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Sanguicin, a Bacteriocin of Oral *Streptococcus sanguis*

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Streptococcus sanguis strain N-2 was found to produce a bacteriocin (sanguicin) which accumulates intracellularly. It was purified by sequential procedures about 98-fold with a recovery of 37% and appeared to be homogeneous on gel electrophoresis. Sanguicin was heat labile and was destroyed by digestion with pronase. The growth of several species of oral indigenous microorganisms was inhibited by sanguicin, of which *Bacteroides melaninogenicus* was most susceptible. Sanguicin acted on susceptible cells as a bacteriostatic agent.

In the human oral cavity, streptococci are the predominant indigenous microorganisms, especially *Streptococcus sanguis,* which is most important in the early stage of plaque formation (2). A growth inhibitory substance from *S. sanguis* is therefore of interest, since such a bacteriocin is one of the conceivable survival stratagems for the organisms in the oral microbial population. We have reported (11) on the formation and partial characterization of sanguicin using crude material, and in this report we describe its purification and more precise properties.

MATERIALS AND METHODS

Bacterial strains and cultivation. The sanguicin-producing strain *S. sanguis* N-2 was isolated from human dental plaque. Laboratory stock culture strain *Bacteroides melaninogenicus* F-3 was used as an indicator strain for assaying the activity of sanguicin. *S. sanguis* N-2 was cultivated at 37°C anaerobically in a glove box (COY Manufacturing Co., Ann Arbor, Mich.) for 4 days in a medium consisting of 17 g of Trypticase (Baltimore Biological Laboratory), 3 g of yeast extract (Difco), 5 g of NaCl, 2.5 g of K_2HPO_4, 0.5 g of sodium-thioglycollate per liter.

Assay of sanguicin activity. Sanguicin activity was assayed as described earlier (11). The indicator strain for bioassay was cultivated in the above described medium supplemented with 5 mg of hemin and 0.5 mg of menadione per liter and solidified with 1.0% agar. The number of sanguicin units per milliliter was defined as the reciprocal of the highest dilution that gave clear zones of growth inhibition of the indicator strain.

Determination of molecular weight. The molecular weight of sanguicin was estimated by the method of Andrews (1) by comparison of its elution volume on Sephadex G-200 gel filtration with those of several proteins with well-characterized molecular weights.

Enzyme treatments. Effect of various enzymes on purified sanguicin was examined by the method described previously (6).

Chemical anaysis. The protein was estimated by the method of Lowry et al. (10) with bovine serum albumin as a reference. For amino acid analysis, a sample of purified sanguicin was exhaustively dialyzed against deionized water, lyophilized, and subsequently hydrolyzed in 6 N HCl at 110°C for 24 h in a sealed, evacuated tube. The amino acid composition was determined with a JEOL amino acid analyzer model JLC-6AH.

RESULTS

Purification of sanguicin. Cells were harvested from 16 liters of culture by centrifugation at 10,000 × *g* for 10 min (yield, 32 g, wet weight), washed three times with 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, and resuspended in 75 ml of 0.05 M phosphate buffer (pH 7.0). The following experiments were conducted at 4°C in phosphate buffer (pH 7.0), if not otherwise specified. The cells were disrupted by ultrasonic treatment at 9 kHz for 20 min, and a clear supernatant fluid was obtained by centrifugation at 100,000 × *g* for 1 h. The pellet was subjected once more to ultrasonic treatment, and the supernatant fluids were combined. In this crude extract, sanguicin activity against several species of microorganisms was demonstrated. Solid ammonium sulfate was added to the crude extract (39 g/100 ml) to give 60% saturation of the reagent, and the precipitate formed was collected by centrifugation at 10,000 × *g* for 10 min and discarded. Ammonium sulfate was added to the supernatant solution to a concentration of 6.9 g/100 ml to bring it to 70% saturation, and the precipitate was collected. The precipitate was dissolved in 13 ml of the buffer, followed by dialysis against 2 liters of the buffer with four changes. When the ammonium sulfate fraction was filtered on a Diaflo membrane XM-50, sanguicin activity did not pass through the membrane and could be recovered in the concentrated unfiltrable fraction without significant loss of the activity. This fraction was applied to a column (2.0 by 30 cm) of diethylaminoethyl-cellulose previously equilibrated

