activity was discarded. The supernatant was then brought to 65% saturation, and the precipitate which contained about 90% of the lytic enzyme was collected for further purification. This step caused a 24-fold purification and a 10% loss of activity.

The 65% ammonium sulfate precipitate was then dialyzed for 96 h against eight changes of 0.05 M phosphate buffer, pH 6.3. This caused a 2% loss of lytic activity and allowed a fourfold purification of the enzyme.

The *S. saprophyticus* lytic enzyme did not bind to the Sepharose 4B-peptidoglycan resin or to chitin under any of the conditions tested. It did bind to the Amberlite IRC-50 ion-exchange resin. Figure 4a shows the elution pattern from the Amberlite column. All enzyme activity was found in the peak corresponding to the major protein peak. The pooled fractions corresponding to the peak of activity were subjected to SDS-polyacrylamide gel electrophoresis and found to contain more than one protein.

The active fractions from the preceding step were submitted to gel filtration through Sephadex G-50. The elution profile from the Sephadex G-50 column is shown in Fig. 4b. The enzyme activity was again found in only one peak, which was also the main protein peak. The fractions corresponding to the top of the peak were concentrated and analyzed again by SDS-polyacrylamide gel electrophoresis. Although one major band was clearly prevalent, a few minor bands were also observed.

To concentrate the enzyme and complete purification, the fractions containing lytic activity of the previous step were collected and subjected to a second chromatography on Amberlite IRC-50. One major and two minor protein peaks were observed (Fig. 4c). All lytic activity was in the major peak.

Criteria for homogeneity of the purified enzymes. The homogeneity of the final enzyme preparations was determined by immunodiffusion and SDS-polyacrylamide gel electrophoresis.

In immunodiffusion tests, the three purified enzymes formed one single precipitation band when tested against antisera raised in rabbits against crude supernatants of cultures of each of the three staphylococcal strains. On the contrary, a large number of bands were formed when these antisera were tested against the pure supernatants.

Figure 5 shows that, after electrophoresis on SDS-polyacrylamide, only one protein band could be seen for each bacteriolytic enzyme.

After this, we also tested the pure lytic enzyme preparations for the presence of other enzymes or toxins which can be produced by staphylococcal strains. The substances tested included coagulase, phosphatase, DNase, hyaluronidase, protease, lipase, and hemolysins. None of these activities was found to be present in any of the three preparations.

Mode of action of the three bacteriolytic enzymes. The appearance of amino and reducing groups during enzymatic digestion of *M. luteus*-purified cell walls was evaluated first. Only reducing groups were found to be released by all three enzymes.

