Purification and the Ultrastructure of a Bacteriocin Produced from Shigella sonnei Strain 100052

KAZUNOBU AMAKO,1* MOTÖYUKI MATSUGUCHI,3 KENJI TAKEYA,2 EMIKO TATSUTA,1 AND YASUYUKI TAKAGI2

Department of Microbiology, School of Medicine, Fukuoka University, Fukuoka 814,1 and Department of Microbiology2 and Department of Biochemistry,2 School of Medicine, Kyushu University, Fukuoka 816 Japan

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A bacteriocin produced by strain 100052 of Shigella sonnei (shigellacin 52) was purified about 80-fold from a mitomycin C-induced culture supernatant. The purified preparation gave a single band on sodium dodecyl sulfate-gel electrophoresis with an approximate molecular weight of 10,000. The negatively stained electron micrograph of the purified bacteriocin preparation revealed the existence of three different particles. They were a small cylindrical tube (4.2 by 6.0 nm), a ring-shaped particle and a filamentous structure. The molecular weight calculated from the shape of the small tube was approximately 50,000. The latter two particles appeared to be constructed from subunits which were morphologically similar to the cylindrical tube. These results suggested that the morphological subunit of shigellacin 52 consisted of five chemical subunits with molecular weights of 10,000 each, and it assembled into two types of polymers, a ring-shaped particle and a filamentous structure, which seemed to be the main components of the purified shigellacin 52 preparation.

Bacteriocins have been classified into two groups on the basis of their molecular weight: a high-molecular-weight class and one of low molecular weight (4). The electron microscopic observations of the high-molecular-weight bacteriocins have revealed that they have a structure similar to bacteriophage or their tail entity (5, 6, 9). This highly organized structure is thought to be the reason for the properties of thermal lability and resistance to digestion by proteolytic enzymes shown by the high-molecular-weight class. On the other hand, the low-molecular-weight bacteriocins are thermally stable and susceptible to proteolysis and are thought to consist of a simple protein (8).

Shigella sonnei strain 100052, one of the standard strains for colicin typing of the sonnei group of Shigella bacilli, produces colicin type VII, and the strain 100033 is the only strain susceptible to this colicin in the set of strains of this typing (1). In preceding papers we reported the purification and some properties of this colicin (here termed shigellacin 52), and assumed that it is a bacteriocin of new type, not included in Bradley's classification (2, 3). Furthermore, we recently found that this bacteriocin was inactivated by heating and was resistant to the treatment of proteolytic enzymes, although its molecular weight seemed to be low. Thus, the nature and properties of this bacteriocin remained obscure and required further study.

The present paper describes a procedure for the further purification of this bacteriocin and electron microscopic studies on the purified preparation.

MATERIALS AND METHODS

Bacterial strains and media. Strain 100052 of S. sonnei and the strain 100033 were both obtained from J. D. Abbott via H. Tokiwa (Fukuoka Environmental Research Center, Fukuoka, Japan) as a set of strains for colicin typing of S. sonnei (1). For the production of the shigellacin 52, strain 100052 was grown in a medium (pH 7.0) containing 10 g of polypeptide, 5 g of yeast extract, and 5 g of NaCl in 1,000 ml of distilled water (PY medium) at 37°C with aeration. When bacteriocin activity was titrated, ordinary nutrient broth and nutrient agar were employed. The agar concentration in the soft agar was 0.7%. The growth of bacteria in PY medium was followed photometrically by absorbance measurement at 660 nm.

Bacteriocin assay. Bacteriocin activity was determined by serially diluting solutions to be tested in nutrient broth with a microdiluter of 0.025 ml (Cooke Co., Texas) and spotting dilutions on a soft agar plate containing approximately 10⁶ cells of freshly grown strain 100033. A unit of bacteriocin activity per milliliter is defined
as the reciprocal of highest dilution giving a clear zone of inhibition of the growth of the indicator strain.

**Electrophoresis.** The molecular weight of bacteriocin was estimated with polyacrylamide gel (10% gel) electrophoresis in the presence of 0.1% sodium dodecyl sulfate as described by Weber and Osborn (10) with a current of 8 mA per tube for 4 h at room temperature in a glass tube of 5 mm in diameter and 100 mm in length.

The following compounds were used as standard markers of molecular weight: cytochrome c (Boehringer Mannheim Corp.; molecular weight, 11,7000), ovalbumin (Sigma Chemical Co.; molecular weight, 43,000), and bovine serum albumin (Sigma Chemical Co.; molecular weight, 68,000).

