Formation of Bacteriolytic Enzymes in Batch and Continuous Culture of Staphylococcus aureus

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The formation of bacteriolytic enzymes of Staphylococcus aureus, with special reference to strain M18, was investigated under a variety of conditions. The bacteriolytic activity was tested by using whole cells of Micrococcus lysodeikticus as a substrate. Complex media were required for production, and a Casein Hydrolysate-Yeast Extract medium (CCY1) was superior to Brain Heart Infusion and Trypticae Soy Broth. The optimal pH level for production was 7.0. Effective oxygenation and exchange of the β-glycerophosphate of the CCY1 medium for glucose increased the rates of growth and autolysis and the rate of appearance of extracellular bacteriolytic enzymes. However, the extracellular lytic activity decreased more rapidly at the end of the growth period than under the standard culture conditions. The appearance of inhibitor(s), probably derived from autolysis, might be responsible for this rapid decrease. The highest yields were obtained in a continuous process in which the activity was almost twice that of batch cultures grown under the same conditions. The bacteriolytic activity produced in continuous culture had a considerably increased stability in the purification process. The advantage of producing unstable bacterial proteins in continuous culture under controlled growth conditions is discussed.

Welsh and Salmon (34) were the first to report the phenomenon of autolysis of Staphylococcus aureus. They made a distinction between an endogenous and an exogenous autolysis, the latter being caused by lytic enzyme(s) liberated into the culture medium. Later Mitchell and Moyle (21) and recently Huff and Silverman (15) and Tipper (32) reported on cell-bound autolytic enzyme(s). However, Richmond (26) described the occurrence of an extracellular enzyme, produced by strain 524 of S. aureus in an amino acid medium, which caused lysis of Micrococcus lysodeikticus. Crude culture filtrates released N-acetylamino sugars and reducing substances which was taken as evidence that a lysozyme (EC 3.2.1.17) was present. The increase of extracellular bacteriolytic activity turned out to almost parallel the increase in the bacterial density during the whole exponential phase of growth. More recently, Billitteri and Bernadini (1) investigated the influence of the composition of a defined medium on the formation of extracellular bacteriolytic enzymes.

A purification procedure for the extracellular lytic enzyme, with the characteristics mentioned, from supernatants of strain 524 was first reported by Hawiger (12). The partially purified product was characterized as a lysozyme. Wadström and Hisatsune (unpublished data) recently purified bacteriolytic enzymes from culture supernatants from several strains of S. aureus, with special reference to the strains M18 and 524. One of the enzymes proved to have an isoelectric point of 9.5. It also showed characteristics typical for a hexosaminidase. The results seemed to agree best with the assumption that it is an endo-β-N-acetylglicosaminidase and not a muramidase.

The production of extracellular bacteriolytic enzyme(s) of this kind seems to be characteristic for some gram-positive bacteria, e.g., Bacillus subtilis and S. aureus (25-27). The appearance of extracellular hexosaminidase of S. aureus was correlated with the production of free coagulase and nuclease and, thus, with the pathogenicity of staphylococci (11, 13, 16). Preliminary experiments of this investigation showed that 50 strains of S. aureus, but only 1 of 15 strains of Staphylococcus albus produced bacteriolytic activity (Wadström and Hisatsune, FEBS Congr. Abstr. 422, Prague, 1968).

Because of low yields of the bacteriolytic enzyme obtained in shake flasks, a more detailed investigation of the influence of cultivation conditions on the formation of this enzyme was performed. Furthermore, production methods on a preparatory scale had to be evaluated, since large amounts of starting material were required for
the purification. The high yields and increased stability of the bacteriolytic activity in the continuous culture enabled the purification of the endo-β-N-acetylgalactosaminidase from strain M18. No systematic study of the conditions for production of a bacteriolytic endo-β-N-acetyl-
galactosaminidase has previously been reported.