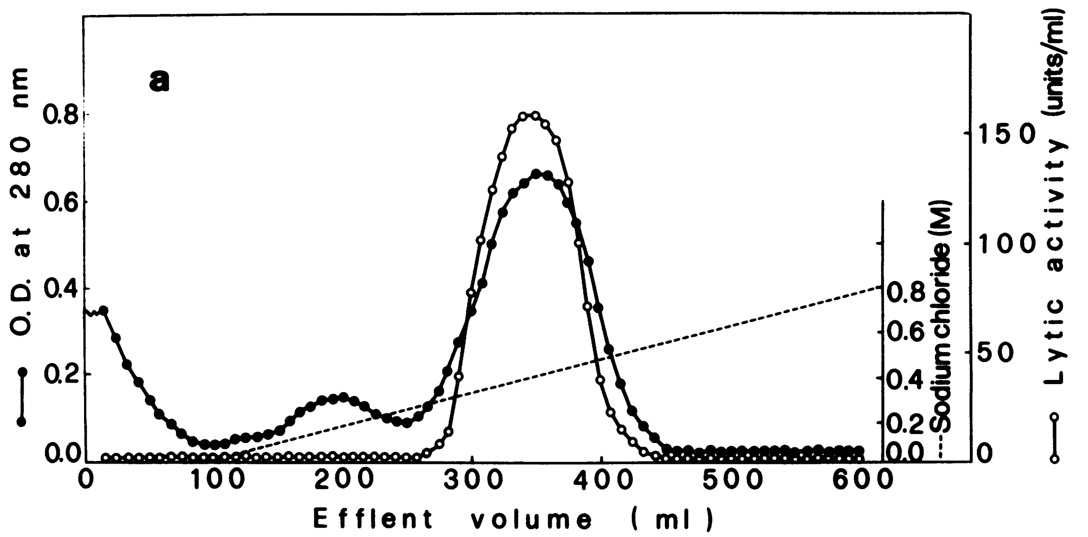
The nature of the reducing groups released was investigated to determine whether muramidase or glucosaminidase activities were involved. Disaccharides were isolated from the peptidoglycan digests (36), where both disaccharides and oligosaccharides are present (18). Samples of the purified disaccharides were reduced with sodium borohydride. Both reduced and unreduced samples were submitted to paper chromatography after acid hydrolysis (2 M HCl at 100°C for 2 h). Glucosamine and muramic acid were treated in the same way and used as controls. The results showed that the disaccharides yielded by digestion with all three enzymes were identical. In all cases, unreduced samples showed spots of muramic acid and glucosamine; quantitative analysis indicated that they were in a 1:1 ratio. In the reduced samples, the glucosamine spot completely disappeared, and a new spot corresponding to glucosaminitol (i.e., the reduction product of glucosamine) became evident. The muramic acid spot remained unchanged in the reduced samples and no muramitol spot appeared. This indicated that glucosamine was at the reducing end of the disaccharides yielded by all three lytic staphylococcal enzymes.

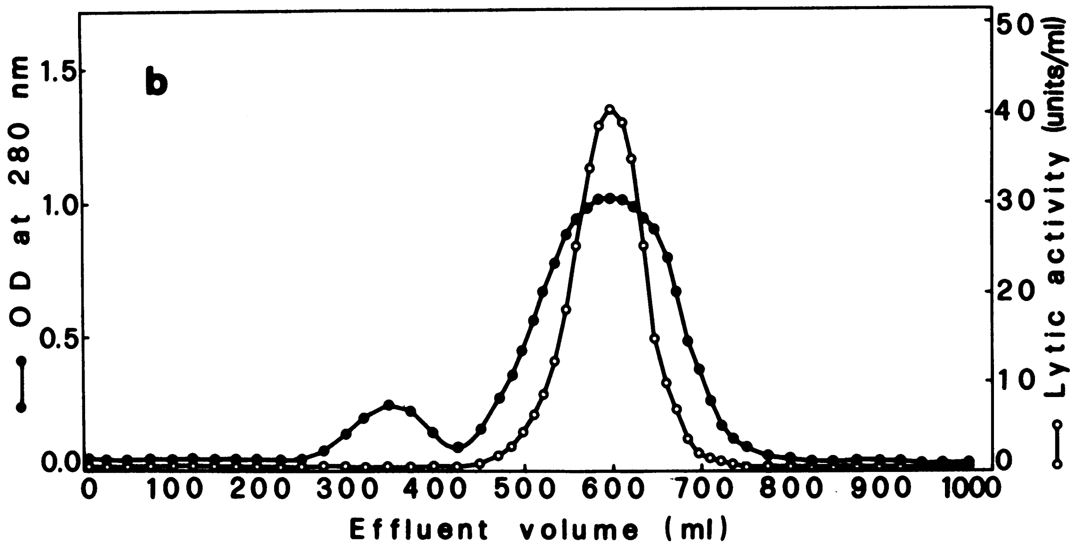
It has been shown by others (19, 36) that, in accordance with β-(1→4) linkages in the glycan chains of bacterial cell wall, both hen egg white lysozyme and the major lytic enzyme excreted by *S. aureus* are much more specifically inhibited by β- than by α-derivatives of 2-acetamido-2-deoxy-D-glucose. We have therefore made pure preparations of such derivatives and compared their inhibitory activity on the three pure staphylococcal lytic enzymes. It was found that all of them were inhibited approximately 10-fold more by the β- than by the α-derivatives.

