

Purification and Properties of a Bacteriocin-Like Substance (Acnecin) of Oral *Propionibacterium acnes*

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Propionibacterium acnes CN-8, isolated from human dental plaque, was grown in a liquid medium, and its bacteriocin-like substance (acnecin) was extracted from the cells by ultrasonic treatment. Acnecin was purified to a homogeneous state with recovery of 47%. Specific activity increased 72-fold in comparison with the crude extract. The properties of acnecin were as follows. (i) Acnecin may consist of five subunits with a molecular weight of about 12,000. (ii) Its isoelectric point was 5.5. (iii) In amino acid composition, aspartic acid, glutamic acid, glycine, and alanine were predominant, whereas cystine was not present. (iv) Acnecin contained 3.3% carbohydrate but was substantially free from lipid. (v) The activity was lost by heating at 60°C or by protease and lysozyme treatments. Acnecin acted bacteriostatically on the indicator strain without killing it. The action spectrum of acnecin was very narrow; it was effective only against strains of non-acnecin-producing *P. acnes* and *Corynebacterium parvum*, a species closely related to *P. acnes*.

Many studies on bacteriocins of oral microorganisms have been made to better understand antagonistic interrelations in oral ecology. Above all, the bacteriocins of *Streptococcus mutans* (6, 10, 15, 16, 21-23, 35) have attracted much interest because of this strain's possible role in formation of dental caries. Other oral streptococci, such as *S. sanguis* (2, 12, 25-27) and *S. mitis* (15), have also been examined for the production and properties of their bacteriocins with respect to establishing and maintaining the predominant microbial flora in the oral cavity.

Diphtheroids including *Propionibacterium acnes* have been detected at about 10^8 to 10^9 per g of human dental plaque or gingival crevice deposits (24). *P. acnes* is considered to be important in the etiology of periodontal diseases (1, 32), but little is known about its bacteriocin. In the course of screening tests of antagonistic activity between oral bacteria, in vitro, a proteinaceous bacteriocin-like substance which we have designated "acnecin" was found in a strain of *P. acnes* isolated from human dental plaque. In this paper we describe the purification and some properties of acnecin.

MATERIALS AND METHODS

Bacterial strains and cultivation. The producer strain of acnecin, *P. acnes* CN-8, identified according to the criteria detailed in *Bergey's Manual* (18), was isolated from human dental plaque. Laboratory stock

culture strain *P. acnes* EXC-1 (32) was used as an indicator strain for assaying the activity of acnecin.

Cultivation was carried out at 37°C anaerobically in an anaerobic glove box (COY Manufacturing Co., Ann Arbor, Mich.) for 4 days in a medium containing 3.7% brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (Difco).

Assay of acnecin activity. The activity of acnecin was measured by the diffusion method on agar plates as described earlier (19). The number of acnecin units per milliliter was defined as the reciprocal of the highest dilution that gave clear zones of growth inhibition of the indicator strain, as in the case of colicin (11). Specific activity was defined as the number of acnecin units per milligram of the protein.

Standard buffer. Standard buffer used mainly in this work was 0.05 M potassium phosphate buffer (pH 7.0).

Polyacrylamide gel electrophoresis. To examine purity of samples, polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide at pH 9.1 according to the technique of Davis (3). After electrophoresis, gels were stained with amido black 10B. Simultaneously, unstained gel was briefly rinsed with the sterile standard buffer and embedded in a brain heart infusion-yeast extract agar plate containing the indicator strain, then incubated to determine the location of acnecin activity in the gel.

Determination of molecular weight. The molecular weight of acnecin was estimated by gel filtration on a Sephadex G-200 column (2.6 by 92 cm). Purified acnecin, blue dextran, and standard proteins (Boehringer-Mannheim, Mannheim, W. Germany) including aldolase, bovine serum albumin, ovalbumin, and cytochrome *c* were applied simultaneously and eluted

with the standard buffer containing 0.2 M NaCl. Coefficient K_{av} was calculated from the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$ (5), where V_e , V_o , and V_t mean elution volume, void volume, and bed volume, respectively. From the plot of K_{av} values against the logarithm of the corresponding molecular weight, the molecular weight of acnecin was determined. Another method employed was polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (34) with the standard proteins of bovine serum albumin, aldolase, chymotrypsinogen, and cytochrome *c* (Boehringer-Mannheim).

