ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1979, p. 504-509
0066-4804/79/04-0504/06$02.00/0

Vol. 15, No. 4

Purification, Properties, and Cytotoxic Effect of a Bacteriocin from *Mycobacterium smegmatis*

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Received for publication 4 January 1979

The bacteriocin produced by *Mycobacterium smegmatis* ATCC 14468 was isolated, and a study was made of its chemical, physical, and biological properties. No appreciable bacteriocin activity was found in the culture supernatant fluids, but it was released in appreciable quantities after disruption of the cells. The material was purified 49-fold by means of chromatography on diethylaminoethyl-cellulose, ammonium sulfate fractionation, gel filtration on Sephadex G-200, and chromatography on diethylaminoethyl-Sephadex A-50. Its molecular weight was determined to be approximately 75,000 from the elution profile on Sephadex G-200 chromatography. The bacteriocin was resistant to deoxyribonuclease, ribonuclease, lipase, ultraviolet irradiation, and freeze-thawing, whereas it was relatively less thermostable and was sensitive to proteolytic enzymes. The lethal effect of the bacteriocin was demonstrated by the decrease in viable counts of the bacteriocin-sensitive indicator strain, *M. diernhoferi* ATCC 19340. The bacteriocin preparation inhibited the growth of HeLa-S3 cells.

A wide variety of bacteria produce bacteriocins, which are bactericidal substances of a proteinaceous nature that are usually active against organisms of the same or closely related species. A number of bacteriocins have been purified and characterized (8, 15, 19).

The first report of bacteriocin-like substances from mycobacteria came from Mora and Eisenstark (Bacteriol. Proc., M81, p. 81-82, 1958). Similar findings were reported by Adámek et al. (1) in a study of rapidly growing mycobacteria. After treatment of a mutant of *Mycobacterium bovis* BCG with mitomycin C, Imaeda and Rieber (7) demonstrated phagelike particles which they categorized as bacteriocins. More recently, Takeya and Tokiwa (20, 21) studied bacteriocins from various mycobacteria and reported that they may be useful for both classification and typing of mycobacteria. However, little is known of the chemical, physical, and biological properties of mycobacteriocins.

This report describes the partial purification and characterization of a bacteriocin from *M. smegmatis*.

MATERIALS AND METHODS

Bacterial strains and media. *M. smegmatis* ATCC 14468 and *M. diernhoferi* ATCC 19340 have already been described (16). For production of bacteriocin, *M. smegmatis* ATCC 14468 was grown in heart infusion (HI) broth (Eikenkagaku, Tokyo; supplemented with 0.1% Tween 80 and 4% glycerol). The bacteriocin-sensitive indicator strain, *M. diernhoferi* ATCC 19340, was grown on 1% Ogawa egg medium (14).

Bacteriocin assay. The procedures used for bacteriocin assay were essentially those described by Gagliano and Hinsdill (5) and Hale and Hinsdill (6). A 3-mm loopful of each dilution was spotted onto a plate of HI agar containing 0.1% Tween 80 and 4% glycerol. Each plate was exposed to chloroform vapor and was overlaid with approximately 10^6 indicator cells. After incubation at 37°C for 72 h, the titer of the assayed preparation was recorded in arbitrary units (AU) per milliliter.

Purification of bacteriocin. Aliquots (100 ml) of HI broth in 300-ml flasks were inoculated with 1 ml of a 24-h culture of *M. smegmatis* ATCC 14468 in HI broth. Cells from a 72-h culture with shaking at 120 rpm in a shaking incubator (type FG-4H, Hirayama Manufacturing Corp., Tokyo) were pelleted by centrifugation and washed three times with 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer containing 0.1 M NaCl and 0.02% NaN_3 (pH 7.5). From this point all operations were performed at 0 to 4°C. Packed cells (120 g [wet weight]) were suspended in 350 ml of buffer and then disrupted with an ultrasonic disintegrator (Insonator, model 200M, Kubota Shoji, Tokyo) at 200 W for 20 min. The supernatant fluid (550 ml) was obtained by centrifugation at 15,000 × *g* for 20 min. The sonicate was applied to a diethylaminoethyl (DEAE)-cellulose (Seikagaku Kogyo, Tokyo) column (5 by 40 cm) previously equilibrated with buffer and was eluted from the column with 0.2 to 0.4 M NaCl. Unless otherwise stated, the buffer used was 50 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM 2-mercaptoethanol. The eluent was placed in Visking tubing (36 mm) and concentrated threefold with solid polyethylene glycol