Finally, the three enzymes were tested for exo-β-*N*-acetylglucosaminidase activity. All of them were found unable to liberate a significant amount of *p*-nitrophenol from *p*-nitrophenyl-β-*N*-acetylglucosaminide.

On the whole, these findings demonstrated that all three staphylococcal bacteriolytic enzymes acted as endo-β-*N*-acetylglucosaminidases.

Enzyme properties. (i) Kinetics of activity. The





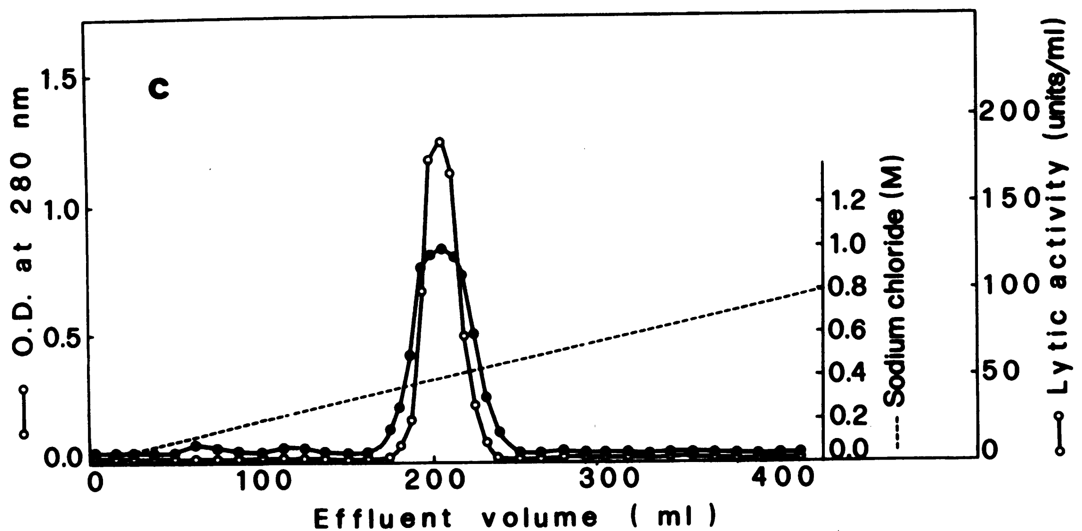


FIG. 4. Chromatographic procedures in the purification of the *S. saprophyticus* bacteriolytic enzyme. (a) First chromatography on Amberlite IRC-50; (b) gel filtration on Sephadex G-50; (c) second chromatography on Amberlite IRC-50. Symbols: OD at 280 nm (●); bacteriolytic activity (○).

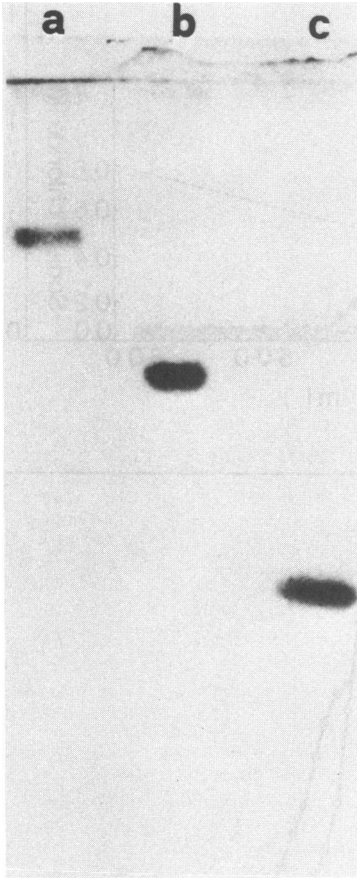


FIG. 5. SDS-polyacrylamide gel electrophoresis of the purified bacteriolytic enzymes of *S. aureus* (a), *S. simulans* (b), and *S. saprophyticus* (c).

kinetics of activity of the three different glucosaminidases on *M. luteus*-purified cell walls are shown in Fig. 6. Reducing group release paralleled turbidity decrease with all three enzymes. Although all enzymes caused complete solubilization of the suspended material within 24 h, the *S. aureus* and *S. simulans* enzymes caused an 85% decrease of the cell wall suspension turbidity within 1 h and showed virtually identical kinetics of OD reduction; the decrease in turbidity caused by the *S. saprophyticus* enzyme was more gradual, with 3 h being necessary to obtain an 85% reduction.

