Bacteriolytic Enzymes from *Staphylococcus aureus* PURIFICATION OF AN ENDO- β -N-ACETYLGLUCOSAMINIDASE

BY T. WADSTRÖM AND K. HISATSUNE*

Department of Bacteriology, Karolinska Institutet, S-104 01 Stockholm, Sweden

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On cultivation of *Staphylococcus aureus* in a complex liquid medium, bacteriolytic activity is found extracellularly. The maximal amount was found at the end of the exponential growth phase in batch culture, but in continuous culture run under similar conditions the yield was doubled. Isoelectric focusing of dialysed crude culture supernatants showed that the bacteriolytic activity of all four strains studied (M18, 524, Wood 46 and Duncan) was heterogeneous. The most alkaline peak of activity (isoelectric point 9.5 ± 0.1) was assayed against *Micrococcus lysodeikticus* turbidimetrically. This bacteriolytic activity was purified more than 70-fold after continuous dialysis by adsorption on CM-Sephadex, precipitation with ethanol, heat purification, isoelectric focusing and Sephadex G-100 chromatography. The purified enzyme (isoelectric point 9.6 ± 0.1) was found to give a single band on polyacrylamide-gel and cellulose acetate electrophoresis and was devoid of all 14 staphylococcal enzymes and toxins assayed for. The molecular weight is $70\,000 \pm 5\,000$ as estimated by Sephadex G-100 and G-200 chromatography. The marked instability of the partially and highly purified enzyme was investigated. The mode of action and some properties of this enzyme are given in the following papers (Wadström & Hisatsune, 1970; Wadström, 1970). These results indicate that this extracellular enzyme which is produced by several strains of *S. aureus* is not a 'lysozyme' (endo- β -N-acetylmuramidase) as previously suggested, but an endo- β -N-acetylglucosaminidase.

The production of bacteriolytic and autolytic enzymes by the genus *Staphylococcus* is well established. Rogers (1954) and Richmond (1959) demonstrated the existence of lytic activity against *Micrococcus lysodeikticus* in culture supernatants from *Staphylococcus aureus*. Cell-bound autolytic enzyme(s) was also found in *S. aureus* (Mitchell & Moyle, 1957; Huff & Silverman, 1968; Tipper, 1969). It is not known if these different activities are caused by one or several enzymes.

Lysis of bacterial cell walls can be brought about by three types of enzymes: hexosaminidases, acetylmuramyl-L-alanine amidases, and endopeptidases (Ghuysen, Tipper & Strominger, 1966; Strominger & Ghuysen, 1967; Ghuysen, 1968). Strains of *S. aureus* and *Staphylococcus epidermidis* seem to possess one or several bacteriolytic and autolytic enzymes belonging to these three classes (Ralston, Baer, Liebermann & Krueger, 1961; Browder, Zygmunt, Young & Tavormina, 1965; Doughty & Mann, 1967; Suganaka, Kashiba,

Amano, Kotani & Imanishi, 1967; Hawiger, 1968a; Tipper, 1969). Huff & Silverman (1968) have reported on autolytic enzyme(s) in culture supernatants and on those released from whole cells by freezing and thawing. Hawiger (1968a) described a procedure based on ethanol precipitation and Sephadex G-100 chromatography for the purification of a lysozyme-like enzyme. High yields of lytic enzymes in batch and continuous cultures of different strains of *S. aureus* have been reported by Wadström & Hisatsune (1968).

By cultivation and purification techniques, described in a series of papers (Vesterberg, Wadström, Vesterberg, Svensson & Malmgren, 1967; Wadström, 1967, 1968; Arvidsson, Holme & Wadström, 1970), a hexosaminidase has been obtained in high yield from strain M18. A high degree of purity after ethanol precipitation, CM-Sephadex chromatography, heat purification, isoelectric focusing and Sephadex G-75 chromatography has been demonstrated by electrophoresis on polyacrylamide gel and cellulose acetate and by immunoelectrophoresis. Attempts have been made to elucidate the mode of action and substrate

* Present address: Department of Food Microbiology, Faculty of Nutritional Sciences, School of Medicine, Tokushima University, Tokushima, Japan.

specificity of the alkaline hexosaminidase, isoelectric point 9.5 (Wadström & Hisatsune, 1970). It proved to be an endo- β -*N*-acetylglucosaminidase. This enzyme is similar to the much more common bacteriolytic endo- β -*N*-acetylmuramidase (muramidase or lysozyme, EC 3.2.1.17) which has been found and purified from a variety of sources, such as animal tissues, plants and bacteria (Chipman & Sharon, 1969). Probably only a single bacteriolytic glucosaminidase has been purified before, from *Streptococcus pyogenes* (Barkulis, Smith, Boltralik & Heymann, 1964), but it has also been reported to occur in cell walls of *S. aureus* strain Copenhagen (Tipper, 1969) and *S. epidermidis* strain KW-61 (Browder *et al.* 1965).