with the buffer. After washing the column with 300 ml of the buffer until the absorbance at 280 nm of the eluate declined to less than 0.05, it was eluted with a stepwise increasing concentration of KCl from 0.1 M to 0.4 M in the buffer at a flow rate of 40 ml/h. Fractions of 3 ml were collected and monitored for absorbance at 280 nm and sanguicin activity. Sanguicin was eluted with 0.2 M KCl, and the active fractions (30 ml) were combined, concentrated with a rotary evaporator in vacuo at 30°C to 2.5 ml, and dialyzed against 2 liters of the buffer containing NaCl. The concentrated material from the DEAE-cellulose column was subjected to gel filtration on Sephadex G-200 column (2.6 by 80 cm) and eluted with the buffer containing 0.15 M NaCl at a flow rate of 15 ml/h. Fractions of 5 ml were collected. As illustrated in Fig. 1, sanguicin emerged as a single peak. Finally, the active fraction was combined, concentrated, and dialyzed against the buffer. The purification is summarized in Table 1.

To examine the purity of sanguicin, polyacrylamide gel (7.5%) electrophoresis at pH 9.1 was carried out by the method of Davis (3). The electrophoresis showed a single band which stained amido black 10B (Fig. 2). The position of the band in the gel coincided with sanguicin activity in mobility as confirmed by bioassay (6).

Properties of sanguicin. The molecular weight of sanguicin was estimated to be about 280,000 from a plot of elution volume versus molecular weight on Sephadex G-200 gel filtration (Fig. 3).

Sanguicin is a heat labile substance, since after heating at 60°C for 10 min, residual activity was no longer detected. Enzymatic digestion with pronase (1.0 mg/ml) at 37°C for 1.5 h caused complete inactivation of activity; however, treatments with other proteolytic enzymes such as trypsin, chymotrypsin, and papain did not affect the activity. Lipase, lysozyme, catalase, ribonuclease, and deoxyribonuclease had no effect on

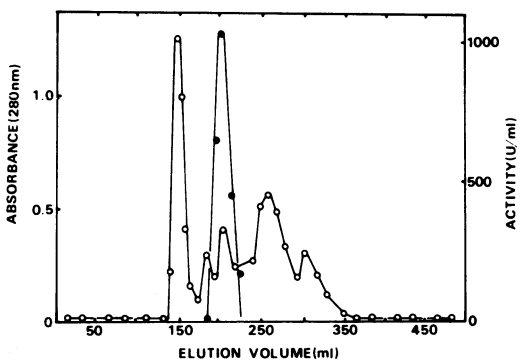

FIG. 1. *Gel filtration of sanguicin on Sephadex G-200. Conditions are as described in the text. Symbols: ●, activity; ○, absorbance at 280 nm.*

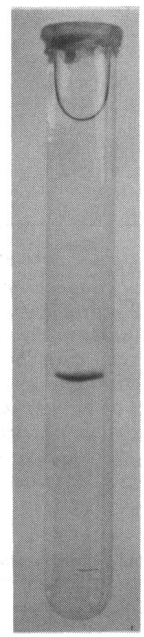

FIG. 2. *Polyacrylamide gel electrophoresis of purified sanguicin. Migration is from top to bottom.*

TABLE 1. *Purification of sanguicin*

Step	Vol (ml)	Protein (mg)	Total activity (units)	Sp act (U/mg of protein)	Purification (-fold)	Yield (%)
Crude extract	115	448	120,900	2.7×10^2	1	100
Ammonium sulfate fraction	15	50	87,200	17×10^2	6	72
XM-50 unfiltrable fraction	3	32	82,000	26×10^2	10	68
Diethylaminoethyl-cellulose column chromatography	30	11	60,500	55×10^2	20	50
Sephadex G-200, concentrate	5	1.7	45,000	265×10^2	98	37

activity. These results suggest that sanguicin is a protein.

Amino acid analysis revealed that lysine, aspartic acid, glutamic acid, alanine, and leucine were predominant. Cystine was not present (Table 2). Among several bacteriocins from different sources such as *Staphylococcus epidermidis* (9), *Serratia marcescens* (5), *Lactobacillus fermenti* (4), *Proteus morganii* (14), colicin E2 and E3 (7), and *Propionibacterium acnes* (6), aspartic acid, glutamic acid, glycine, and alanine were predominant. Their combined contents were 41, 49, 44, 42, 45, 45, and 43%, respectively. However, with sanguicin the combined content of their four amino acids was lower, representing 35%.

