Lytic Enzyme Produced by *Myxococcus xanthus*

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ABSTRACT

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Lytic enzyme produced by *Myxococcus xanthus*. J. Bacteriol. 92:1632–1637. 1966.—Strain FBa of *Myxococcus xanthus* releases into its culture medium an enzyme capable of lysing *Micrococcus lysodeikticus* cells and of releasing N-acetyl amino sugars from their cell walls. The lystin is stable at pH values near neutrality and at temperatures below 50 C. It lyzes a number of bacterial species sensitive to egg-white lysozyme, and fails to lyse lysozyme-resistant species. Sensitivity of *M. lysodeikticus* cell walls to FBa lysein and to lysozyme is changed in a similar manner by addition and removal of O-acetyl groups. We describe methods for obtaining 190-fold purification of the lysein from culture filtrates, using acetone and ammonium sulfate precipitations and filtration through diethylaminoethyl cellulose. We also describe a gel filtration method for separating the lysein from a protease which is also produced by strain FBa.

During the past few years, there have been a number of reports on enzymes, elaborated by myxobacteria, that cause the lysis of various microorganisms. Bender (1) characterized a lytic enzyme produced by a strain of *Myxococcus xanthus*. He reported that this lysein did not cause the release of N-acetyl amino sugars from substrate cell walls, and he concluded that it did not have a lysozyme-like activity. Our report describes a lytic enzyme produced by another strain of *M. xanthus* which acts differently from the enzyme studied by Bender. We also discuss the origin of the lysein, conditions for its optimal production, a spectrum of its lytic activity against various microorganisms, and procedures for its partial purification.

MATERIALS AND METHODS

Organism. *M. xanthus* strain FBa was selected in this laboratory from strain FB, originally obtained from Martin Dworkin. It is distinguished by its ability to form fruiting bodies more readily than strain FB on synthetic media.

Medium. Cultures were grown in Casitone broth made of 20 g of Casitone (Difco), 1.515 g of K2HPO4, 0.174 g of KH2PO4, and 1 liter of distilled water. The generation time of FBa in this medium was about 5 hr. Stock cultures were grown on Casitone-agar slants, consisting of Casitone broth solidified with 1.5% agar (Difco). The stock cultures were incubated at 30 C for 36 hr and then stored at 4 C; fresh transfers were made weekly. All incubations were carried out at 30 C. Unless otherwise stated, the buffer used throughout was 0.025 M sodium phosphate at pH 7.5.

Preparation of culture filtrate. A 250-ml Erlenmeyer flask with 25 ml of Casitone broth was inoculated with a loopful of cells from a 36-hr slant. The flask was shaken for 24 hr on a reciprocal shaker. Then a series of 250-ml flasks, each containing 25 ml of broth, was inoculated with 1 ml of the starting culture. After 24 hr of incubation, these cultures were used to inoculate 2-liter flasks, each containing 250 ml of Casitone broth. Each 2-liter flask was inoculated with enough of a 24-hr culture to bring its optical density to 0.10, as measured with a Bausch & Lomb Spectronic-20 colorimeter at 570 μm. This optical density corresponds to about 7 x 10⁷ cells per milliliter. The flasks were incubated on a reciprocal shaker for 26 to 28 hr. The *M. xanthus* cells were then removed by centrifugation at 14,500 x g for 60 min at 4 C. The growth liquor was decanted and filtered by passing it first through a membrane filter (Millipore Filter Corp., Bedford, Mass.) with a pore size of 1.2 μ and then through one with a pore size of 0.45 μ. The filtered material was stored at 4 C or —15 C.

Enzyme preparation for gel filtration. The enzyme preparations used in experiments involving gel filtration were prepared in the following manner. The lytic agent in a culture filtrate was precipitated by the addition of solid ammonium sulfate to give 45% saturation. The precipitate was allowed to settle overnight at 4 C. It was removed by passing the suspension through a pad of Celite and Whatman no. 1 filter paper in a large Büchner funnel. It was then extracted from the pad with buffer. A fivefold concentration of this extract was achieved by exposure to Carbowax 4000 (Union Carbide Corp., New York, N.Y.) in a
cellulose acetate sac at 4 C. The concentrated material was then dialyzed against buffer at 4 C for 36 hr.

