Purification of a Streptococcal Bacteriocin (Viridin B) and Its Separation from Alpha-Hemolysin

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Viridin B, a bacteriocin produced by *Streptococcus mitis*, copurified with the alpha-hemolysin after ammonium sulfate precipitation and gel filtration on Sephadex G-200. The bacteriocin and hemolysin were separated in some instances by ion-exchange chromatography on diethylaminoethyl-Sephadex A-50, but the two substances were shown to be distinct after polyacrylamide gel electrophoresis. Attempts at recovery of nonhemolytic or nonbacteriocinogenic mutants were unsuccessful after exposure to mutagenic agents. The molecular weight of viridin B was determined to be approximately 87,000.

Viridin B, a bacteriocin produced by *Streptococcus mitis* (*S. mitior*) strain 42885, has been described previously (3-6). The earlier studies were done by using a partially purified preparation which contained bactericidal and hemolytic activities (5). Both activities coexisted in successive purification steps.

Brock and Davie (2) described a bacteriocin from *Streptococcus faecalis* subsp. *zymogenes* strain X-14 which had hemolysin activity associated with the bactericidal activity. They used genetic and physiological evidence to show that the hemolysin was probably the same molecule as the bacteriocin.

We investigated whether the hemolytic and bactericidal activities from *S. mitis* strain 42885 were identical or distinct products. Data are presented in this report which show that the two activities are the result of two separate proteins. Viridin B was highly purified, and its molecular weight was determined.

MATERIALS AND METHODS

Bacterial strains. *S. mitis* (*S. mitior*) strain 42885, which produces viridin B, and *Neisseria sicca* strain 15362, which is killed by viridin B, have been previously described in detail (4).

Media and chemicals. Both *S. mitis* and *N. sicca* were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). Phosphate buffer (0.067 M; Na_2HPO_4 plus KH_2PO_4) was used at pH 7.2. Phosphate-buffered saline contained 0.01 M sodium phosphate and 0.145 M NaCl at pH 6.8. Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) was suspended in phosphate buffer, packed into a column (1.5 by 90 cm) at 4°C, and stored at that temperature. Diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia Fine Chemicals) was suspended in 0.067 M ethylenedia-

minetetraacetic acid, pH 7.0. A column (1.0 by 30 cm) was packed and stored at 4°C.

Bacteriocin preparation. *S. mitis* was grown at 37°C for 24 to 48 h. Cells from 6 liters of broth were collected by centrifugation at $2,500 \times g$ for 20 min, washed in phosphate buffer, and resuspended in 50 ml of the same buffer. The cells were then stirred vigorously at 4°C on a magnetic stirring platform for 24 to 48 h. The mixture was centrifuged at $13,000 \times g$ for 90 min. The supernatant fluid was brought to 80% ammonium sulfate saturation and then centrifuged at $13,000 \times g$ for 60 min. The resulting precipitate was dissolved in 25 ml of phosphate buffer, dialyzed for 18 h at 4°C against the same buffer, and sterilized by filtration through a 0.45- μm membrane filter (Millipore Corp.). Such preparations had both bacteriocin and hemolysin activities and were stored at -20°C until used.

Mutagenesis. The mutagenic agents used were 1-methyl-3-nitro-1-nitrosoguanidine at 100 to 500 $\mu\text{g}/\text{ml}$ (8), 1 M nitrous acid (11), and ultraviolet light at a dose allowing approximately 1% survival. *S. mitis* cells grown in broth to a logarithmic phase were exposed to the various mutagenic agents. Cells were then subcultured onto sheep blood agar plates. After 18 to 24 h of incubation, colonies were screened for alpha-hemolytic activity. For each mutagenic agent 1,000 to 10,000 colonies were screened. Similarly, 100 to 200 colonies were tested for bacteriocin production after exposure to each mutagen by using the simultaneous antagonism technique (4).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (10). Sodium dodecyl sulfate and β -mercaptoethanol were omitted from the gels and buffer when assay for the bacteriocin was made, because both substances were inhibitory to the indicator strain. Generally, gels with a final acrylamide concentration of 5% were used with 0.1 M sodium phosphate, pH 7.2, as the electrophoresis buffer. Samples (5 to 50 μl and containing 25 to 100 μg of protein)

of the bacteriocin preparations at each stage of purification were tested. Electrophoresis was carried out at room temperature for 4 h. Gels were stained with Coomassie brilliant blue (Bio-Rad Laboratories, Richmond, Calif.) or sliced into 1.5-mm slices and tested for bacteriocin or hemolysin activity as described below.