(ii) Spectrum of lytic activity on different substrates. The three enzymes displayed significant differences in their ability to lyse heat-killed cells of different bacteria. Cells of *Micrococcus* sp. X60 were rapidly lysed by the *S. simulans* glucosaminidase, whereas they were almost completely resistant to the *S. aureus* enzyme. Cells of *M. luteus* AH47 were fourfold more sensitive to the *S. saprophyticus* than to the *S. aureus* enzyme. Cells of *Micrococcus* sp. X15, which were very sensitive to both *S. aureus* and *S. saprophyticus* glucosaminidases, were completely resistant to the *S. simulans* enzyme.

(iii) Molecular weight. The molecular weights of the three enzymes were evaluated both in the native and in the denatured proteins by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively. With the latter procedure, molecular weights of 80,000, 45,000, and 31,000 were calculated for the glucosaminidases of *S. aureus*, *S. simulans*, and *S. saprophyticus*, respectively. By the gel filtration method, respective values of 79,000, 46,000, and 30,000 were obtained. The very good agreement between the results achieved by the two procedures indicated that all three glucosaminidases were made up of only one polypeptide chain.

(iv) Stability. The stability of the three enzymes was heavily influenced by both temperature and pH. The *S. saprophyticus* glucosaminidase was the most labile to heating. Exposures for 5 min at 70°C caused 4, 23, and 58% inhibition of the *S. aureus*, *S. simulans*, and *S. saprophyticus* enzymes, respectively. As for pH, the greatest stability of the three gluco-

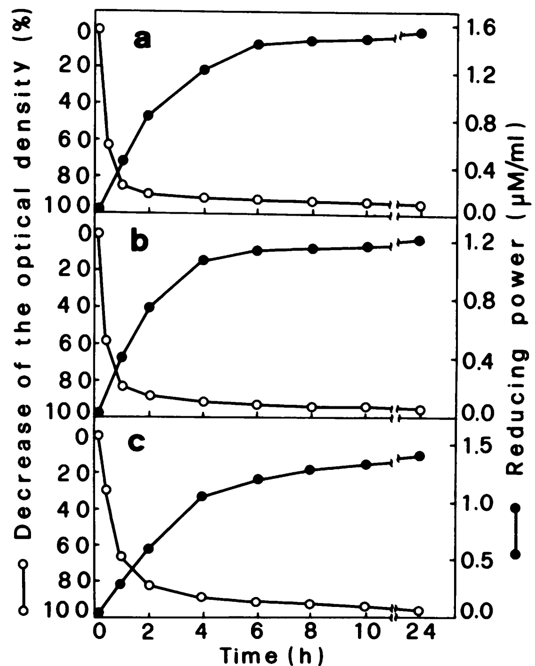


FIG. 6. Time-course relationship of OD decrease (O) and reducing power release (●) during digestion of *M. luteus* cell walls with purified bacteriolytic enzymes from *S. aureus* (a), *S. simulans* (b), and *S. saprophyticus* (c). Enzymatic digestion was performed at 37°C in 0.078 M phosphate buffer, pH 6.5.

saminidases was between 7.5 and 8.5 for *S. aureus*, 6.5 and 7.5 for *S. simulans*, and 7.0 and 8.0 for *S. saprophyticus*.

Effects of physical and chemical factors on enzyme activity. (i) pH. The optimal pH values for enzyme activity were 7.0, 6.0, and 6.5 for the *S. aureus*, *S. simulans*, and *S. saprophyticus* glucosaminidases, respectively.