Assay procedures. Except where specifically mentioned, assays of lytic enzyme activity were performed with crude culture filtrates by use of suspensions of ultraviolet-irradiated, lyophilized Micrococcus lysodeikticus (Difco) as substrate. A stock suspension, containing 2 mg/ml of substrate in distilled water, was prepared and stored at 4 C. For the assay, 1.0 ml of enzyme preparation was mixed with 0.5 ml of a distilled water dilution of the stock substrate suspension (1:3) in a 1-cm absorption cell. This gave an optical density of 0.65 at 650 μm. The decrease in turbidity in the cuvette was measured in a Beckman model DU spectrophotometer, equipped with a Gilford model 210 automatic cuvette positioner. The resultant decline in absorbance was followed on a Hewlett-Packard model 153 X 58 Electronik Recorder for a 15-min period. Optical density was recorded every 5 sec at a chart speed of 30 inches per hr. The optical density usually decreased linearly with time, until a very low optical density was reached. Occasionally, when very low enzyme concentrations were used, an initial lag in lysis was observed. For this reason, the graph was examined during the 5-min interval when the greatest decline in optical density was recorded. One unit of lytic activity was defined as a change in absorbance of 0.01 at 650 μm in 5 min at 45 C. Assays were made at pH 7.5, unless otherwise noted. The number of units thus measured was directly proportional to enzyme concentration, at least when the limits were 5 and 50 units/ml. Highly active preparations were diluted with buffer. Because of the dilution with water-suspended substrate, the sodium phosphate buffer concentration in the cuvette was 0.0167 m.

The protease assay substrate was a 2% solution of denatured hemoglobin (Nutritional Biochemicals Corp., Cleveland, Ohio) which had been dialyzed exhaustively against buffer. Samples (1 ml) of enzyme and 0.5-ml amounts of substrate were used in the assay system described by Herriot (8). One unit of protease activity was defined as the release of 10⁻⁴ meq of trichloroacetic acid-soluble tyrosine from 0.5 ml of hemoglobin solution, in 20 min, per ml of enzyme. All assays were performed at 40 C.

Protein was determined by the method of Lowry et al. (9). Specific activity of protease or lysis is defined as the number of units per milligram of protein.

Determinations of free amino group and N-acetyl amino sugar were made according to the procedures of Ghuysen and Strominger (6). In N-acetyl amino sugar determinations, experimental samples were heated at 100 C in sodium borate buffer for 30 min. Cell walls of M. lysodeikticus prepared by the method of Park and Hancock (10) were used for these assays.

Results

Origin of the lytic activity. A flask containing 100 ml of Casitone broth was inoculated with enough cells of strain FBa to give an initial concentration of 7 x 10⁵ cells per milliliter. After 28 hr of incubation on a reciprocal shaker, samples of the culture (with about 10⁹ cells per milliliter) were subjected to various treatments to determine whether the lytic activity was cell-bound. One 25-ml culture sample was subjected to sonic treatment for 10 min at 4 C at 2.5 amp with a Branson Sonifier equipped with a microprobe. This treatment was sufficient to disrupt all of the vegetative cells of strain FBa. The sonic-treated culture was dialyzed against buffer for 24 hr at 4 C. In another 25-ml sample, the cells were removed by centrifugation at 34,800 x g for 15 min at 4 C. The cells were resuspended in 5 ml of buffer, and both the cell suspension and the supernatant fraction from the centrifugation were subjected to the sonic treatment. After dialysis against buffer, lysis assays were made. No lysis was released from sonic-treated cells; sonic-treated cultures (cells plus supernatant fluid) had the same lytic activity as the supernatant fraction alone. Sonic treatment did not affect the lysis under the conditions used. We thus concluded that the lysis was extracellular, and that no appreciable amount of it was contained within the cells.

Conditions for optimal lysis production. Lysin production paralleled the growth cycle. The greatest enzymatic activity was in the latter portion of the exponential phase and in the beginning of the stationary phase of growth. For maximal production of the lysin, a starting cell concentration of 7 x 10⁸ cells per milliliter of a 24-hr culture was used. Reduction of the Casitone content from 2 to 1% caused a marked decrease in lysis formation. The addition of substrate, in the form of 0.4 mg/ml of ultraviolet-irradiated M. lysodeikticus cells, to the Casitone broth at either concentration did not cause a stimulation of lysis production.