6000. Saturated ammonium sulfate solution was slowly added to the material with constant stirring until 35% saturation was obtained. The resulting precipitate was centrifuged off at 15,000 × *g* for 30 min. The supernatant fluid was placed in Visking tubing and soaked into 85% saturated ammonium sulfate solution with constant stirring until inner fluid reached 85% saturation. The resulting protein precipitate was collected by centrifugation, dissolved in a minimal volume of buffer, and dialyzed overnight against buffer. The dialysate was placed on a Sephadex G-200 (Seikagaku Kogyo) column (2.5 by 75 cm) previously equilibrated with buffer and was eluted with the same buffer at a flow rate of 20 ml/h. Effluent fractions of 10 ml each were collected, and peak fractions (70 ml) of bacteriocin activity were pooled. The sample was applied to a DEAE-Sephadex A-50 (Seikagaku Kogyo) column (2.5 by 60 cm) previously equilibrated with buffer. The column was washed with 100 ml of equilibration buffer, and a linear NaCl gradient was begun (400 ml of equilibration buffer in the mixing chamber and 400 ml of the buffer containing 1.0 M NaCl in the reservoir; flow rate, 15 ml/h). The most active fractions (20 ml) were stored at −80°C.

Protein was determined by the method of Lowry et al. (10), with bovine serum albumin fraction V (Wako Pure Chemicals, Osaka, Japan) as a standard.

Molecular weight determination. A mixture of 0.5 ml of native bacteriocin (320 AU/ml) and 1 mg of standard proteins (aldolase, bovine serum albumin, chymotrypsinogen A, and cytochrome *c*; Boehringer Mannheim, West Germany) was applied on the Sephadex G-200 column (2.0 by 40 cm) prepared in 50 mM Tris-hydrochloride buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and eluted with the same buffer at a flow rate of 9 ml/h in the cold; fractions of 3 ml were collected. Both the bacteriocin activity and the absorbance at 500 nm were measured for each fraction collected.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out essentially by the method of Maizel (11) and Studier and Maizel (18), except for the electrode buffer. Samples of the highest peak fraction from a DEAE-Sephadex A-50 column were diluted in a minimal volume of 0.01 M Tris-hydrochloride buffer (pH 7.5). The coincidence of the position of a band that stained with Coomassie brilliant blue with the position of the gel slices containing bacteriocin activity was studied. A 50-μl sample of native bacteriocin was layered on top of the 7.5% gels (0.5 by 10 cm) without sodium dodecyl sulfate. The gels were placed at 4°C and subjected to a current of 3 mA/gel for 30 h, using the electrode buffer (0.1 M Tris-hydrochloride buffer, pH 7.5) without sodium dodecyl sulfate. The electrophoresed gels were sliced transversely into 0.5-cm slices. Bacteriocin activity could be leached from gel slices by soaking at 4°C for 24 h with 0.5 ml of 50 mM Tris-hydrochloride buffer (pH 7.5).