(ii) Temperature. All three enzymes were most active at 37°C. However, the *S. simulans* enzyme was more active than the others at temperatures below 30°C, whereas temperatures above 40°C caused a much greater reduction in its activity than observed with the *S. aureus* or *S. saprophyticus* glucosaminidases. Of the latter two, only the *S. aureus* enzyme showed good activity at 60°C.

(iii) NaCl concentration. The *S. aureus* and *S. simulans* glucosaminidases were more heavily influenced by NaCl concentration than was that of *S. saprophyticus*. The optimal NaCl concentrations for enzyme activity were 175 mM for the former two glucosaminidases and 210 to 240 mM for the latter.

(iv) Mg^{2+} ions. Of several divalent cations tested, Mg^{2+} ions were those which influenced the lytic activity of the three enzymes to the greatest extent. A concentration of 30 mM Mg^{2+} ions, which had virtually no effect on the *S. saprophyticus* enzyme, caused a threefold increase in the activity of the *S. simulans* glucosaminidase and 75% inhibition of the *S. aureus* enzyme activity.

DISCUSSION

The data reported demonstrate that the three bacteriolytic enzymes have been purified to apparent homogeneity. In each enzyme preparation, only one precipitation line was observed in the immunodiffusion tests and one single protein band was obtained by fractionation based on the molecular weight (i.e., SDS-polyacrylamide gel electrophoresis). Moreover, other enzymes and toxins which can be excreted by staphylococcal strains were not detectable in the lytic enzyme preparations.

The pure enzymes, although all active as endo-β-*N*-acetylglucosaminidases, have proved to be different proteins. This is clearly supported by the differences observed in molecular weight and the other properties tested and by the great differences in their capability of binding to different affinity adsorbents or digesting different substrate cells. This suggests that, despite the fact that the same chemical bond is split, the activities of the three enzymes strongly depend on specific features of the substrates. It is interesting to recall that different endo-β-*N*-acetylmuramidases (lysozymes) isolated from different sources have been shown to degrade a given substrate in quite different ways (25), confirming that enzymes splitting the same glycosidic bond can markedly differ in other aspects of their activity.

The demonstration that the bacteriolytic enzymes excreted by different staphylococcal species are different proves the validity of the assay system previously proposed for the identification of staphylococci based on their bacteriolytic activity patterns (31, 33). This system, in fact, succeeded in separating different lytic enzymes although they shared the same mode of action. This indicates that differences relevant to the properties of the enzymes rather than to their synthesis or excretion were evidenced by this system.

That the staphylococcal glucosaminidases differ in several properties is interesting also because such enzymes come from strains of one bacterial genus whose species differ, among other characteristics, in the biochemical composition of the cell wall (13, 27). It seems likely that the different properties of such enzymes reflect an evolutionary adaptation of the bacterial autolysins to changes in the chemical cell wall composition. The possibility of a substrate-induced evolution has recently been proposed for lysozymes (25). The staphylococcal lytic enzymes could provide a useful starting point for tracing the sequence of the evolutionary steps in the development of the different staphylococcal species. The genus *Staphylococcus*, whose species are now well characterized not only for their biochemical cell wall composition but also for properties of their main bacteriolytic enzymes, should be regarded as a unique system for studying the mechanisms of cell wall extension, septum formation, and cell division, particularly through the analysis of their evolution in related species.

That different staphylococcal species both possess specific cell walls and produce specific cell wall-degrading enzymes suggests that each *Staphylococcus* species carries a specific cell wall-autolysin complex, which is likely to correlate with specific differences in the mechanisms of cell wall growth and cell division. The latter, which are not dispensable bacterial functions and involve the coordinate and critical action of products of many genes, appear to be among the most genetically stable characters in bacteria. Therefore, bacterial properties related to such functions are likely to have a much higher taxonomic value than properties most often used in bacterial taxonomy, such as sugar reactions or production of enzymes of unknown physiological significance, which mostly reflect the action of the product of a single gene and are dispensable for normal bacterial physiology. Analysis of the cell wall composition is already considered

an essential taxonomic tool (26), and analysis of the bacteriolytic activity as well should be regarded as another tool of primary importance in bacterial taxonomy. We have shown its utility previously not only in staphylococcal classification (32–34), but also in the separation, within *Micrococcaceae*, of staphylococci from micrococci and planococci (24, 30) and shall describe in forthcoming papers its usefulness in the taxonomy of *Streptococcaceae* and *Pseudomonadaceae*.