Bacteriocin assay. Viridin B was assayed after polyacrylamide gel electrophoresis by slicing the gel into 1.5-mm sections. The slices were placed on a lawn of *N. sicca* strain 15362, and the plates were incubated at 37°C. A clear zone of inhibition around the gel slice indicated the presence of viridin B. Column chromatography fractions were assayed for viridin B activity, and arbitrary units were determined as described earlier (4).

Hemolysin assay. The hemolysin assay was a modification of that reported by Basinger and Jackson (1). Human erythrocytes were washed three times with phosphate-buffered saline and suspended at 0.12% (vol/vol) in phosphate-buffered saline. Gel slices, as described above, were each placed into 0.8 ml of erythrocyte suspension. The samples were incubated for 18 h at 37°C and then centrifuged at $3,000 \times g$ for 3 min. The supernatant fluid was read at 541 nm to detect released hemoglobin. Fractions after DEAE-Sephadex chromatography were also assayed for hemolysin activity. A 0.5-ml portion of each fraction was dialyzed against phosphate-buffered saline for 4 h, mixed with an equal volume of erythrocyte suspension, and incubated at 37°C for 30 min. The samples were then centrifuged, and the supernatant fluid was read at 541 nm. Sephadex G-200 fractions were assayed for hemolysin activity by an identical procedure. A hemolytic unit is the reciprocal of the highest dilution contained in 1 ml of a preparation producing detectable hemolysis (i.e., an optical density reading of ≥ 0.1 at 541 nm).

Protein determination. Protein content after each purification step was determined by the method of Lowry et al. (7).

RESULTS

No nonhemolytic or nonbacteriocinogenic mutants could be detected after exposure of *S. mitis* to the various mutagens. Therefore, physicochemical methods were used to separate the hemolysin and the bacteriocin.

Chromatographic purification of viridin B. The 80% ammonium sulfate precipitate containing bactericidal and hemolytic activity was further purified by column chromatography on a Sephadex G-200 column (Fig. 1). The bactericidal activity was detected in most fractions showing hemolysis. Both activities were separated, however, from a sizable portion of extraneous protein. Fractions showing hemolytic and bactericidal activities (fractions 33 through 42) were pooled and lyophilized.

Viridin B was further purified by chromatography of the pooled fractions from the Sephadex

G-200 column on DEAE-Sephadex A-50 (Fig. 2). The bactericidal activity occurred in a single peak. Hemolysin activity was present in the same fractions as the bactericidal activity in

FIG. 1. Gel filtration on Sephadex G-200 of the 80% ammonium sulfate precipitate. Protein content (●), hemolytic activity (○), and bacteriocin activity (shaded area) were determined in each fraction (1.5-ml samples). O.D., Optical density; A.U., arbitrary units.

FIG. 2. Ion-exchange chromatography (DEAE-Sephadex A-50) of pooled fractions after Sephadex G-200 gel filtration. A continuous ionic-strength gradient of 0.5 to 1.0 M NaCl was used (vertical arrow). Each fraction (1.0-ml samples) was assayed for protein content (●) and bacteriocin activity (shaded area). O.D., Optical density; A.U., arbitrary units.

approximately one-half of the chromatographs. In those instances where no hemolysin activity was associated with the bactericidal activity, the hemolysin could not be recovered from any fraction and could not be eluted off the column. The fractions (Fig. 2) containing bacteriocin activity were pooled and lyophilized.

A summary of the purification steps of a preparation containing viridin B and hemolysin is given in Table 1.