Isoelectric focusing. To determine the pI value of acnecin, isoelectric focusing (110-ml column; LKB Produkter, Stockholm, Sweden) was used according to the method of Vesterberg et al. (33). The sample was dialyzed against a 1% glycine solution overnight at 4°C before use. Ampholite (LKB Produkter) concentration was 1% (vol/vol), and the pH range was from 3.5 to 10. Focusing was done for 24 h under constant voltage (300 V), and the column was maintained at about 4°C with a circulating bath.

Enzyme treatment. Effect of six enzymes on purified acnecin activity at a concentration of 2 mg/ml was individually examined. Pronase (Kaken Chemicals, Tokyo, Japan), trypsin (Sigma Chemical Co., St. Louis, Mo.), and lipase (Sigma Chemical Co.) were dissolved in 0.05 M tris(hydroxymethyl)amino-methane-hydrochloride buffer (pH 8.0) containing 0.01 M CaCl₂. Papain (Merck, Darmstadt, W. Germany), lysozyme (Sigma Chemical Co.), and catalase (Sigma Chemical Co.) were in 0.05 M potassium phosphate buffer (pH 7.2). Acnecin (60 U)-enzyme mixtures were incubated for 1.5 h and assayed.

Chemical analysis. The protein was estimated by the method of Lowry et al. (17) with bovine serum albumin as a reference. Carbohydrate content was determined using anthrone (9,10-dihydro-9-oxanthracene; Nakarai Chemicals LTD, Kyoto, Japan) (28) with glucose as a standard. For amino acid analysis, a sample of purified acnecin was dialyzed against deionized water exhaustively and lyophilized. The powder of acnecin was dissolved in 6 N HCl and hydrolyzed at 110°C for 24 h in a sealed, evacuated tube. Amino acid composition was determined with a JEOL automatic amino acid analyzer, model JLC-6AH.

RESULTS

Purification of acnecin. The purification of acnecin is summarized in Table 1. All procedures were performed at 4°C, if not otherwise specified.

(i) **Extraction of acnecin.** Inhibitory activity could not be demonstrated in the culture supernatant or in the 10-fold-concentrated culture supernatant. No significant amount of the activity was detected in the extracts from the intact cells by 1 M NaCl in the standard buffer or in 0.05 tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.5), and 8 M urea solution. Only ultrasonic treatment of the cells was effective to obtain the activity. Cells from an 8-liter culture of *P. acnes* were harvested by centrifugation at 15,000 × *g* for 15 min (yield, 34 g [wet weight]), washed three times with the standard buffer containing 0.9% NaCl, and suspended in 50 ml of the standard buffer. The cells were then broken by ultrasonic treatment using a Kubota Insonator, model 200 M. A clear supernatant fluid was obtained by centrifugation at 100,000 × *g* for 1 h.

(ii) **Ammonium sulfate precipitation.** Solid ammonium sulfate was slowly added to the crude extract to 60% saturation with constant stirring. The mixture was further stirred for 1 h. The resultant precipitate was collected by centrifugation at 20,000 × *g* for 30 min and dissolved in 15 ml of the standard buffer. Then the solution was dialyzed against 1 liter of the standard buffer with several changes of the buffer. Precipitate formed during dialysis was removed by centrifugation at 20,000 × *g* for 10 min.

(iii) **Fractionation by negative adsorption to DEAE-cellulose.** The salting-out fraction was applied to a column (2.6 by 35 cm) of diethylaminoethyl (DEAE)-cellulose (DE 32) previously equilibrated to the standard buffer. When the column was eluted with the standard buffer, almost all activity was recovered in the washes and was not adsorbed to the column under these conditions. The washings were concentrated in vacuo by a rotary evaporator at 35°C, followed by dialysis against the standard buffer containing 0.2 M NaCl.