Relationship of proteolytic activity to lytic activity. The culture filtrate possessed proteolytic activity as well as lytic activity, since it was able to catalyze the release of trichloroacetic acid-soluble tyrosine from denatured hemoglobin. It was possible that lysis of cells and protein breakdown were catalyzed by a single enzyme with broad activity. However, strong evidence of the presence of two distinct enzymes was obtained when both lytic and proteolytic activities were determined throughout the following purification scheme.

Fivefold concentration of the culture filtrate was achieved by placing the fluid in cellulose acetate sacs and exposing the entire surface of the sacs to Carbowax 4000 for 8 to 12 hr at 4 C. The concentrated material was then dialyzed exhaustively against buffer at 4 C.

The concentrated, dialyzed preparation was subjected to acetone precipitation. First, 1.5 volumes of acetone, precooled to −15 C, was added slowly with vigorous stirring. After 60 min, the
precipitate was removed by passing the suspension through a pad of Celite and Whatman no. 1 filter paper in a large Büchner funnel. The precipitate on the pad, which contained most of the lytic activity, was dissolved in cold buffer.

The active fraction obtained from the acetone treatment was precipitated by the addition of solid ammonium sulfate to give 45% saturation. The precipitate was allowed to stand overnight at 4 C for optimal settling. It was then removed by centrifugation at 34,800 × g for 30 min. The precipitate was dissolved in buffer and dialyzed against the same buffer for 24 hr at 4 C.

The dialyzed solution of enzyme was then allowed to pass by gravity filtration at 4 C through a pad of diethylaminoethyl (DEAE)-cellulose and Whatman no. 1 filter paper on a small Büchner funnel. (The DEAE-cellulose had been previously equilibrated with buffer, and excess moisture had been removed by suction on a Büchner funnel.) After 30 min, the enzyme solution remaining on the pad was drawn through by suction. Some residual enzyme was washed from the pad by passing 1 volume of buffer through it. The solutions which had passed through the pad were pooled to give the partially purified enzyme preparation.

A summary of the overall purification procedure is given in Table 1. A 190- to 200-fold purification of the lysin was realized. Because the various fractions in the purification scheme contained different ratios of proteolytic and lytic activities (see Table 1), we concluded that two separate enzymes were involved.

Separation of the enzymes. A 1-ml amount of partially purified concentrated enzyme (see Materials and Methods) containing 10 mg/ml of protein was placed on a Bio-Gel-P 60 (Calbio-chem) column (2.5 × 25 cm), which had previously been equilibrated with buffer. By means of continuous elution of protein from the column, separation of the two enzymatic activities was achieved (Fig. 1). Fractions 2 and 3 from this separation were pooled and used for tests in which it was desirable to have protease activity uncontaminated by lysin. Similarly, fractions 12 to 18 were pooled and used for tests requiring lysin free from proteolytic activity.

Heat inactivation of the lysin. In experiments designed to test the effect of temperature on the FBa lysin, the test system was maintained at various temperatures over a range of 25 to 70 C. The lysin displayed maximal activity at 55 C and virtually complete inactivity at 65 and 70 C. Heating enzyme preparations at 50 C for 10 min prior to assaying caused loss of about 15% of the activity; treatment at 60 C for 10 min caused loss of more than 90% of the activity.

Effect of pH on the lysin. The FBa lysin was shown to have measurable activity between pH 4 and 10; maximal activity was observed at pH 7.5. In other experiments, enzyme preparations were adjusted to various pH values for 10 min and then readjusted to a pH of 7.5. All of the original activity was regained after 10-min exposures to pH values between 6 and 8.

Spectrum of lytic activity. A highly active culture filtrate was tested against cell suspensions of various living microorganisms to obtain a spectrum of lytic activity. All the organisms tested were obtained from the Cornell University Stock Culture Collection. They had been kept on nutrient agar slants and were subcultured three times prior to testing on a medium having the following composition (% w/v) in distilled water.