Electrophoretic separation of hemolytic and bactericidal activities. Preparations after the various purification steps were placed on three parallel polyacrylamide gels not containing sodium dodecyl sulfate or β -mercaptoethanol. After electrophoresis, one gel was used for bacteriocin assay, another gel was used for hemolysin assay, and the third gel was stained for protein. When 15-mm slices from a gel were placed on the surface of a tryptic soy agar plate previously seeded with *N. sicca*, the bacteriocin could be detected in one slice (Fig. 3). The hemolytic activity could also be detected in one or two slices from another gel. In a typical electrophoresis, the hemolysin was detected 18 mm from the origin, whereas the bacteriocin was located 15 mm further. Figure 4 shows polyacrylamide gel electrophoresis patterns for three preparations after different purification procedures. It is evident that the bacteriocin and hemolysin are two distinct moieties.

The molecular weight of viridin B after DEAE-Sephadex A-50 chromatography was determined to be $87,000 \pm 3,000$ by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (10). The protein markers used were human immunoglobulin G (molecular weight, 160,000), bovine serum albumin (68,000), the H chain of immunoglobulin G (50,000), and the L chain of immunoglobulin G (22,000).

DISCUSSION

Since the identity of a bacteriocin with a hemolysin has been suggested in a strain of *Streptococcus faecalis* (2), it was of interest to clarify such a possible relation for our strain. In the

present study, the bacteriocin and hemolysin produced by *S. mitis* strain 42885 were shown to be two distinct products. Although the two substances copurified after ammonium sulfate precipitation and gel filtration on Sephadex G-200, the bacteriocin and hemolysin were separated by polyacrylamide gel electrophoresis.

Attempts at finding nonhemolytic mutants or mutants that do not produce the bacteriocin were unsuccessful. Several mutagenic agents were used. It is possible that the rate of mutation is very low and that screening of even larger numbers of cells may have resulted in selection of an appropriate mutant. Alternately, *S. mitis* strain 42885 may have a very stable genome, or the control of the hemolysin and bacteriocin may be by extrachromosomal factors. Further exploration of these possibilities may be warranted.

The molecular weights of bacteriocins of gram-positive bacteria are quite variable and range from 8,000 to more than 4.0×10^7 (9).

FIG. 3. Demonstration of bacteriocin activity after polyacrylamide gel electrophoresis. Slices (1.5 mm each) were placed on a lawn of indicator strain. Inhibition can be seen surrounding slice 18 but not around adjacent slices.

TABLE 1. Steps in viridin B and hemolysin purifications

Procedure	Protein (mg/ml)	Viridin B		Hemolysin	
		Arbitrary units	Sp act ^a	Hemolytic units	Sp act ^b
Ammonium sulfate (80%)	3.80	160	42	256	67
Sephadex G-200	0.33	20	61	64	194
DEAE-Sephadex	0.06	20	333	32	540 ^c

^a Arbitrary units per milligram of protein.

^b Hemolytic units per milligram of protein.

^c Hemolysin was present after DEAE-Sephadex chromatography in only one-half of the runs.

FIG. 4. Polyacrylamide gel electrophoresis of preparations at various purification steps, showing bacteriocin (B) and hemolysin (H) activities from the origin (O). Gel 1 is that of the 80% ammonium sulfate precipitate; gel 2 is the preparation after Sephadex G-200 filtration; and gel 3 is after DEAE-Sephadex chromatography.

Viridin B was shown to have a molecular weight of approximately 87,000, placing it at the intermediate position. Compared with bacteriocins of similar molecular weights, viridin B has several unusual properties, including heat lability, narrow pH range of activity, and either a bactericidal or bacteriostatic effect, depending on the indicator strain (3, 4).

Although bacteriocin production has been reported for a large number of bacteria generally

and for streptococci specifically (9), only a few bacteriocins have been highly purified. A major problem in bacteriocin purification is the increasing lability upon increased purification (9). The addition of albumin or other proteins to a bacteriocin preparation during purification steps has enabled increased stability. Viridin B did exhibit marked lability upon purification; however, it was highly purified and was separated from most other extracellular products.

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