(iv) **Gel filtration.** The concentrated material from the DEAE-cellulose column was subjected to gel filtration on a Sephadex G-100 column (2.6 by 91 cm) and eluted with the standard buffer containing 0.2 M NaCl. Frac-

TABLE 1. Purification of acnecin

Step	Volume (ml)	Protein (mg)	Total activity	Sp act	Purification (fold)	Yield (%)
Ultrasonic treatment supernatant	45	1,104	11,500	10.4	1	100
Ammonium sulfate precipitation	18	556	9,200	16.5	1.6	80
DEAE-cellulose	55	122	8,800	72.1	6.9	77
Sephadex G-100, concentrated	8.5	7.2	5,400	750	72.1	47

tions of 5 ml were collected and monitored for absorbance at 280 nm and acnecin activity. One major and one minor peak of the UV absorbance emerged from the column (Fig. 1). The activity was found in the latter fraction. This fraction was combined as indicated in the figure, concentrated, and dialyzed against the standard buffer. This sample was the purified acnecin.

Criteria of purity. Polyacrylamide gel electrophoresis of the purified acnecin showed a single band, which corresponded to the activity in mobility (Fig. 2).

Molecular weight. The molecular weight was estimated to be about 60,000 from a plot of K_{av} versus molecular weights in Sephadex G-200 gel filtration. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, acnecin migrated with nearly the same mobility as cytochrome *c* and gave an approximate molecular weight of 12,000. These results suggest that acnecin consists of five subunits, assuming that acnecin dissociated to subunits with a similar molecular size in the presence of sodium dodecyl sulfate.

Isoelectric point. The profile of isoelectric focusing showed that acnecin activity was found in a single peak with a pI value of 5.5 (Fig. 3).

Chemical nature. Table 2 summarizes the amino acid composition of acnecin. Acidic amino acids (aspartic acid and glutamic acid), glycine, and alanine were predominant. The sum of these four amino acid contents represented 43% of the total amino acid residue. Carbohydrate content was 3.3%. When the polyacrylamide gel after electrophoresis of the purified acnecin was stained with oil red to detect lipid (9), no stained band was observed over the whole gel.

Action of acnecin. Whether acnecin acts on the indicator strain as a bacteriostatic or bactericidal agent was examined. Acnecin and a suspension of the indicator strain were mixed, and a drop of the mixture was spotted on a brain heart infusion-yeast extract agar plate. After

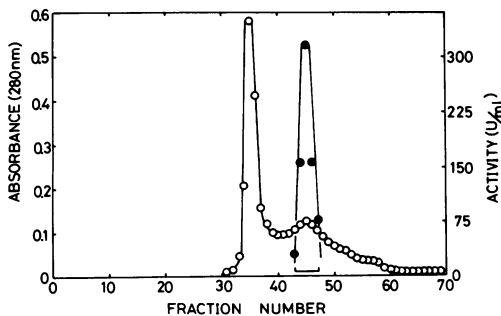


FIG. 1. Sephadex G-100 gel filtration of acnecin. Conditions are described in the text. Active fractions 43 to 47 were combined. Symbols: (●) activity; (○) absorbance at 280 nm.

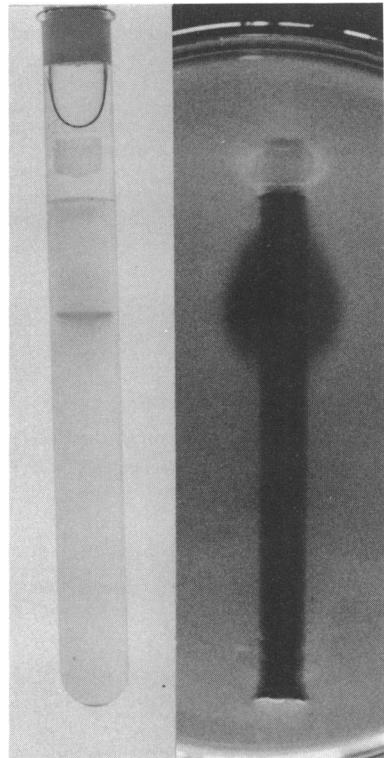


FIG. 2. Polyacrylamide gel electrophoresis of the purified acnecin. Gels were stained for protein (left) and bioassayed acnecin activity (right).

incubation for 3 days (no growth was observed), a sterile loop was streaked across the spot. After further incubation for 3 days, growth was seen where the cells had been spread from the spot (Fig. 4). This is indicative of a bacteriostatic effect of acnecin. This was quantitatively confirmed by viable count of cells treated with acnecin. In time-course measurements of colony-forming units of the indicator strain with 60 U of acnecin or without acnecin control, it was evident that cells treated with acnecin did not lose viability as compared to the control cells.