MATERIALS AND METHODS

Materials. Lysozyme grade III (EC 3.2.1.17), catalase stock no. C-40, ovalbumin, myoglobin (horse heart), ox heart cytochrome *c*, dithioerythritol and freeze-dried whole cells of *M. lysodeikticus* were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Human serum albumin and human γ -globulin were from KABI, Stockholm, Sweden. Sephadex was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Columns for isoelectric focusing and carrier ampholytes, i.e. Ampholine, were purchased from LKB-Produkter, Stockholm-Bromma, Sweden, polyacrylamide from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., $(\text{NH}_4)_2\text{SO}_4$, ultrapure grade, from Mann Research Laboratories, New York, N.Y., U.S.A., and agarose from Industrie Biologique Française, S.A., Gennevilliers, Seine, France. Polypropylene glycol (polyglycol P2000) was purchased from Dow Chemical International S.A., Stockholm, Sweden, polyvinylpyrrolidone (commercial grade) from Thomas, Philadelphia, Pa., U.S.A., and polyethylene glycol 20 M (commercial grade) from KEBO, Stockholm, Sweden. Equipment for ultrafiltration (Diaflow) was purchased from Amicon N.V., The Hague, Holland. If not otherwise stated, all chemicals were of analytical grade. A polyvalent staphylococcal antiserum '011' was generously supplied by the Commonwealth Serum Laboratories, Melbourne, Vic., Australia (batch 011).

Bacterial strains. Specimens of several strains of *S. aureus* were kindly provided as follows: strain M18 from Dr J. Schmidt and two different specimens of strain 524 from Dr J. Hawiger and Dr H. J. Rogers (524; micromanipulated).

Culture conditions. The different strains of *S. aureus* were grown in a CCY medium (Gladstone & van Heyningen, 1957), modified to meet the demand of increased yields of cells and extracellular proteins (referred to below as CCY₁ medium) (Arvidsson *et al.* 1970). A stirred fermentor (Biotec FL 103, Bromma, Sweden) with a working volume of 2.2 litres was used. Foam was controlled by manual or intermittent automatic addition of 0.5 ml volumes of polypropylene glycol. In batch cultures the cells were grown to the end of the exponential phase, resulting in 8–10 g dry wt./l and a high yield of bacteriolytic activity in the medium compared with the yield obtained in shaken flasks or on CCY₁-agar

covered with a membrane (Arvidsson *et al.* 1970). Continuous-culture experiments were performed in the same type of vessel. The cells were grown to 6–8 g dry wt./l before the continuous feed was started. A dilution rate of 0.5 h^{-1} was used, i.e. about a 40 litre volume was harvested during a 36 h experiment. A detailed description of the equipment, cultivation and harvesting conditions is given by Arvidsson *et al.* (1970).

Continuous dialysis. Samples (4–10 l) of the crude culture supernatant were dialysed by a continuous procedure with an 'artificial kidney' (AB Nycotron, Oslo, Norway) as described by Kiil (1960). The dialyser was equipped with thin films of Cuprophane membranes (150 PTM, Bemberg, Wuppertal, Germany) with an effective dialysing area of 0.67 m^2 (Wadström & Vesterberg, 1970). A peristaltic pump (Sigma) made it possible to dialyse at a rate of 1800 ml/h against three times the volume of deionized water and to lower the ionic strength from 0.3 to less than 0.05. The calculation was estimated from a standard curve for solutions of NaCl of different ionic strengths (LKB Conductolizer). The dialysed material was subjected to rotary evaporation (Rotavapor R, Büchi; Vesterberg *et al.* 1967) or adsorbed on CM-Sephadex C-25. After a concentration of the material to one-tenth of the initial volume it was dialysed again before further treatment.

Sephadex chromatography. Samples (10 litres) of the dialysed material (step 1) were applied to columns of CM-Sephadex C-25 equilibrated with 0.05 M-sodium phosphate buffer, pH 6.5 (step 2, Table 2). The columns (6 cm $\times$ 100 cm) were packed to a height of 10–20 cm; this permitted adsorption of the lytic activity from a 50 litre culture volume. After washing with 1 litre of 0.05 M-phosphate buffer, pH 6.5, the lytic activity was eluted with the same volume of 0.2 M-sodium phosphate buffer, pH 6.5.

Ethanol precipitation. Precipitation with ethanol was performed at -5 to 0°C (step 3, Table 2). The temperature was carefully controlled during the whole procedure. The lytic activity as well as the staphylococcal hyaluronate lyase was precipitated at 45% (v/v) ethanol. After 1 h at 0°C the voluminous white precipitate was collected on a filter on a Büchner funnel. Elution was carried out by washing with five 100 ml portions of 0.1 M-sodium phosphate buffer, pH 7.0.

Heat purification (step 4). The partially purified enzyme obtained after CM-Sephadex chromatography and ethanol precipitation was dialysed for 4–6 h against 0.1 M-sodium acetate buffer, pH 5.0. This material was heated in 50 ml portions in a boiling-water bath for 5 min and was then rapidly cooled in an ice-water bath. Coagulated protein was separated by centrifugation at 15000 g for 30 min at 0°C . The supernatants were passed through a fine sintered-glass filter and concentrated against polyethylene glycol, if necessary, to a volume of less than 150 ml, dialysed against glycine (1%, w/v) containing dithioerythritol (0.1 mM) and then subjected to isoelectric focusing in a 440 ml column.

Isoelectric focusing (step 5). This was carried out as described by Vesterberg & Svensson (1966) and Vesterberg *et al.* (1967). Carrier ampholytes were used in a final concentration of 1% (w/v) to establish the pH gradient. Rotary-evaporated material was first subjected to focusing in the pH range 1–12, followed by a high-resolution focusing

in the pH range 8–11. This gradient was obtained by the use of carrier ampholytes 8–10 (1%, w/v) and different amines: diethanolamine, 0.1 ml; triethanolamine, 0.2 ml; arginine, 200 mg (Flatmark & Vesterberg, 1966). These refocusing experiments were run for 36–48 h at 4°C with a potential of 600–700 V (1–2 mA) in a 110 ml column equipped with a double cooling-jacket (LKB-Produkter).