MATERIALS AND METHODS

Bacterial strains. S. aureus strain M18 was obtained from J. Schmidt, University of Leipzig, Germany; strain V8 from G. P. Gladstone, University of Oxford, England; strain Wood 46 (NCTC 10344) from A. W. Bernheimer, New York University, New York, N.Y.; strain Wood 46 (NCTC 10345) from E. Kjems, Statens Seruminstitut, Copenhagen, Denmark; strain R1 from C. Cheesbro, Rutgers University, New Brunswick, N.J.; strain Foggie from G. Ommen, NIH, Bethesda, Md.; and strain 524; from J. Hawiger, Institute of Hygiene, Warsaw, Poland. Another specimen of the same strain (524α), once isolated from one single organism, was obtained from H. J. Rogers, Mill Hill, London, England.

Culture media. Except for some preliminary experiments, a medium (CCY1) based on the Woodin medium supplemented with "CCY"-peptone (described by Gladstone and van Heyningen (10) was used. If not otherwise stated, it had the following composition: casein hydrolysate (Oxoid), 40 g; yeast extract (Difco), 10 g; sodium β-glucosephosphate, 20 g; sodium lactate (50%), 10 ml; NaHPO4·2H2O, 1 g; KH2PO4, 0.4 g; (NH4)2SO4, 1 g; DL-tryptophan, 80 mg; L-cystine, 100 mg; thiamine, 2 mg; nicotinic acid, 4 mg; MgSO4·7H2O, 20 mg; MnSO4·4H2O, 10 mg; FeSO4·7H2O, 6 mg; citric acid, 6 mg; distilled water to a final volume of 1,000 ml. The vitamins were heat-sterilized separately at pH 4.5 in a 100-fold concentrated solution. The trace elements were also sterilized separately in a 100-fold concentrated solution. The bulk of the medium was sterilized at 120°C for 20 min. Polypropylene glycol P-2000 (PPG, Dow Chemical Co., Midland, Mich.) was added as an antifoam agent before sterilization to a concentration of 0.05 ml/liter. Other complex media, such as Trypsine Soy Broth (TSB; BBL) and Brain Heart Infusion broth (BHI; Difco) were tested in shake flask cultures. A defined medium prepared by the method of Mah et al. was also used (19). It contained 11 amino acids, nicotinic acid, and thiamine. The solution of trace elements for the CCY1 medium was added to this medium. The media were sterilized at 120°C after addition of the antifoam (PPG).

Cultivation techniques. The microorganisms were grown on a rotary shaker in 1-liter indented Erlenmeyer flasks with a culture volume of 100 ml and in stirred fermentors with working volumes of 2.5 and 8 liters (Biotec FL 103 and FL 110, Stockholm, Sweden). The FL 103 vessel was used both for batch and continuous cultures. The pH of the cultures was maintained constant by automatic control. The temperature was regulated at 37 ± 0.2°C. The impeller speed was 800 rev/min and the air flow was 0.5 liter/min. The cultures were grown batchwise to a wet weight of approximately 30 g/liter, which corresponds to 6 g (dry weight)/liter. In the standard procedure, the continuous feed was started after 5 to 7 hr of growth with a dilution rate of 0.5/hr. Oxygen limitation of growth was demonstrated by using the oxygen electrode described by Borkowski and Johnson (2).

Assay for bacteriolytic activity. Lyophilized whole cells of M. lysodeikticus were suspended in 3% NaCl solution to a concentration of 0.6 mg (dry weight)/ml. Part of the suspension was diluted with two parts of 0.15 M tris(hydroxymethyl)aminomethane (Tris)-hyd-
drochloride buffer (pH 7.0) giving 20% transmittance (714 m). The test specimen was mixed with 2 ml of this "cell-buffer suspension." The rate of clearance was measured in a Beckman spectrophotometer (model DB-G) with an attached Beckman recorder. The increase of the transmittance was recorded for 5 to 15 min at 37°C after an initial lag phase of 2 to 5 min. One unit of enzymatic activity is defined as the amount of test specimen which gives a linear increase in transmittance at 640 nm of 1% per minute at 37°C.