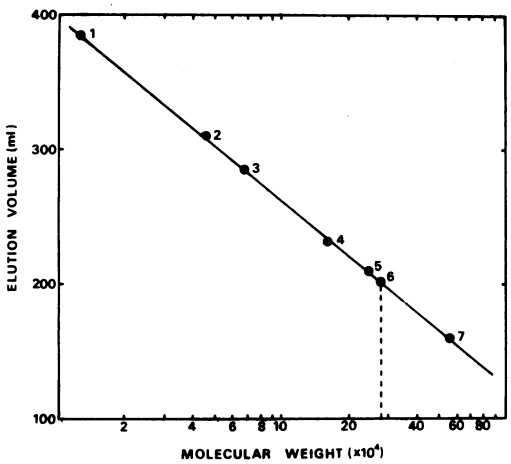

FIG. 3. *Estimation of molecular weight of sanguicin. Sanguicin and 2 mg of each standard protein were mixed and chromatographed on a Sephadex G-200 column (2.6 by 80 cm) with the buffer containing 0.15 M NaCl. The elution profile was determined by absorbance at 280 nm or 415 nm (cytochrome c). The elution volume of sanguicin was determined by bioassay. 1, cytochrome C; 2, ovalbumin; 3, bovine serum albumin; 4, aldolase; 5, catalase; 6, sanguicin; 7, ferritin.*

TABLE 2. *Amino acid composition of sanguicin*

Amino acid	Content[a]
Lysine	83
Histidine	28
Arginine	55
Aspartic acid	97
Threonine	62
Serine	48
Glutamic acid	97
Proline	41
Glycine	76
Alanine	83
Cystine	0
Valine	55
Methionine	21
Isoleucine	62
Leucine	110
Tyrosine	41
Phenylalanine	41

[a] Values are expressed in numbers of residues per 1,000 amino acids.

Examination of the inhibitory spectrum of sanguicin against some oral microbial species revealed that *B. melaninogenicus* and other species of *Bacteroides, Corynebacterium parvum* ATCC 11829, *Actinomyces israelii* ATCC 12102, and *Actinomyces naeslundii* ATCC 12104 were susceptible. Of these species, *B. melaninogenicus* was found to be the most susceptible. *Actinomyces viscosus* ATCC 19246, *Propionibacterium acnes* ATCC 11828, *Bacterionema matruchotii* ATCC 14266, *Fusobacterium nucleatum* ATCC 25286, *Streptococcus mutans* Ingbritt, *S. sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 9759, and *Streptococcus mitis* ATCC 9811 were not susceptible.

When a mixture of sanguicin and susceptible cells was spotted on an agar plate and incubated for 3 days, no growth was seen on the plate. A sterile loop was then streaked across the susceptible cell spot, and the plate was incubated for a further 3 days. Growth was seen where the cells had been translocated away from the sanguicin spot. However, cells in a control spot still could not grow. These results indicate that sanguicin is not bactericidal for the susceptible indicator cells; it only inhibits their growth.

DISCUSSION

Schlegel and Slade (12) found a bacteriocin present in aerobic cultures of *S. sanguis* and purified it by a combination of ammonium sulfate precipitation and Sephadex G-100 gel filtration. However, its properties are different from those described here for sanguicin. First, they found it in the surrounding broth medium, and its action mode was bactericidal for susceptible cells. On molecular weight determination they observed two molecular size forms: a 110,000-molecular-weight form appeared in low ionic strength and a neutral pH buffer system, and a 28,000- to 30,000-molecular-weight form appeared in high ionic strength and an alkaline pH buffer system. They later concluded that the bacteriocin induced a change in membrane permeability (13). Holmberg and Hallander (8) have observed that *S. sanguis* inhibits the growth of *A. naeslundii* in stab culture, and that these organisms reduced the colony-forming units of *A. naeslundii* in mixed liquid culture. We could not demonstrate sanguicin activity in the culture filtrate. Since this may be partly due to the difference of the cultivation methods, including aerobic versus anaerobic cultivation

and shaking versus stationary culture under aerobic conditions, these variations were tested for the production of sanguicin. These variations yielded only poor cell densities, and no significant amount of sanguicin activity was detected in culture filtrate.

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