After an extensive pre-purification step and dialysis against glycine (1%, w/v), isoelectric focusing in pH range 8–11 could be performed in the first run (900 V; 1–2 mA). Dithioerythritol, final concentration 0.1 mM, was usually added both to the dense and to the less dense solution to increase the recovery of the glucosaminidase. The contents of the column were collected in 2 or 3 ml fractions; after primary electrofocusing in the 110 ml and the 440 ml columns the contents were collected in 4 and 10 ml fractions respectively. Immediately after pH determination of each fraction the pH was adjusted to 6.0–6.5 with conc. NH_3 (diluted 1:10) or conc. acetic acid (diluted 1:10). Isoelectric focusing was also performed in pH gradients containing carrier ampholytes in 5% (w/v) final concentration and in pH gradients containing 4 M-urea and 1% (w/v) *n*-butanol. If not otherwise stated, density gradients of 0–50% (w/v) sucrose were used, but gradients of 0–40 and 0–60% sucrose and 20–70% (w/v) glycerol were also used in some experiments.

Sephadex G-75 chromatography. Either two or three 10 ml fractions (pH values 9.3–9.6) obtained from isoelectric focusing in the 440 ml column were pooled after pH adjustment, dialysed against 0.1 M-ammonium acetate buffer, pH 6.5, for 6 h, concentrated by polyethylene glycol or freeze-dried. Freeze-dried material was dissolved in 0.2 M-sodium acetate buffer, pH 6.5, and subjected to Sephadex G-75 chromatography. Columns were packed (2 cm $\times$ 110 cm; Wadström, 1968) and equilibrated with 0.2 M-sodium acetate buffer, pH 6.5. Between the separations the columns were stored with buffer containing 1% (v/v) *n*-butanol to prevent bacterial growth.

Molecular-weight determination. Portions of Sephadex G-100 and G-200 were equilibrated and packed in columns (1.5 cm $\times$ 160 cm) and calibrated with several reference proteins of known molecular weight. The molecular weights were calculated as described by Andrews (1965) and Determan (1967).

Electrophoresis on supporting media. Electrophoresis on cellulose acetate strips (average temperature 10°C) was performed in a Beckman Microzone cell (model R-101) at constant voltage (Duostat). The strips were stained with Ponceau S.

After each step of purification samples from fractions containing the maximum amount of lytic activity were subjected to electrophoresis in 6 and 7.5% (w/v) polyacrylamide gels (0.6 cm $\times$ 6 cm) (Davis, 1964; Ornstein, 1964) in a Buchler model DEC-12 gel cell (Buchler Instruments Inc., Hicksville, N.Y., U.S.A.) equipped with a cooling jacket. A continuous buffer system (Hjertén, 1963; Hjertén, Jerstedt & Tiselius, 1965), with 0.05 M-tris-acetate buffer, containing 0.05 M-tris final concentration and adjusted to pH 7.4 with acetic acid (S. Jerstedt, personal communication) was used. The degree of homogeneity of the purified glucosaminidase was also tested in a similar manner in 7.5, 10 and 15% (w/v) gels at pH 4.5 in β -alanine-acetate buffer (Reisfeld, Lewis & Williams,

1962) and at pH 8.3 in lutidine-glycine buffer (Flatmark, 1964). The gels were photopolymerized and used for electrophoresis (4–8 mA/tube; 1–2 h) without prior removal of the polymerizing catalyst (riboflavin). The temperature in this water-jacketed model was kept below 10°C by circulation of water (0°C) during the whole experiment. The viscosity of the samples was increased by addition of a few mg of dry Sephadex G-25 (coarse grade) and the samples were stored for 15 min at 4°C. A portion (0.1 ml) of this solution was then layered on to the top of each gel by injection through the upper buffer reservoir with a Jencon 1 ml automatic syringe (Jencon Ltd., Hemel Hempstead, Herts., U.K.).

The samples were usually run in duplicate; one gel was stained with Amido Black or Coomassie Brilliant Blue and destained in acetic acid (7%, v/v) and the other gel was rapidly frozen (–60°C), later cut with a razor blade into 1–1.5 mm slices, which were separately eluted in sodium phosphate buffer (0.1 M; pH 7.0) and assayed for bacteriolytic activity. The gels intended for slicing were also supplied with 0.1 mg of ox heart cytochrome *c*, isoelectric point 10.8, as a reference protein (Flatmark & Vesterberg, 1966).

Assay of lytic activity. A modification of the method described by Jollés (1962) and Parry, Chandan & Shahani (1965) was used. Freeze-dried whole cells of *M. lysodeikticus* (15 mg dry wt.) were suspended in 25 ml of 3% (w/v) NaCl solution. The suspension was stable for 24 h at 20°C. This suspension was diluted (1:2, v/v) with 0.15 M-tris-HCl buffer, pH 7.0, giving 20% transmittance ($T_{540\text{nm}}^{1\text{cm}}$). A sample (0.1 ml) of the test specimen was mixed with 2 ml of this 'cell buffer suspension'. The rate of clearance was measured in a Beckman DB-G spectrophotometer equipped with a Beckman recorder. The increase in transmittance was recorded for 5–15 min at 37°C after an initial lag phase of 2–5 min. A standard muramidase solution, prepared by dissolution of crystalline hen's-egg-white lysozyme (3 $\times$ crystallized; Sigma) in 0.15 M-sodium phosphate buffer, pH 6.2, was assayed in a similar test system except that 0.15 M-sodium phosphate buffer, pH 6.5, was used. The rate of decrease of turbidity was linearly proportional to the enzyme concentration in the range 0.1–10 μg . A specimen containing 1 μg gave an increase of transmittance of 1%/min at 37°C. A unit of lytic activity is defined as the amount of the test lytic activity required to give a linear increase of 1%/min at 37°C.