Autolysis. Samples for dry-weight determination and autolysis experiments were taken every hour. Cells were washed and suspended in 0.01 M phosphate buffer (pH 7.0) to a density of A400 of 0.6, corresponding to a dry weight of 0.7 g/liter. A Beckman spectrophotometer (model B) was used. The rate of autolysis at 37°C was linear during the first 2 hr and was calculated in percent decrease of the initial absorbancy per hour. Autolysis in the cultures was estimated in 10-ml culture supernatants pre-

cipitated by 10% (v/v) perchloric acid (PCA). The precipitate was resuspended in 1 ml of 0.01 M phosphate buffer (pH 7.0). The deoxyribose content was esti-

mated by the diphenylamine method of Burton (3).

Detection of cell-bound and intracellular enzyme. Suspensions of whole cells were washed with 0.01 M phosphate buffer (pH 7.0) at 4°C. The cells were suspended in the same buffer to one-third of the initial volume and disintegrated by the freeze-press technique (X-press, Biotec, Stockholm, Sweden) of Edabo (7). The disintegrated material was centrifuged at 4,000 × g for 10 min to remove whole cells. The supernatant was then centrifuged at 10,000 × g for 30 min. The supernatant from the second centrifugation, the cytoplasmic fraction, was tested for bacterio-
ytic activity. The pellet, i.e., the cell wall fraction, was treated with sodium phosphate, sodium citrate, ribonucleic acid (RNA) from yeast (commercial grade, Sigma Chemical Co., St. Louis, Mo.), and 1 M KCl as described previously (4, 5, 20) to release enzymatic activity. Similar experiments were performed on whole cells after the first washing. Selective release by sudden cooling of the cells was also tried (30), as was release or activation of cell-bound enzyme by treatment with trypsin by the method of Conover et al. (6).

RESULTS

Fifty different strains of S. aureus were cul-
vatated in the CCY1 medium in shake flasks to find high-producing strains. The results obtained
with some strains, previously used for the production of various extracellular proteins, are summarized in Table 1. A great variation between the ability of different strains to produce bacteriolytic enzymes was found. This difference was verified in experiments with the Biotec fermentors for some of the strains. Strain M18 was superior in both cases in the production of extracellular lytic activity to the other strains investigated, whereas very low activities were obtained in both types of cultures of strain V8. Strain M18 was chosen for further studies under controlled conditions of pH, temperature, aeration, and agitation. For all strains, the 24-hr samples showed less than one-tenth of the highest activity which was obtained after 6 to 10 hr of cultivation.

Medium composition and pH. Strain M18 was cultivated in CCY1, BHI, TSB, and a defined medium in flasks on a rotary shaker. Both the highest yield of cells and bacteriolytic activity were obtained in the CCY1 medium (Table 2). When the yeast extract was omitted from this medium (Cas, Table 2), the cell yield decreased, and the lytic activity was practically absent. The yield of cells was low in the defined medium, and no lytic activity was detected.

To optimize the production of bacteriolytic activity, the CCY1 medium was modified in some experiments. Cultivation in a medium with the concentration of all constituents increased by 50% gave a higher yield of cells [14 g (dry weight)/liter] and a simultaneous increase in bacteriolytic activity (50 units/ml; Fig. 1a, compare Fig. 2). The exclusion of the β-glycerophosphate (20 g/liter), the main carbon source of the CCY1 medium, and an increase in the lactic acid con-

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**TABLE 1. Bacteriolytic activity in shake flask cultures of different strains of Staphylococcus aureus after 8 hr of cultivation in the CCY1 medium**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell yield (g/liter)</th>
<th>Bacteriolytic activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V8</td>
<td>9</td>
<td>&lt;2</td>
</tr>
<tr>
<td>R1, Foggie, Newman</td>
<td>6-9</td>
<td>3-5</td>
</tr>
<tr>
<td>Wood 46, 524, 524ii</td>
<td>6-9</td>
<td>5-8</td>
</tr>
<tr>
<td>M18</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