**Enzymes.** Trypsin (bovine pancreas) was the product of P-L Biochemical Inc. Chymotrypsin (bovine pancreas), deoxyribonuclease I (bovine pancreas) and ribonuclease A (bovine pancreas) were purchased from Sigma Chemical Co.

**Ultrafiltration.** Ultrafiltration of shigellacin 52 was carried out with XM100A or UM10 membranes using a cell (model 202; Amicon Co., Ltd.) in a refrigerator. The filtration rate was controlled by altering the pressure of nitrogen gas.

**Electron microscopy.** Specimens were examined by using a negative staining technique with 2% potassium phosphotungstate (pH 7.2) or 1% uranyl acetate (pH 7.2). The pH of the uranyl acetate solution was adjusted to neutral with 12 mM oxalate and diluted ammonium hydroxide. For fixation, specimens were mixed with an equal volume of 1% glutaraldehyde solution for 20 min at 4°C. The dimensions of the specimens were calibrated by the lattice spacing of catalase crystals (Boehringer Mannheim, bovine liver) (11). For examination, a Hitachi HU-12A electron microscope was used at 75 kV.

**RESULTS**

**Growth and induction of the bacteriocinogenic strains.** PY medium (10 ml) was inoculated with 1 ml of freshly grown strain 100052 and incubated at 37°C with continuous shaking. When the absorbance at 660 nm of the culture reached 100 to 140 Klett units, mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Japan) was added to a final concentration of 0.5 μg/ml. A marked reduction of viable cells was observed after exposure to the antibiotic, whereas the absorbance increased continuously over the following 2 h and then declined gradually (Fig. 1). However, the bacteria did not lyse completely. The bacteriocin activity appeared in the culture fluid about 2 h after the addition of mitomycin C with 60 U of activity per ml finally detected. But when mitomycin C was not added to the culture, the bacteriocin activity did not increase over 8 U/ml.

**Purification of shigellacin 52.** (i) Ammonium sulfate fractionation. To 10 liters of mitomycin-induced culture fluid, after removing the cells by centrifugation at 10,000 × g for 30 min, finely powdered ammonium sulfate (164 g/liter) was added gradually with stirring over a 30-min period at room temperature. After an additional 60 min of stirring, the resulting precipitate containing 40 to 60% of the bacteriocin activity was collected by centrifugation at 10,000 × g for 30 min and dissolved in 100 ml of 0.01 M phosphate buffer (pH 6.5). Then, the bacteriocin-containing fraction was clarified by centrifugation at 5,000 × g for 30 min.

(ii) Phosphocellulose column chromatography. The shigellacin fraction of 100 ml was dialyzed against 0.01 M phosphate buffer (pH 6.5) overnight in a refrigerator and applied to a phosphocellulose column (9 by 300 mm, Whatman P11) previously equilibrated with the same buffer. The column was washed with the same solution. The bacteriocin activity was found in fractions together with material adsorbing light at 280 nm. No additional activity could be eluted from the column by adding NaCl to the washing solution.

(iii) Ultracentrifugation. To separate active material from other particles (2), the pooled fractions from the phosphocellulose column were centrifuged at 100,000 × g for 120 min after being concentrated to 5 ml with UM10 ultrafiltering membrane.
(iv) **Sepharose 4B gel filtration.** The supernatant fluid from the centrifuged bacteriocin (step iii) was applied to a column (24 by 1,000 mm) of Sepharose 4B (Pharmacia, Sweden) previously equilibrated with 0.01 M phosphate buffer (pH 6.5) containing 0.1 M NaCl at a flow rate of about 5 ml/h. The bacteriocin activity was eluted in a single but broad peak (Fig. 2). Fractions containing most of bacteriocin activity (fraction no. 21 to 28) were pooled and concentrated with a UM10 membrane. The concentrated Sepharose 4B fractions represents an 80-fold purification over the starting culture fluid (Table 1), and this fraction, referred to as purified shigellacin 52, was used in all the experiments to be described.

**Molecular weight determination.** Purified shigellacin 52 showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3B), and its molecular weight was estimated to be approximately 10,000 by comparison of the mobility and the molecular weights of standard proteins (Fig. 3A).