Other assays. α -, β - and δ -Haemolysin, protease, nuclease, phosphatase, leucocidin, hyaluronate lyase, staphylokinase, lipase, sphingomyelinase and lecithinase were assayed as described by Vesterberg *et al.* (1967), Wadström (1968) and S. Arvidsson, T. Holme & T. Wadström (unpublished work). Strain M18 was also shown not to produce enterotoxin B (H. O. Hallander, personal communication).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) or by measurements at 280 nm in a Zeiss PMQ spectrophotometer.

Spectrophotometry and pH determinations. These were performed as described by Vesterberg *et al.* (1967).

RESULTS

In previous experiments 50 different strains of *S. aureus* were investigated for the production of

extracellular bacteriolytic activity for *M. lysodeikticus* (Wadström & Hisatsune, 1968; Arvidsson *et al.* 1970). All strains except two produced the activity. The best producers (strains M18, 524 and Wood 46) were cultivated under controlled conditions in a Biotec FL 103 vessel and found to yield 10–20 units/ml and a cell mass of 4–6 g dry wt./l in the CCY₁ medium (Arvidsson *et al.* 1970). On increasing the amount of carbon source and oxygen supply, the yield of cells (8–10 g dry wt./l) and lytic activity increased remarkably (20–30 units/ml). In a continuous-cultivation process in the CCY₁ medium and in the same vessel, the yield (30–40 units/ml) exceeded that in a similar batch culture, although the bacterial cell mass remained the same. Strain M18 was found to give the highest yield of bacteriolytic activity in continuous culture and was therefore chosen for purification of the endo- β -N-acetylglucosaminidase.

Isoelectric focusing of crude culture supernatants. Samples (40 ml) of supernatants from different strains (M18, 524, Duncan and Wood 46) were dialysed against glycine (1%, w/v) and subjected to isoelectric focusing. All four strains gave separation patterns similar to that described by Wadström & Hisatsune (1968) when tested against a suspension of *M. lysodeikticus* (Fig. 1).

The rotary-evaporation method has been found suitable for concentration of several staphylococcal exoproteins (Vesterberg *et al.* 1967; Wadström, 1968). A continuous dialysis procedure performed before the evaporation step was found to prevent precipitation of material even at a 20-fold concentration. Crude samples of supernatant (6 litres) were concentrated to 300 ml, extensively dialysed

against glycine (0.5%, w/v) and finally decreased in volume to about 200 ml, with 70% recovery of activity. When the material was subjected to isoelectric focusing in a 440 ml column precipitates formed in the range pH 2–5. Chemical analyses indicated that they contained nucleic acid and polysaccharide material, probably released by autolysis during cultivation. The formation of precipitates is prevented by the addition of 4–6 M-urea to the standard columns. The yield of enzymic activity was, however, only 20–50%, possibly owing to the formation of cyanate (Stark, Stein & Moore, 1960). Despite the fact that a high degree of purity of the glucosaminidase (isoelectric point 9.5 ± 0.1) was obtained after isoelectric focusing of crude culture supernatant, a pre-purification was necessary to avoid losses due to precipitate-formation.

CM-Sephadex chromatography (step 2, Table 1). The high ionic strength of the culture medium necessitated an efficient dialysing procedure before ion-exchange chromatography could be used to adsorb the lytic activity in crude culture supernatants (Wadström & Vesterberg, 1970). Batchwise addition of CM-Sephadex (1 g dry wt./l of supernatant) or column chromatography on CM-Sephadex (step 2) were both found to be effective for fixation of approx. 80% of the lytic activity. The remaining 20% was adsorbed on DEAE-Sephadex equilibrated with 0.05 M-tris-HCl buffer, pH 8.3. A scheme for the purification of 10 litres of supernatant is described below (see Table 1). Most (65%) of the activity was eluted in 1 litre of 0.2 M-sodium phosphate buffer, pH 7.0, and another 10% was recovered after stepwise elution with 0.2 M- and 0.4 M-sodium chloride.

A smaller column (2 cm $\times$ 6 cm) was packed for

Fig. 1. Isoelectric focusing of crude culture supernatant (40 ml; strain M18). The experiment was performed in a 110 ml column containing carrier ampholytes 3–10 (1%, v/v) for 36 h at 400–600 V. Fractions (4 ml) were collected, the pH of each fraction was measured (●), bacteriolytic activity against *M. lysodeikticus* was assayed (○), and the protein content of each fraction was determined after extensive dialysis (▲).

adsorption of the dialysed ethanol precipitate after step 3 (Table 1). Elution with a linear salt gradient (0.1–0.7M-sodium chloride; Varigrad type; Parr, 1954) in 0.1M-sodium phosphate buffer, pH 6.5, gave a recovery of less than 20% in several experiments. This yield is low compared with that obtained by a stepwise elution procedure (60–80%). Addition of thiol compounds, such as dithioerythritol (1 mM), the blowing of N₂ on to the surface or the use of another impeller to prevent a vortex formation did not totally prevent this inactivation. Thus the stepwise elution procedure was preferred. A combination of a salt and a pH gradient did not give a much higher resolution when investigated by disc electrophoresis. Five bands were seen after staining with Coomassie Brilliant Blue (Plate 1).