**TABLE 2. Influence of the medium composition on the yield of cells and bacteriolytic activity of strain M18 in shake flask experiments**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell yield (g/liter)</th>
<th>Bacteriolytic activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB</td>
<td>2</td>
<td>2-5</td>
</tr>
<tr>
<td>BHI</td>
<td>4</td>
<td>5-7</td>
</tr>
<tr>
<td>CCY1</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Cas</td>
<td>2.5</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Aa</td>
<td>1.5</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

*Maximal value of bacteriolytic activity was obtained at different times of cultivation in the different media (6 to 10 hr).

**FIG. 1. Relationship between bacterial growth and production of bacteriolytic enzymes in a stirred fermentor. Cultivation was performed in CCY1 medium with all ingredients increased 50%. (a) Standard aeration conditions; (b) aeration with 40% oxygen; (c) aeration with 40% oxygen in a modified CCY1 medium containing glucose (40 g/liter) instead of β-glycerophosphate. Symbols: cell dry weight (▲), bacteriolytic activity (●).**
centration from 10 to 30 g/liter resulted in a low yield of cells and bacteriolytic activity. When β-glycerophosphate was replaced by glucose (20 or 40 g/liter), no significant difference in the enzyme yield was obtained but a more rapid decrease in enzymatic activity was noticed (Fig. 1c). A decline to half of the maximum value was found after 30 to 120 min. This decreased stability was also noted for the bacteriolytic activity in the crude supernatants stored at different temperatures (−20, 4, 20, and 37 C).

Strain M18 was grown at different pH levels in the stirred fermentor equipped with automatic pH control. The highest activity was achieved at pH 7.0 after 6 to 8 hr of growth.

Relationship between bacterial growth and formation of bacteriolytic enzymes. Preliminary investigations showed that a high bacteriolytic activity was obtained in stirred fermentors and in baffled shake flasks which gave a high aeration efficiency. The exponential growth phase lasted for 4 hr and was followed by a period of oxygen-limited, linear growth, which lasted for 2 to 4 hr (Fig. 1 and 2). The dissolved oxygen concentration reached zero at a cell concentration of approximately 4 g/liter in stirred fermentor cultures gassed with air. The increase in bacteriolytic activity was found to parallel the growth curve. A maximum value was obtained during the later part of the period of linear growth. Although the cell dry weight remained constant after this period, a rapid decrease of lytic activity was observed.

Oxygen at a concentration of 40% in air resulted in an increased growth rate (Fig. 1b). The decline in lytic activity was then more rapid than under standard conditions. When a culture with glucose as the carbon source was aerated with the oxygen-enriched air, the rate of growth increased even more, and a very rapid decrease of extracellular activity was obtained (Fig. 1c). The rate of autolysis during the period of linear growth was also increased (see below).

Autolysis. A maximal rate of autolysis was observed in cell samples taken during the period of linear growth. The tendency to autolyze decreased rapidly for cells taken from the retardation phase. The autolytic activity was very low in cells from the stationary phase (Fig. 2). No significant differences between the rate of autolysis in samples from batch cultures in shake flasks or in the fermentors were observed. At the maximal rate of autolysis, 10 to 15% of the cells lysed per hour upon incubation at 37 C, whereas cells from continuous culture lysed more slowly (5 to 10%/hr). When strain M18 was grown in CCY1 medium containing glucose instead of β-glycerophosphate, the maximal rate of autolysis increased to 20 to 25%/hr. In all experiments, the tendency to autolysed was highest during the period of linear growth, independent of the culture conditions and medium composition (Fig. 2). Autolysis was estimated by measuring liberated nucleotides in culture supernatants from a number of batch and continuous cultures. There was a good correlation between the amounts of liberated nucleotides and the autolytic tendency, determined as described above.