Action of chemical and physical agents on bacteriocin activity. Except for lipase R, all enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. Enzyme stock solutions were added to samples of bacteriocin at a final concentration and pH as follows: 200 μg of trypsin, α-chymotrypsin, and lipase R (Seikagaku Kogyo) per ml in 50 mM Tris-hydrochloride buffer (pH 7.5); 200 μg of pepsin per ml in 50 mM citrate buffer (pH 6.0); 50 μg of deoxyribonuclease and ribonuclease per ml in 50 mM Tris-hydrochloride buffer (pH 7.5). Enzyme-treated samples were incubated at 37°C for 30 min and assayed for residual bacteriocin activity. Controls consisting of bacteriocin and buffered enzyme solutions alone were also assayed. Resistance to ultraviolet irradiation was determined by exposure of samples of bacteriocin to ultraviolet irradiation from a Mitsubishi Electric 15-W lamp at a distance of 25 cm for 20 min.

To test for heat resistance, bacteriocin was heated in 50 mM Tris-hydrochloride buffer (pH 7.5) to the temperature indicated, maintained at that temperature for 10 min, and then cooled by tap water.

The effect on bacteriocin activity of freezing (−20°C) and thawing (room temperature) three times was determined.

Bactericidal effect. A bacterial suspension of *M. diernhoferi* ATCC 19340 was prepared from 3-day-old cultures on 1% Ogawa egg medium by grinding the organisms in HI broth to a concentration of 5×10^6 viable units per ml; 0.9 ml of this suspension was incubated at 37°C with or without 0.1 ml of bacteriocin solution (32 and 64 AU/ml). At the end of the time specified, a 0.2-ml amount of a series of 10-fold dilutions in 50 mM Tris-hydrochloride buffer, pH 7.5, was pipetted in triplicate onto the HI agar plate surface and spread evenly with a sterile glass rod. The mean viable count was determined after 5 days of incubation.

Cytotoxic effect. HeLa-S3 cells were cultured in Eagle minimal essential medium supplemented with 10% calf serum (Wako Pure Chemicals) at 37°C for 3 days. Five milliliters of the cell suspension (10^5 cells per ml) was plated in triplicate onto plastic tissue dishes (60 mm; Wako Pure Chemicals) or petri dishes (60 mm) and incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2. After incubation, the cells were washed three times with 5 ml of minimal essential medium and then exposed to 0.2 ml of the native or heat-inactivated bacteriocin (64 and 128 AU/ml) or minimal essential medium at 37°C for 3 h. The cells were washed and cultured in 5 ml of the above growth medium. At various time intervals, the cytotoxic effect was determined by counting the adherent cells with a hemocytometer.

Morphological changes of HeLa-S3 cells induced by treatment with the bacteriocin were examined. The cells (10^5) were grown on glass cover slips (9 by 32 mm) in Leighton tubes at 37°C for 3 h. At various time intervals, the cover slips were washed three times with saline, fixed by methanol, and stained with Giemsa solution.

RESULTS

Isolation and purification. No appreciable bacteriocin activity was found in the culture supernatant fluid from *M. smegmatis* ATCC 14468. Extraction of the cells with 1 M NaCl or 7 M urea failed to liberate much of the activity (data not shown). When the cells were sonicated, appreciable quantities of bacteriocin were released into the clear brownish-yellow superna-

tant fluid. This was used as the starting material for bacteriocin purification. The results of the purification of bacteriocin 14468 are summarized in Table 1. The whole procedure yielded an overall recovery of 15% of bacteriocin, with about a 50-fold increase in the specific activity.

Molecular weight. The molecular weight was estimated to be approximately 75,000 by an experiment in which bacteriocin was run in Sephadex G-200 gels together with the standard protein.

Electrophoretic homogeneity. Electrophoresis of the bacteriocin on 7.5% polyacrylamide gels at pH 7.5 showed a major rapidly migrating and a minor slowly migrating band staining with 0.25% Coomassie brilliant blue (Fig. 1). When bacteriocin activity was measured in eluates of gel slices, the activity coincided with the position of the major band, which stained with Coomassie brilliant blue.