Ethanol precipitation. The optimum conditions for ethanol precipitation were investigated. They were found to be similar for crude material, i.e. culture supernatant, and eluate of Sephadex C-25, but the yield was superior in the first case (see the Discussion section). It was important to keep the temperature between 0 and –10°C. Precipitation at 45% (v/v) ethanol and pH 7.0 gave a better recovery than at pH 4.5, 6 or 9. After extraction with 0.2M-sodium phosphate buffer, pH 7.0, 40–80% of the bacteriolytic activity was recovered. The precipitates were extracted with four–eight 60ml portions of this buffer. The eluates were pooled and dialysed against 0.1M-sodium acetate buffer, pH 5.0, with a loss of activity of about 10% before the next step of purification.

Heat purification. The heat treatment resulted in formation of a white flocculent precipitate. After centrifugation and filtration a drastic decrease in

Table 1. Purification of an extracellular endo-β-N-acetylglucosaminidase (isoelectric point 9.5) from *S. aureus* strain M18

Step	Culture supernatant (10 litres)
1	Continuous dialysis ('artificial kidney') 1800 ml/h
2	CM-Sephadex C-25 chromatography (elution: 0.2M-sodium phosphate buffer, pH 7.0)
3	Ethanol precipitation (45%, v/v; –5°C; pH 7.0) (elution: 0.2M-sodium phosphate buffer, pH 7.0) Concentration by polyethylene glycol to approx. 200ml Dialysis against 0.1M-sodium acetate buffer, pH 5.0
4	Heat purification (100°C, 5 min, pH 5.0–5.5) Dialysis against glycine (1%, w/v)
5	Isoelectric focusing (Ampholine, pH 8–10; pooling of fractions, pH 9.3–9.6) Concentration by polyethylene glycol or freeze-drying
6	Sephadex G-75 chromatography (0.1M-ammonium acetate buffer, pH 6.5) Freeze-drying

Table 2. Purification of endo-β-N-acetylglucosaminidase (isoelectric point 9.5) from *S. aureus* strain M18

Step	Fraction	Vol. (ml)	Protein		Glucosaminidase		Specific activity† (units/mg)	Yield (%)	Purification
			(mg/ml)*	(mg)	(units/ml)	(total units)			
1	Culture supernatant	10000	1.2†	12000	25	250000	21	100	—
2	Eluate from CM-Sephadex	600	4.8	2880	530	318000	110	127	5
3	Eluate from ethanol precipitation (45%, v/v)	400	1.5	600	440	176000	294	70	14
4	Heat purification (100°C, 5 min, pH 5.5)	440	0.7	310	395	174000	560	70	27
5	Isoelectric focusing (440 ml column)	30	2.2	66	3500	105000	1600	42	77
6	Sephadex G-75	60	1.0	60	1300	78000	1300	31	—

* Mean value in a pooled dialysed material after the different purification steps (steps 1–4) determined by the method of Lowry *et al.* (1951).
† Protein content before dialysis in the artificial kidney was 1.8 mg/ml.
‡ Calculated on pooled material after the separate steps of purification.

the protein content of the supernatant and an increase in specific activity was achieved without a loss of glucosaminidase activity (Table 2).

Isoelectric focusing. The heat-treated material was dialysed against glycine (1%, w/v) and concentrated, when necessary, against polyethylene glycol to approx. 200ml. The material was then subjected to isoelectric focusing in a narrow pH range (pH 8–11) in a 440ml column, by using Ampholine 8–10, triethanolamine, diethanolamine and arginine to extend the pH gradient in the alkaline range (Fig. 2). Under standard conditions separations were performed in a sucrose gradient (0–50%, w/v) with the addition of dithioerythritol (0.1mM final concn.). From a stock solution (0.01M) 0.2ml was added every 10h at the cathode, i.e. at the top of the column. This was done as dithioerythritol is partly dissociated at high pH and will migrate towards the anode. The total yield was then increased to 70–100%. In a parallel run without any added dithioerythritol a yield of 40% was obtained. All of the activity was found in three 2ml fractions (isoelectric point 9.5 ± 0.1). These were pooled, adjusted to pH 6.5 with 0.5M-acetic acid and concentrated to approx. 3ml with polyethylene glycol. This sample was then finally separated by Sephadex G-75 chromatography (Fig. 3).

Experiments were also performed in glycerol gradients and in inverted pH gradients, i.e. with the cathode at the top and the anode at the bottom. The isoelectric-point determination did not seem to be dependent on these variations of the standard procedure.