Continuous culture experiments. For the production of large quantities of staphylococcal exoproteins, a process for a short-term continuous cultivation was developed. During the first 4 to 6 hr after the start of the continuous feed, a yield of bacteriolytic activity equivalent to that in batch cultures was obtained. After this time there was an increase of the activity, and the enzyme yield then remained at the higher value (Fig. 3). The steady-state values in four continuous cultures ranged between 45 and 55 units/ml.

Stability of the enzymatic activity. Samples from both batch and continuous cultures were momentarily cooled in ice-water, centrifuged at 4 C, and assayed for lytic activity at different times. Enzyme from the logarithmic phase of growth was less labile than that from the period of linear growth. The half-life at 4 C was in both cases less than six hr. Samples from a continuous culture possessed better stability with a half-life of 24 to 36 hr. The activity in similar crude preparations was labile to storage at −20 C, and repeated freezings and thawings inactivated the enzyme. The stability of the bacteriolytic activity obtained from the early stationary phase varied depending on the culture conditions. In cultures
with glucose as the main energy source, the inactivation of the enzyme in the supernatant was very rapid; the half-life at 4 and 37°C was less than 1 hr.

Distribution of bacteriolytic activity. To estimate the proportion of cell bound and extracellular lytic activity, samples were removed at intervals from cultures of strain M18, and the washed bacteria were disintegrated. None of the methods tested released from whole cells or cell walls more than a few percent of the total extracellular activity. No activity was detected in the cytoplasmic fraction. The supernatants of autolysates after centrifugation (10,000 g, 30 min) contained less than 1% of the lytic activity found in the supernatant from the same culture volume. Thus, the bacteriolytic activity seems to be due to extracellular enzymes by the definition of Pollock (22).

DISCUSSION

No systematic study of the influence of the medium composition on the yield of bacteriolytic enzymes from *S. aureus* has been reported earlier. Defined media, according to Gladstone (10) and Mah et al. (19), gave low yields of cells and bacteriolytic activity in spite of the fact that bacteriolytic activity has been reported to be produced in such media (1, 26). To increase the enzyme yields, different media were tested. This study shows the importance of the choice strain, medium composition, and cultivation conditions to obtain a yield high enough to permit purification of the unstable extracellular bacteriolytic enzymes. A continuous culture process, which was found satisfactory for production of other extracellular staphylococcal proteins (Holme and Wadström, unpublished data), was found to be superior to a similar batch process. In the former process, a considerable increase in the yield was obtained with a modified CCY medium (CCY1), whereas none of a number of other extracellular toxins or enzymes studied from strain M18, Wood 46, or V8 increased. Owing to these findings, the conditions for the formation of high bacteriolytic activity were investigated more extensively.

The endo-β-N-acetylmuramidase with an isoelectric point of 9.5 is one of two or more extracellular bacteriolytic enzymes of strain M18 which lyse whole cells of *M. lysodeikticus*. The partially purified glucosaminidase also contains a cell wall-degrading amylase and probably also a staphyloendopeptidase (Wadström and Hisatsune, unpublished data) which are bacteriolytic enzymes previously reported to occur in *S. aureus* (10, 33, 34). The presence of so many enzymes complicates the interpretation of results obtained in cultures grown under different physiological conditions. However, the amidase and peptidase activities were low compared with the activity of the hexosaminidase(s) when assayed against *M. lysodeikticus*.

Bacteriolytic enzymes are probably involved in cell wall synthesis and autolysis (28, 33). Therefore, the need for these enzymes should be greatest during the exponential growth phase. An autolytic muramidase from *Streptococcus faecalis* has been separated from cell wall polysaccharides by ion-exchange chromatography. The purified enzyme proved to be much more labile than the cell-bound activity (29). It has previously been proposed that bacteriolytic enzymes are labile to be rapidly inactivated after having fulfilled their physiological function (28).