**Table 1. Lysin and protease activities during purification**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Type of assay</th>
<th>Activity (units/ml)</th>
<th>Total activity (units)</th>
<th>Specific activitya</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Carbowax exposure</td>
<td>L</td>
<td>340</td>
<td>120,000</td>
<td>71</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>79</td>
<td>27,720</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>After acetone (1.5 vol) precipitation</td>
<td>L</td>
<td>1,125</td>
<td>79,000</td>
<td>662</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>88</td>
<td>6,300</td>
<td>52</td>
<td>3</td>
</tr>
<tr>
<td>After (NH₄)₂SO₄, 45%, precipitation</td>
<td>L</td>
<td>1,375</td>
<td>48,000</td>
<td>4,910</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>122</td>
<td>4,284</td>
<td>437</td>
<td>27</td>
</tr>
<tr>
<td>After diethylaminoethyl cellulose filtration</td>
<td>L</td>
<td>1,100</td>
<td>21,750</td>
<td>13,750</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>22</td>
<td>428</td>
<td>270</td>
<td>17</td>
</tr>
</tbody>
</table>

* Units of activity per milligram of protein.
* L = lytic activity. One unit of lytic activity is defined as a change in absorbancy of 0.01 at 650 μm in 5 min at 45 C.
* P = protease activity. One unit of protease activity is defined as 10⁻⁶ meq of trichloroacetic acid-soluble tyrosine released from 0.5 ml of hemoglobin solution in 20 min at 40 C.
water: peptone, 1.0; yeast extract, 0.5; NaCl, 0.5; glucose, 1.0. The pH of the medium was adjusted to 7.5. The organisms were all harvested in the exponential phase of growth. The cells were washed three times in buffer and then tested for susceptibility to lysis. The results are summarized in Table 2. All of the gram-negative organisms tested were resistant to lysis. Among the gram-positive bacteria tested, only those sensitive to egg-white lysozyme were susceptible to the FBa lysin. The one species of yeast tested was resistant. Lysozyme free from protease activity, obtained from the gel filtration separation, was tested against representative species used in the determination of the lytic spectrum. The results were identical to those recorded when culture filtrates were employed.

Effect of O-acetylation of M. lysodeikticus cell walls. Brumfit et al. (2) reported that the lysozyme sensitivity of M. lysodeikticus cell walls could be altered by changing their O-acetyl content. To test the effect of this treatment on the sensitivity of M. lysodeikticus to the FBa lysin, commercially prepared M. lysodeikticus cells were acetylated with acetic anhydride and pyridine for 2 hr at 37°C and washed according to the methods of Brumfit et al. Then an assay for lytic activity was run with these acetylated cells as substrate, and with either FBa lysin (free from proteolytic activity) from the gel filtration separation, or egg-white lysozyme, as enzyme. Untreated cells were used as a control. The acetylated cells were completely resistant to the action of 50 units of lysin or of lysozyme. Complete susceptibility of the M. lysodeikticus cells to both enzymes was restored by the removal of the O-acetyl groups with glycine-NaOH (pH 10.6), followed by repeated washing (2).

In an earlier attempt to acetylate M. lysodeikticus cells, the treatment with acetic anhydride-pyridine had been for only 1 hr. A lysin preparation, containing 49 units of activity when measured against untreated cells, gave 16 units of activity against these partially acetylated cells—a reduction of 67% in the cells' sensitivity to lysin. A preparation of commercial egg-white lysozyme, containing 54 units of lytic activity against untreated M. lysodeikticus cells, showed 16 units of activity when tested against the partially acetylated cells—a reduction in cell sensitivity of 70%. Partial removal of the O-acetyl groups from these cells restored their sensitivity to lysin and to lysozyme to similar extents: the lysin preparation gave 29 units of activity against the partially decorolized cells; the lysozyme preparation gave 38 units of activity. It appears that the addition and removal of O-acetyl groups changes the sensitivity of M. lysodeikticus to lysin and to lysozyme to similar extents.

Substrate specificity of the lysin. The release of N-acetyl amino sugars and free amino groups during the solubilization of M. lysodeikticus cell walls by the FBa lysin, obtained from the gel filtration separation, was followed over a 3-hr period with 120 units of lysin and 1 mg of cell walls. N-acetyl amino sugars were released from the substrate cell walls at a rapid linear rate for 2 hr, followed by a leveling off; 60 mmol of N-acetyl sugars per mg from

### Table 2. Spectrum of lytic activity

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lytic activity* (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micrococcus lysodeikticus</td>
<td>72</td>
</tr>
<tr>
<td>Sarcina lutea</td>
<td>48</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>38</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Saccharomyces cerevisae</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as a change in absorbancy of 0.01 at 650 mµ in 5 min at 45°C.
this batch of cell walls.) No free amino groups were liberated from the cell walls by the lysin. From these data, we conclude that the FBa lysin acts by cleaving the polysaccharide "backbone" of the cell walls.