Sephadex G-75 chromatography. The use of ^{14}C -labelled carrier ampholytes has shown that Sephadex G-50 is effective in separating several proteins from carrier ampholytes (Vesterberg, 1969). Both

Sephadex G-50 and G-75 proved suitable for this purpose. Ammonium acetate buffer (0.1M, pH 6.5), a volatile buffer, was used to permit freeze-drying of eluted active fractions and to obtain a good recovery after this process (Fig. 3). Disc electrophoresis performed on the pooled fractions showing bacteriolytic activity (isoelectric point 9.5) after dialysis (0.2M-sodium phosphate buffer, pH 7.0) gave a homogeneous product on 7.5% (w/v) polyacrylamide gel in all three buffer systems used (see the Materials and Methods section). Gel filtration did not increase the specific activity either. On the contrary, owing to the marked instability at this high degree of purity, there was never a recovery of more than 40–70%. Serum albumin (0.5mg/ml), glycine (1%, w/v), dithioerythritol (0.1mM) and calcium chloride (0.1mM) were all separately found to increase the recovery. However, because we wished to characterize the enzyme physicochemically and to study the kinetics of the release of reducing power and free amino groups, addition of these compounds was avoided.

Criteria of homogeneity. (a) Analytical electrophoresis. Progress in the purification of the enzyme was followed by subjection of samples from the active fraction of each purification step (Tables 1 and 2) to electrophoresis on polyacrylamide gel (Plate 1) and on cellulose acetate membranes.

A crude supernatant, concentrated ten times by rotary evaporation or by freeze-drying gave ten distinct bands and four to eight faint bands after disc electrophoresis in the β -alanine buffer system and staining with Amido Black or Coomassie Brilliant Blue. Samples of the dissolved precipitate after ethanol precipitation gave three distinct bands, whereas the eluate containing the lytic activity from CM-Sephadex C-25, when used as a first step of

Fig. 2. Isoelectric focusing of pre-purified (steps 1–4, Table 1) glucosaminidase (isoelectric point 9.5) from 10 litres of culture supernatant, strain M18. Symbols are the same as those given in Fig. 1.

EXPLANATION OF PLATE I

Analytical polyacrylamide-gel electrophoresis of bacteriolytic activity from steps 1-6 (Table 1). (A) Culture supernatant was concentrated 50 times by polyethylene glycol. (B) CM-Sephadex C-25 eluted enzyme (step 2). (C) Ethanol-precipitated material (step 3). (D) Heat-purified enzyme (step 4). (E) Pooled fractions (isoelectric point 9.4 ± 0.1) after isoelectric focusing in a narrow pH gradient (step 5) and chromatography on Sephadex G-75 (step 6).

EXPLANATION OF PLATE 2

(a) Immunodiffusion of the endo- β -*N*-acetylglucosaminidase after the different steps of purification against a polyvalent staphylococcal antiserum (011). For symbols and experimental data see Plate 1(A-D). Peak fraction (isoelectric point 9.5) after isoelectric focusing (step 5) undiluted in (E) and crude material diluted 1:5 in (F). (b). Immunoelectrophoresis of crude (A) and purified endo- β -*N*-acetylglucosaminidase (B) against the '011' serum.

Fig. 3. Sephadex G-75 chromatography of purified glucosaminidase (isoelectric point 9.6, step 5, Table 1). The fractions (Fig. 2) containing more than 50% of the activity recovered after isoelectric focusing were pooled and applied to a G-75 column equilibrated with 0.1M-ammonium acetate buffer, pH 6.5. The protein content ($\blacktriangle$) and the lytic activity against *M. lysodeikticus* ($\bullet$) were estimated for each 5 ml fraction.

purification, yielded six to eight bands. When the CM-Sephadex chromatography preceded the ethanol precipitation step as described in Table 1, only one distinct and two faint bands were observed in all the buffer systems and gel concentrations. The pooled peak fractions (two or three each of 2 ml) from isoelectric focusing were also subjected to disc electrophoresis after dialysis or chromatography on Sephadex G-75 to remove the carrier ampholytes. Otherwise, these were found to interfere with the staining procedure. A too short dialysing time gave broadened bands both on polyacrylamide gel and cellulose acetate electrophoresis. However, all these experiments indicated the presence of a single protein band.

Gel cutting and elution were performed with gels containing bacteriolytic material after each step of purification. All activity was recovered from the eluate of one to three adjacent 1–1.5 mm thick gel slices. The location of these was always in accordance with the most heavily stained band in a parallel gel. However, the total recovery was always less than 50% and decreased to about 30% after step 2. An increase to about 60% was found when 0.1 mM-dithioerythritol was included in the gels and tray buffer solutions. Cytochrome *c* was used as a reference substance in these experiments. The homogeneous band containing the lytic activity was located somewhat closer to the anode, which is thus in accordance with the isoelectric point of these proteins when determined by the method of isoelectric focusing (Flatmark & Vesterberg, 1966).

(b) Immunodiffusion and immunoelectrophoresis. Samples from the different steps of purification were subjected to immunodiffusion against a polyvalent

staphylococcal antiserum, '011' (Plate 2a). The purified enzyme (stage 6) and the crude material were also subjected to immunoelectrophoresis against the same antiserum (Plate 2b). A single line of antigen-antibody reaction was obtained for the purified glucosaminidase, whereas up to 21 different precipitates were visible for the crude material.

(c) Assays for enzymes and toxins. Highly purified glucosaminidase (stage 6) was assayed for most of the extracellular proteins produced by strains of *S. aureus*. Nuclease (deoxyribonuclease and ribonuclease activity), α -, β - and δ -haemolysin, leucocidin, lipase, alkaline phosphatase, protease, hyaluronate lyase, staphylokinase, enterotoxin B, sphingomyelinase and lecithinase activity were not detectable under the assay conditions used.