Purification and characterization of bacteriolytic enzymes from *S. capitis* (lyogroup III), *S. epidermidis* (lyogroup V), and *S. hominis* (lyogroup VI) are in progress in our laboratory. In preliminary experiments, these enzymes have displayed marked differences from the three lytic enzymes from *S. aureus*, *S. simulans*, and *S. saprophyticus* described here.

The potential of pathogenicity is known to differ among the different staphylococcal species, with *S. aureus* being regarded as the most pathogenic. Bacteriolytic activity, when only *S. aureus* was thought to produce it, has been regarded as a property of pathogenic staphylococci (9, 12). This concept, even though all staphylococci are currently known to produce bacteriolytic enzymes (23), may now be taken again into consideration in light of the fact that each different staphylococcal species produces a different lytic enzyme. The specific enzymes might interact in different ways with host cells and tissues (as suggested by the differences that emerged in the bacteriolytic spectrum and affinity for specific ligands) and thus play a different role in pathogenicity. We have shown elsewhere that the bacteriolytic enzyme purified from *S. aureus* stimulates DNA synthesis, anchorage to plastic surface, and migration of human fibroblasts (21, 22) and inhibits phagocytosis by human neutrophils and mitogen responsiveness by human lymphocytes (C. Pruzzo, P. E. Varaldo, S. Valisena, and G. Satta, Int. Congr. Immunol., 4th, Paris, 1980, abstr. 11.3.19 and 20.80). Preliminary investigations indicate that the bacteriolytic enzymes purified from *S. simulans, S. saprophyticus*, and *S. epidermidis* under identical experimental conditions display effects different from those caused by the *S. aureus* enzyme. These findings support the possibility that bacteriolytic enzymes excreted by staphylococci may not only contribute to staphylococcal pathogenicity but also account for some special differences in the pathogenicity of the different staphylococcal species.

Glycoproteins are proving to be more and more important components of eucaryotic cells. In particular, they often enter in the composition of membrane organs which are essential in the interactions of the cells with the environment and in the regulation of cell physiology (4). Glycosidases with different substrate specificities are needed for characterizing the polysaccharidic moieties of these molecules. Both *S. aureus* and *S. simulans* strains appear to be convenient sources of easily purifiable glycosidases. In this paper, we have actually presented a method by which *S. aureus* and *S. simulans* lytic enzymes can be purified to apparent homogeneity by only one major purification step. Such a rapid and efficient purification was probably achieved due to a few important factors. (i) A growth medium made up exclusively of dialyzable products was used. (ii) Culture supernatants were collected when autolysis was still negligible. Under these conditions the crude supernatants contained only the dialyzable components of the medium and the proteins excreted by the cells, which are believed to be some 30 in *S. aureus* and even less in *S. simulans* (37). (iii) A convenient affinity chromatography procedure was used. That purification of *S. saprophyticus* was more complex and required several steps could be both because cultures were collected when cells had already entered into the stationary growth phase and production of the enzyme was much lower than in the *S. aureus* or *S. simulans* strains. Even more important, however, was the lack of such a specific purification step as affinity chromatography.

Chitin columns were not used previously to purify lytic enzymes of bacterial origin. A solubilized peptidoglycan resin was previously used by others who showed its potential of utility for purification of bacteriolytic enzymes (38). However, in those studies only muramidases or uncharacterized lysozymes were dealt with. The present work shows that solubilized peptidoglycan resins can successfully be used for glucosaminidases as well. The bacteriolytic enzyme of *S. aureus* was previously purified by others with different methods (10, 35). The method presented here appears to give the same or an even higher degree of purity, it is much simpler and more rapid, and it allows a greater recovery of enzyme activity.

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