Substrate specificity of the protease. The protease, free from lysin activity, hydrolyzed denatured hemoglobin, but it was unable to cause the release of free amino groups or of N-acetyl amino sugars from _M. lysodeikticus_ cell wall preparations. It therefore is unlikely that the protease plays any role in cell wall lysis.

**DISCUSSION**

The present study was undertaken to examine the nature of the enzyme produced by _Myxococcus xanthus_ FBa, which lyses living _Micrococcus lysodeikticus_ and causes a reduction in the turbidity of their isolated cell walls. However, the presence of proteolytic activity in the filtrate of FBa cultures made it necessary to determine its relationship to the lysin.

Purification data indicated the presence of two distinct enzymes which were subsequently separated by gel filtration. The FBa lysin solubilizes cell walls by cleaving the polysaccharide "backbone"; its enzymatic action on walls results in the release of _N_-acetyl amino sugars. The protease had no effect on cell walls. It thus can be concluded that the lysis of some microorganisms by _M. xanthus_ FBa is the result of an enzyme which lyses their cell walls, and that the protease plays no part in this phenomenon.

In nature myxobacteria seem to be predatory on eubacteria. It is known (3, and studies in our laboratory) that strain FBa can grow in a medium containing only amino acids and salts. At least eight amino acids are required for growth, and several others are stimulatory. We can, therefore, speculate that the role of the lysin in nature is to attack the cell walls of sensitive bacteria, and that the role of the protease is to digest cell proteins of host bacteria into small peptides and amino acids, which can then diffuse back to the myxobacterium.

The spectrum of lytic activity of the FBa lysin was found to be very similar to that of egg-white lysozyme. Only gram-positive microorganisms were attacked. _Staphylococcus aureus_, _Streptococcus faecalis_, and all gram-negative organisms tested were resistant to the actions of lysozyme and the FBa lysin.

There are obviously many features which determine the sensitivity of cell walls to enzymes capable of cleaving the polysaccharide moiety. In an examination of a lysozyme-resistant mutant of _M. lysodeikticus_, Brumfitt et al. (2) found that resistance was due to the presence of about one _O_-acetyl group for each muramic acid residue in the cell walls of the mutant. The lysozyme-sensitive, parental strain had fewer than 1% as many _O_-acetyl groups in its cell walls. Brumfitt and his co-workers showed that sensitive cell walls of _M. lysodeikticus_ could be rendered resistant to lysozyme by acetylation, and made sensitive again by removal of the _O_-acetyl groups with alkali. We have found that _O_-acylated _M. lysodeikticus_ cells are also resistant to FBa lysin, and that they regain sensitivity upon removal of these acetyl groups, in a manner parallel to egg-white lysozyme.

Bacteriolytic enzymes have been isolated from culture filtrates of a variety of myxobacteria. The FBa lysin appears to be different from those that have been well characterized.

Bender (1) studied a lytic enzyme produced by another strain of _M. xanthus_. Unlike the FBa lysin, this enzyme does not release _N_-acetyl amino sugars from substrate cell walls. Bender described his lytic enzyme as an amidase on the basis of its release of alanine, glutamic acid, and lysine from _Bacillus subtilis_ cell walls. Unfortunately, his lytic enzyme preparation was contaminated with proteolytic activity, making it difficult to draw conclusions from his data.

An enzyme described by Ensigh and Wolfe (4, 5), from an unidentified nonfruitering myxobacterium, hydrolyzed both proteins and cell walls of _M. lysodeikticus_, _Arthrobacter_ sp., _Corynebacte-
rium diphtheriae, and Staphylococcus aureus. The enzyme was reported to solubilize cell walls by hydrolyzing peptide bonds; purified preparations were strongly proteolytic. This enzyme is clearly different from the FBA lysin, since the FBA lysin attacked the polysaccharide "backbone" of the cell wall, not the peptide moiety, and since it did not show any proteolytic activity.

A lytic system produced by Sorangium sp., described by Gillespie and Cook (7), was shown to act on isolated cell walls of Arthrobacter globiformis. Substances giving the reactions of amino sugars were release from the walls as the result of enzymatic action. However, this enzyme did not lyse M. lysodeikticus, a fact which suggests important differences between it and lysozyme-like enzymes.

ACKNOWLEDGMENTS

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LITERATURE CITED