Stability. The instability of the crude bacteriolytic activity on storage at 4 or -20°C was even more pronounced after different purification procedures. A half-life of a few days at 4°C has been calculated both for a crude product and for the activity after steps 1–3. More extensive purifications by isoelectric focusing, Sephadex G-75 or G-100, and disc electrophoresis all gave an enzyme that was remarkably unstable and had a half-life of 24–48 h. Storage of crude and purified material at 4, -20 and -70°C at different enzyme concentrations and in different buffers, e.g. citrate, acetate, phosphate, tris, imidazole, maleic acid and glycine, was tried. In general, storage at -70°C was better than at -20°C . This in turn was better than storage at 4°C .

Concentration of partially purified bacteriolytic enzyme(s) mostly resulted in great loss of activity. This was the case for rotary evaporation, for an ultrafiltration procedure (Diaflow) and for concentration in dialysing bags with powdered polyvinylpyrrolidone on the outside. Polyethylene glycol for the same purpose proved to be milder for purified materials. Freeze-drying after a continuous dialysis of crude supernatant solutions against 0.02M-ammonium acetate buffer, pH 6.5, gave a yield of 80%. However, when dialysis was omitted the yield was 20–40%. Freeze-drying after dialysis against a volatile buffer was also found advantageous for purified enzyme preparations.

Various stabilizers. As both crude and purified preparation fractions with bacteriolytic activity showed a marked instability on storage under different conditions, a variety of compounds were tested as stabilizers. Crude and purified preparations (20 and 200 units/ml) were dialysed against 0.05M-sodium phosphate buffer, pH 6.5, and stored for 48 h at 4°C . Ca^{2+} , albumin (1 mg/ml), glycerol (5–50%, w/v), sucrose (10–50%, w/v), dimethyl sulphoxide (5–10%, v/v), glycine (1–10%, w/v), and carrier ampholytes (1 and 5%, w/v) were all

Fig. 4. Determination of molecular weight of endo- β -*N*-acetylglucosaminidase by chromatography on Sephadex G-100 and G-200. Reference proteins: (1) cytochrome c; (2) myoglobin; (3) ovalbumin; (4) serum albumin; (5) γ -globulin; (6) catalase. Each line represents four experiments; the cross-hatched area depicts the elution behaviour of the glucosaminidase.

found to increase the half-life of the enzyme. Thiol compounds, such as β -mercaptoethanol (1 mM) and dithioerythritol (0.1 mM), calcium chloride (1 mM) and albumin (0.5 mg/ml) were found suitable under certain conditions, e.g. in Sephadex G-100 chromatography for molecular-weight determination.

Addition of several components increased the half-life significantly. On repeated freezing and thawing the activity decreased rapidly; this could be partly prevented in a buffer containing glycerol. Dithioerythritol was always used as a stabilizer after the first step of purification and glycerol (25%, w/v, final concentration) was used for storage of the purified enzyme. This was usually stored in a frozen state at -70°C in glycerol or freeze-dried after dialysis against 0.1 M-ammonium acetate buffer, pH 6.5.

Molecular weight. The molecular weight of crude and purified endo- β -*N*-acetylglucosaminidase was estimated by plotting the ratio of the elution volume (V_e) and the void volume (V_0) against the molecular weight of some reference proteins (Fig. 4). A mol.wt. of $70\,000 \pm 5\,000$ was calculated. This value was calculated from five experiments on the crude and purified enzyme in a tris-HCl buffer system, pH 8.4. Separation in phosphate and acetate buffers gave the same value, within experimental error.

DISCUSSION

Production of extracellular 'lysozyme' proved to be a frequent property of staphylococcal strains and was reported to be strongly correlated to pathogenicity (Hawiger, 1968b; Kashiba, Niizu, Tanaka, Nozu & Amano, 1959; Jay, 1966; Grossgebauer, Schmidt & Langmaack, 1968). The enzyme from strain 524 was studied by Richmond (1959) and by Hawiger (1968a). Release of reducing power and material reacting as *N*-acetylamino sugar from *M. lysodeikticus* whole cells (Richmond, 1959) and cell walls and mucopeptide from *S. aureus* strain Zak (Hawiger, 1968a) were demonstrated. These results indicated that a crude product (Richmond, 1959) or a partially purified enzyme (Hawiger, 1968a) were lysozymes similar to hen's-egg-white lysozyme or muramidase, i.e. mucopeptide *N*-acetylmuramylhydrolase or endo- β -*N*-acetylmuramidase, in mode of action.

In this laboratory several strains of *S. aureus* have been grown under different culture conditions to investigate pH, aeration and medium composition in relation to enzyme and toxin production. Freezing and thawing and disintegration of whole cells harvested in early, middle and late exponential growth phase have been performed to compare the yields of cell-bound and extracellular bacteriolytic activity. In no case was more than a few per cent of the total activity found to be intracellular or attached to the cell walls. In a continuous culture process a tenfold higher extracellular activity than the one obtained in cultures on a rotary shaker was achieved under the same conditions (Arvidsson *et al.* 1970). The increased yield and the less pronounced instability of enzyme produced in continuous culture facilitated the purification processes. Also a large volume (1 litre/h) is easily produced in a comparatively short time; this remarkably simplified purification of the enzyme on a large scale.