Chemical and physical properties. Chemical and physical properties are summarized in Table 2. A slight loss of bacteriocin activity occurred when it was heated at 56°C for 10 min, whereas a marked loss of activity was noted when it was heated at 100°C for 10 min. Neither ultraviolet irradiation nor freeze-thawing three times caused a reduction in activity. Treatment of the bacteriocin with trypsin, α-chymotrypsin, and pepsin under the experimental conditions described resulted in a loss of 90% or more of its activity, whereas the compound was resistant to the action of lipase R, deoxyribonuclease and ribonuclease.

Bactericidal activity. Figure 2 shows the bactericidal effect of bacteriocin on sensitive indicator cells. There was a slow loss of viability of the indicator cells upon incubation with the bacteriocin. When the indicator cells were treated with a bacteriocin solution at a final concentration of 32 AU/ml, the number of colony-forming cells did not apparently decrease until after 3 h of incubation. In 12 h, the number of survivors was approximately 18%, and in 24 h, it was 6%. When a bacteriocin solution at a final concentration of 64 AU/ml was used, there was a more rapid loss of viability; at 2 h, the loss of viability started, and the numbers of survivors after 6-, 12-, and 24-h incubations were approximately 3, 0.2, and 0.05%, respectively.

Cytotoxicity to HeLa cells. The results of treatment of HeLa-S3 cells with bacteriocin are

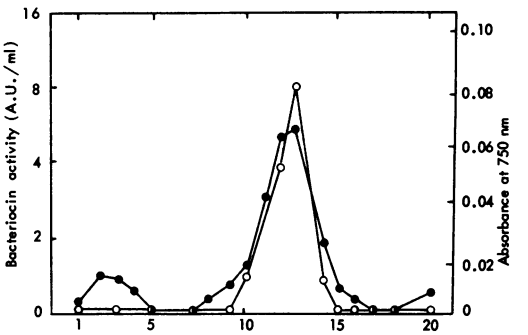

FIG. 1. *Activity of fractions collected from crude bacteriocin electrophoresed on a 7.5% polyacrylamide gel. A 50-μl quantity of the highest peak fraction from a DEAE-Sephadex A-50 column was applied to the top of a 7.5% polyacrylamide gel (0.5 by 10 cm) containing 0.1 M Tris-hydrochloride buffer (pH 7.5). The current was adjusted to 3 mA/gel at 4°C for 30 h. The electrophoresed gel was sliced transversely into 0.5-cm slices. Each slice was soaked in 0.5 ml of the above buffer and kept at 4°C for 24 h to leach out the bacteriocin. The bacteriocin activity* (○) *and the amount of protein* (●) *of each slice were measured as described in the text.*

TABLE 2. *Effect of various agents on activity of partially purified bacteriocin 14468*

Treatment	Bacteriocin activity (AU/ml)	
	Control	Treated
Heat		
10 min at 56°C	320	160
10 min at 80°C	320	40
10 min at 100°C	320	20
Ultraviolet irradiation (15 W, 25 cm, 20 min)	320	320
Freeze-thawing (3 times)	160	160
Trypsin (200 μg/ml)	640	10
α-Chymotrypsin (200 μg/ml)	640	20
Pepsin (200 μg/ml)	640	40
Lipase R (200 μg/ml)	640	640
Deoxyribonuclease (50 μg/ml)	640	640
Ribonuclease (50 μg/ml)	640	640

TABLE 1. *Purification of bacteriocin 14468 from M. smegmatis*

Purification step	Vol (ml)	Activity (AU/ml)	Protein (mg/ml)	Sp act (AU/mg)	Yield (%)	Fold purification
Disrupted supernatant fluid	550	320	31.2	10	100	1
DEAE-cellulose column 35–85% $(NH_4)_2SO_4$ pellet	700	160	4.1	39	64	4
	20	2,560	26.4	97	29	10
Sephadex G-200 column (peak fractions)	70	320	2.3	139	12	14
DEAE-Sephadex A-50 column (peak fractions)	20	1,280	2.6	492	15	49