The bacteriolytic activity of *S. aureus* both in crude and purified state is highly unstable, which has delayed the development of a successful purification procedure. Incubation of crude culture supernatant at different temperatures verified the instability of these enzymes. The rapid decline of activity during the last part of active growth shows that it was also very unstable in the culture. The appearance of inhibitors of the bacteriolytic activity during the period of a high rate of autolysis (Fig. 2) might also be responsible for this rapid decrease in activity. Recently, Pryme, Joner, and Jensen showed that an acidic inhibitor of T2 and T4 Lysozyme was of mucopolysaccharide origin and could be separated from the enzyme by chromatography on a chitin column (25, 26). Fractions with low isoelectric points (pI 3 to 5), obtained from crude culture supernatants of strain M18 by isoelectric focusing, were found to
inhibit the separated endo-β-N-acetylglucosaminidase (pl 9.5). Resistance to digestion with nucleases and several proteases and to boiling indicates that these inhibitors might also be polysaccharides, probably derived from autolysed cells.

The rate of autolysis in cell samples obtained from the continuous culture was lower than in cells from batch cultures. The amount of PCA precipitable nucleotides was also lower than in samples from batch cultures grown to the same bacterial density. Thus, it is possible that the increased enzyme yield in the chemostat could be explained by a smaller amount of inhibitor(s) than in batch cultures. The higher yield could also be due to an increased stability of the extracellular bacteriolytic enzymes formed in the continuous cultures. In the crude culture supernatants, the bacteriolytic activity was much more stable upon storage at different temperatures than this activity from batch cultures.

Altered conditions for aeration and the choice of carbon source were previously found to be important for production of extracellular bacteriolytic enzymes. Sudden removal of oxygen from aerated log-phase cultures probably activated the autolysins of B. subtilis (17) and B. megaterium (18), whereas violent foaming rapidly destroyed the extracellular bacteriolytic activity of B. subtilis (25). Active aeration of cultures of Escherichia coli was recently shown to cause the culture to terminate in extensive lysis when grown on glucose-based media, whereas there was almost no lysis in lactic acid-based media (14).

The maximal yield in bacteriolytic activity in cultures of S. aureus was always found during the later part of the oxygen-limited, linear growth period. The decrease in activity was found to be most rapid in a medium containing glucose and aerated with 40% oxygen. The rate of growth and autolysis were more rapid in these cultures, and the formation of foam was also pronounced. These results support the assumption that the decrease of the accumulated activity was caused both by denaturation of the enzymes in the culture and the appearance of heat-stable acidic inhibitors released by autolysis.

Production by a short-term continuous process permitted purification of an unstable bacteriolytic endo-β-N-acetylglucosaminidase. This process was suitable for production of several extracellular toxins and enzymes by three different strains of S. aureus (Holme and Wadström, unpublished data). Constant levels were obtained after 4 hr for bacterial dry weight and for all the activities studied except hyaluronate lyase and lipase.

However, none of all these proteins showed the peculiar increase during the first hours after the continuous feed was started. The conditions chosen for continuous culture gave a cell density of 6 to 7 g/liter (dry weight) which was close to the maximal value for cell production when oxygen was the growth limiting factor (Holme and Wadström, unpublished data). Limitation of growth by a nutrient factor in the medium was not easily applied, since a defined medium was not adequate for the production of bacteriolytic enzymes.

Compared with production in batch culture, the continuous culture produced an increased yield, a better stability of the crude bacteriolytic activity, and a process easier to scale-up. The holding time in the continuous culture for all the components of the culture system is constant but dependent on the dilution rate, whereas in batch culture more than half of the enzymes accumulate during the last hour of production. Production by a continuous process might thus also be interesting in a more general aspect for other unstable bacterial proteins.

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LITERATURE CITED