Certain difficulties are encountered in the purification of this bacteriolytic enzyme (isoelectric point 9.5). The most serious of these difficulties is its instability. Adjustment of the pH of the crude culture supernatant with 0.1 M-phosphoric acid to pH 3, 4, 5 and 6 did not increase the half-life significantly as would have been expected from the increased heat-stability of the crude and purified enzyme in an acidic environment. The instability becomes more pronounced after a certain degree of purification, i.e. after step 3 in Table 1. Several experiments were made in which one of the steps outlined in the purification procedure was left out or exchanged for another. Crude rotary-evaporated culture supernatants were directly subjected to isoelectric focusing. The total recovery was about 80% in five different experiments with about one-third to one-half of the lytic activity found in

fractions with an isoelectric point of 9.5 ± 0.1 . These fractions were pooled, and the activity was separated from carrier ampholytes by Sephadex G-50 or G-75 chromatography; one to three bands were then observed in analytical disc electrophoresis. However, after another separation in a more shallow pH gradient, with carrier ampholytes 8-10, only one band was detected in the different gels and buffer systems. The recovery after this experiment and after the subsequent Sephadex chromatography diminished significantly.

Ammonium sulphate precipitation proved an unsatisfactory method of concentrating the enzyme, even in the presence of EDTA which often increases the recovery of metal-sensitive proteins (Dixon & Webb, 1964). Continuous thin-film dialysis (Vesterberg & Wadström, 1970; Wadström & Vesterberg, 1970) lowered the ionic strength for 1800 ml of supernatant from 0.3 to 0.05 M, which made adsorption on CM-Sephadex C-25 possible. Adjustment of the pH to 8.3 and attempts to adsorb the eluted material or a sample of a crude dialysed supernatant on DEAE-Sephadex were also made. The bacteriolytic activity adsorbed has not yet been further studied. It might be the same as one or more of the components found in the acidic pH range after isoelectric focusing of a crude material (Fig. 1).

Precipitation by ethanol (45%, v/v; 0°C to -5°C; pH 7.0) was found suitable as a first step of purification for bacteriolytic activity and hyaluronate lyase with a 70-100% recovery for both (O. Vesterberg, unpublished work) but was not convenient on the large scale. It was therefore used only after preliminary concentration on CM-Sephadex. Precipitation was not observed in 10% (v/v) ethanol, pH 3.8, as described by Hawiger (1968a). A co-precipitation of the activity with acidic medium constituents of the brain-heart infusion, and perhaps also other acidic material such as products of lysis, could explain the discrepancy in these results, since it was confirmed that no precipitate formed after similar cultivations in other media (J. Hawiger, personal communication). A high degree of purification was achieved by ethanol precipitation of crude supernatants, as shown by the disc electrophoresis experiments.

Heating of the crude culture supernatant at pH 5 resulted in a complete loss of activity. However, this procedure gave considerable purification when applied to the ethanol precipitate. The same isoelectric point, movement in disc electrophoresis and elution volume in Sephadex G-75 chromatography suggested that the enzyme was undenatured, although it had been freed of all other enzymic and toxic activities except a trace of nuclease (isoelectric point 10.1; Wadström, 1967). Without heat purification the enzyme still contained amidase

and peptidase activities since staphylococcal peptidoglycan was digested and *N*-terminal glycine and alanine were produced. Dinitrophenylation revealed that the spots of DNP-glycine and DNP-alanine disappeared on t.l.c. when peptidoglycan was digested with heat-treated enzyme. The time-course of digestion of *M. lysodeikticus* cell walls also showed the release of more free amino groups by partially purified enzyme than by glucosaminidase (steps 1-6) and hen's-egg-white lysozyme.

An isoelectric point of 9.5 ± 0.1 for the glucosaminidase was confirmed in many experiments in sucrose- and glycerol-density gradients. The recovery varied in different experiments between 20 and 80% for the pre-purified enzyme (steps 1-4). Adjustment of the pH to 6.5 increased the recovery and proved important for the stability of the enzyme on storage. When trying to remove the carrier ampholytes, Sephadex G-50 or G-75 chromatography was used since it is superior to dialysis (Vesterberg, 1969). The lytic activity was more unstable after all procedures that followed CM-Sephadex chromatography and ethanol precipitation. The final product after isoelectric focusing and chromatography on Sephadex G-75 showed only one band after electrophoresis on polyacrylamide gel at three different pH values and also on cellulose acetate. Elution from the polyacrylamide gels showed that the protein band contained the lytic activity. The enzyme was somewhat less cationic than ox heart cytochrome *c* (isoelectric point 10.8; Flatmark & Vesterberg, 1966). The material purified by CM-Sephadex chromatography gave six or eight bands. Only three bands were visible for crude ethanol-precipitated material. Thus a combination of these methods was used (Table 1, steps 1 and 3). Samples of this material (steps 1-3) showed three bands in disc electrophoresis, whereas heat-purified material (step 4) showed one strong band and one faint band containing staphylococcal nuclease (isoelectric point 10.1; Wadström, 1967). After isoelectric focusing and Sephadex G-75 chromatography only one band was observed in all three buffer systems as well as in cellulose acetate electrophoresis in 0.05 M-veronal buffer, pH 8.6.

On digestion of cell walls of *M. lysodeikticus* with an enzyme (isoelectric point 9.5) purified by the general procedure, reducing groups and acetylhexosamine reactive material were released (Wadström & Hisatsune, 1970). These results indicate that the enzyme is a type of bacteriolytic hexosaminidase. Wadström & Hisatsune (1970) have isolated a disaccharide in the cell-wall digest and analysed it by paper chromatography in three solvent systems. The reducing end was found to be glucosamine and not muramic acid, which indicates that this enzyme is a type of endo- β -*N*-acetylglucosaminidase.

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