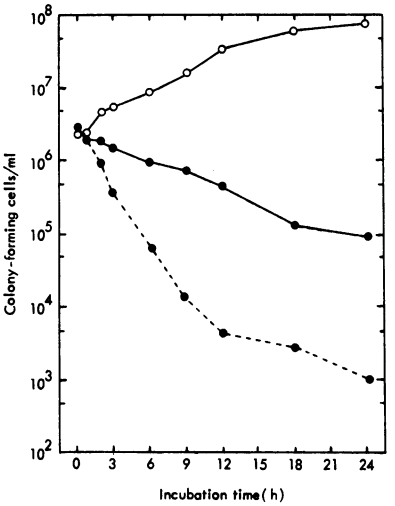

FIG. 2. *Bactericidal activity of bacteriocin for indicator cells. A 0.9-ml amount of bacterial suspension of M. diernhoferi ATCC 19340 grown in HI broth containing 4% glycerol and Tween 80 (5 × 10⁸ viable units per ml) was incubated with or without 0.1 ml of bacteriocin solution (final concentrations, 32 and 64 AU/ml). At indicated intervals, a portion of the mixture was withdrawn, and the colony-forming cells were assayed as described in the text. Symbols: Untreated cells* (○); *cells + 32 AU of bacteriocin* (-●-); *cells + 64 AU of bacteriocin* (- -●- -).

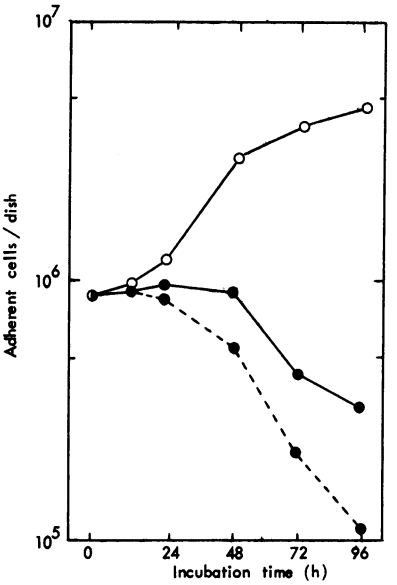

FIG. 3. *Inhibitory effect of bacteriocin on growth of HeLa-S3 cells. Five milliliters of the cell suspension (10⁵ cells per ml) was plated onto plastic tissue or petri dishes (60 mm) and then incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2. The cultivated cells were treated with 0.2 ml of native or heat-inactivated bacteriocin at 37°C for 3 h. The cells were washed and cultured in 5 ml of fresh Eagle minimum essential medium supplemented with 10% calf serum and further incubated at 37°C. At indicated intervals, the residual adherent cells were counted with a hemocytometer. Symbols: Cells + no or heat-inactivated bacteriocin* (○); *cells + 64 AU of bacteriocin* (-●-); *cells + 128 AU of bacteriocin* (- -●- -).

shown in Fig. 3. The number of adherent cells treated with 64 AU of bacteriocin solution per ml after 48 h of incubation was almost the same as that at the start of incubation; thereafter, it decreased slowly with the passage of time. In the case of treatment of the cells with 128 AU of bacteriocin solution per ml, the decrease in adherent cells was more conspicuous. After 96 h of incubation, the number of adherent cells was 9.8 × 10^6 in the control and 5.4 × 10^5 and 1.3 × 10^5 in the groups treated with 64 and 128 AU of bacteriocin solution per ml, respectively. Morphological changes of cells induced by treatment with 64 AU of bacteriocin solution per ml at 37°C for 3 h revealed that after 3 days of incubation, the cells showed striking morphological alterations, such as marked shrinking and many vacuoles in their cytoplasma. In contrast, the control cells treated with heat-inactivated bacteriocin solution showed good growth without any sign of morphological changes.