**Electron microscopic observation of pu**
rified shigellacin 52. The electron micrograph of negatively stained shigellacin 52 is shown in Fig. 4. Two types of particles were observed: a ring-shaped particle of 13.0 nm in diameter, and a long filamentous structure of variable length. In addition to these structures, when the specimen was observed by high magnification, the presence of small particles of a diameter of 4.2 nm were noted. The detailed observation of 4.2-nm particles revealed that they have the structure of small cylindrical tubes of the size of 4.2 by 6.0 nm with a hole of 1.8 nm in diameter in the middle of the tube (Fig. 5, arrows). This structure was the smallest found in the purified bacteriocin preparation.

The ring-shaped particles and the filamentous structure seemed to be constructed from subunits similar to the 4.2-nm particles. When the specimens were fixed with glutaraldehyde before negative staining, more ring-shaped particles or the filamentous structures and few 4.2-nm particles were detected. This implied that the ring-shaped particle or the filament is fragile and tends to disintegrate into its subunits during the negative staining process.

Ultrafiltration of shigellacin 52. The structure of bacteriocin observed in the electron micrographs appeared to be larger than that expected from the molecular weight obtained by sodium dodecyl sulfate-gel electrophoresis. Therefore, the purified bacteriocin was subjected to ultrafiltration with an XM-100A membrane which retains materials with molecular weights larger than 100,000.

The amount of bacteriocin activity passing through the membrane was less than 1% of the total bacteriocin activity, and the amounts of protein filtered were 4% of the total protein content.

It was therefore suggested that most of shigellacin 52 in purified preparation has a molecular weight larger than 100,000.

Properties of shigellacin 52. Shigellacin 52 gave an UV light absorption curve typical for protein and was inactivated by heating at 80°C for 5 min, but survived after heating at 60°C for 30 min. It was refractory to ribonuclease, deoxyribonuclease, and chymotrypsin (Table 2). In addition to these properties, it was stable to UV irradiation.

DISCUSSION

The bacteriocin induced from strain 100052 of S. sonnei was purified, and on electron microscopy three different types of particles were detected. The smallest was a cylindrically shaped particle of 4.2 nm in diameter, and the other two were ring-shaped and filamentous particles. The
In this micrograph, it appears that the images of negatively stained bacteriocin appear black in the micrograph. Arrows indicate 4.2-nm particles. Specimen was stained with uranyl acetate without fixation.

**TABLE 2. Some properties of shigellacin 52**

| Treatment | Bacteriocin activity (U/ml) |
|-----------|-----------------------------|
|           | Nontreated | Treated |
| Trypsin (200 µg/ml)* | 64 | 64 |
| Chymotrypsin* (200 µg/ml) | 64 | 64 |
| DNase* (100 µg/ml) | 64 | 64 |
| RNase* (100 µg/ml) | 64 | 64 |
| Heating 37°C for 60 min | 128 | 128 |
| 60°C for 30 min | 128 | 128 |
| 60°C for 60 min | 128 | 32 |
| 80°C for 30 min | 128 | 0 |

* Purified shigellacin 52 was incubated with the enzyme at 37°C for 60 min. DNase, Deoxyribonuclease; RNase, ribonuclease.

Ring-shaped particle is constructed from a few (four to six) subunits, and the filamentous one appears to be formed by arranging the subunits in linear or helical fashion. Because the shape and dimensions of the subunits in these structures appeared to be similar to those of the 4.2-nm particle, it might be possible that they are polymerized forms of the 4.2-nm particle.

The molecular weight of the 4.2-nm particles could be estimated to be approximately 51,000, if it is assumed to have a cylindrical structure of 4.2 nm in diameter and 6.0 nm in length with a hole of 1.8 nm in diameter in it and the specific gravity of the protein is taken as 1.30. However, on sodium dodecyl sulfate-gel electrophoresis, the molecular weight of bacteriocin was shown to be about 10,000. Therefore, the 4.2-nm particle should consist of at least five protein molecules each with molecular weights of 10,000, and this structure could be the morphological subunits of the shigellacin 52.

The result of the ultrafiltration and gel filtration experiment indicate that most of the bacteriocin activity is associated with material of molecular weight larger than 100,000. If the molecular weight of the 4.2-nm particle was taken as 50,000, the ring-shaped particles are more than 200,000. From the electron micrograph of glutaraldehyde-fixed bacteriocin preparations, which showed the presence of more polymerized particles than that of a nonfixed specimen, we might be able to assume that most of shigellacin 52 has the polymerized structure in the purified preparation, and during the process of negative staining they are disintegrated into their subunits.

Some of the properties of shigellacin 52 resembled those of high-molecular-weight bacteriocins, although its molecular weight was low. The polymerized structure of this bacteriocin as revealed in this study might be the reason for the unique biological properties of this bacteriocin.

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