DISCUSSION

Many bacteriocinogenic organisms elaborated maximum quantities of bacteriocins only after induction with ultraviolet light, metabolic inhibitors such as mitomycin C, or other agents known to induce lysogenic phages (13).

Bacteriocin from *M. smegmatis* ATCC 14468, like a bacteriocin from *M. fortuitum* ATCC 1701 (22), could not be found in significant concentrations in the supernatant fluid of broth cultures, nor was it inducible by addition of an inducing agent, mitomycin C (0.1 μg/ml). Moreover, extraction of the cells with 1 M NaCl or 7 M urea failed to yield enhanced titers (data not shown).

Bacteriocin was released in appreciable quantities after disruption of the cells. As with small quantities of the bacteriocin, the processing of large volumes of materials requires an efficient scheme to purify the active principal. To obtain purified bacteriocin, we attempted to use the purification procedures described herein. The work presented here represents the first step towards a goal of full characterization of several mycobacteriocins. The supernatant of the disrupted cells was first subjected to ion-exchange chromatography (DEAE-cellulose) to remove other cell components, and then the crude bacteriocin preparation was concentrated by fractional precipitation with ammonium sulfate.

Subsequent purification steps used were gel filtration on Sephadex G-200 and ion-exchange chromatography on DEAE-Sephadex A-50. The specific activity of the highest peak fraction of the active principal from the DEAE-Sephadex A-50 was 492 AU/mg of protein. It was purified 49-fold, with a yield of 15%.

The molecular weight of bacteriocin which was eluted from the Sephadex G-200 column with the marker proteins was estimated to be 75,000.

The electrophoresis of partially purified bacteriocins on 7.5% polyacrylamide gels at pH 7.5 gave a major and a minor band. The bacteriocin activity was present only in the major band.

Partially purified bacteriocin was attacked by proteolytic enzymes, but not by deoxyribonuclease, ribonuclease, and lipase R. The activity was not reduced by ultraviolet irradiation and freeze-thawing. The activity was significantly lowered by heating at 80°C for 10 min and boiling for 10 min.

Bradley (2) separated bacteriocins into two distinct categories. Mycobacteriocin 14468 clearly fell within one group consisting of bacteriocins of small molecular size, which are sensitive to the action of proteolytic enzymes but slightly less thermostable.

Addition of bacteriocins from most of the gram-positive and gram-negative organisms to exponential-phase cultures of the indicator cells results in an abrupt cessation of growth and, thereafter, a marked decrease in viable cell numbers within minutes. In contrast, mycobacteriocin 14468 exhibited a different bactericidal activity: the lengthy time required for cessation of growth and decrease in the number of sensitive cells was characteristic. The reasons for this are not clear and the matter is under investigation.

Several studies have suggested that only certain regions of the bacterial cell membrane offer potential sites of bacteriocin attachment (9, 17). It has also been reported that receptors for several bacteriocins may correspond in function to receptors for various materials such as vitamin B_{12} (3) and polymyxin B (12) on cell membranes of *Escherichia coli*. Farkas-Himsley (4) reported that vibriocin, the bacteriocin from *Vibrio cholerae*, has a cytotoxic effect on HeLa and L cell lines. In the present study, the growth of HeLa cells was also inhibited by mycobacteriocin, and marked morphological changes were noted in treated cells. Recently, we found that cultured simian virus 40-transformed BALB/c mouse tumor cells are more sensitive to the mycobacteriocin than are nontransformed BALB/3T3 cells established from BALB/c mice. Furthermore, herpes simplex virus type 2-transformed hamster tumor cells and human gastric cancer cells were less sensitive to the mycobacteriocin (unpublished data). This will be reported separately.

ACKNOWLEDGMENTS

We thank George P. Kubica, Center for Disease Control, Atlanta, Ga. for critical review of this manuscript. We also thank Yoshihiro Ito, Shimane Prefectural Institute of Public Health, Shimane, Japan.

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