Stability. Acnecin activity was completely lost by heating at 60°C for 10 min. Its activity was reduced to half the initial activity on storage at -20°C for a month. Treatment with Pronase, trypsin, and papain caused complete inactivation. Lysozyme also reduced the activity, but catalase had no effect on the activity.

Inhibitory spectrum of acnecin. Of 10 species from other genera tested for susceptibility to acnecin, only *Corynebacterium parvum* was found to be inhibited. When the effect of acnecin on the growth of 12 strains of *P. acnes* isolated from human dental plaque was examined, the strains that produced inhibitory factor (moni-

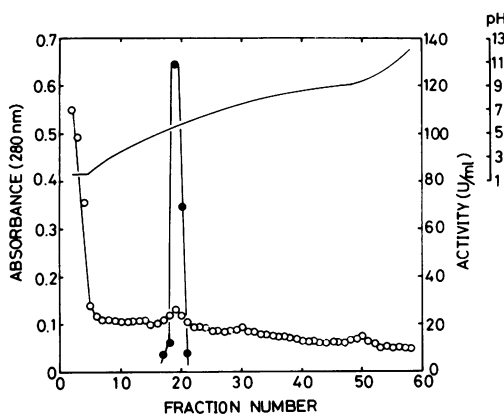


FIG. 3. Isoelectric focusing of the purified acnecin. Symbols: (●) activity; (○) absorbance at 280 nm; (—) pH.

TABLE 2. Amino acid composition of acnecin

Amino acid	Content
Lysine	70
Histidine	10
Arginine	42
Aspartic acid	95
Threonine	67
Serine	59
Glutamic acid	129
Proline	69
Glycine	104
Alanine	102
Cystine	0
Valine	83
Methionine	15
Isoleucine	45
Leucine	76
Tyrosine	19
Phenylalanine	15

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

tored by growth inhibition on *P. acnes* EXC-1) were not affected. On the other hand, the strains that did not produce the inhibitory factor were susceptible to acnecin. Resistant to acnecin were *S. mutans* Ingbritt, *S. sanguis* (ATCC 10556, ATCC 10557), *S. salivarius* (ATCC 9759), *S. mitis* (ATCC 9811, ATCC 9895), *Actinomyces naeslundii* (ATCC 12104), *A. viscosus* (ATCC 19246), *Fusobacterium nucleatum* (ATCC 25286), *Bacterionema matruchotii* (ATCC 14266), and *Staphylococcus aureus* (FDA 209P).

DISCUSSION

The bacteriocin-like substance found in supernatant fluid of ultrasonic treatment of *P. acnes* CN-8 was purified to a homogeneous state by a combination of ammonium sulfate precipi-

tation, ion exchange chromatography, and gel filtration. In the purification steps, fractionation by negative adsorption to DEAE-cellulose is considerably advantageous, since many other substances were removed, judged by polyacrylamide gel electrophoresis (data are not shown), by this easy technique without great loss of activity.

The purified acnecin was inactivated readily by heating at 60°C or by protease digestion, suggesting that a site that is essential for the activity is likely to be protein in nature, but no explanation can be provided presently for inactivation by lysozyme treatment. Heterogeneity in isoelectric point as demonstrated in colicin E2 (11) was not found in acnecin. Its relatively low isoelectric point (pI 5.5) is reflected in its higher contents of the acidic amino acids. The molecular weight of native acnecin was about 60,000, whereas results obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggest that acnecin may consist of subunits having a molecular weight of 12,000. Foulds reported (8) that the bacteriocin from *Serratia marcescens* readily aggregated to a large molecular size of over 10^6 . These aggregates could be dissociated with 0.4 M NaCl to monomeric form, but such an effect of NaCl was not observed in acnecin.

The possibility that the entity of acnecin is a phage particle or lytic enzyme can be excluded for the following reasons. (i) Acnecin was not

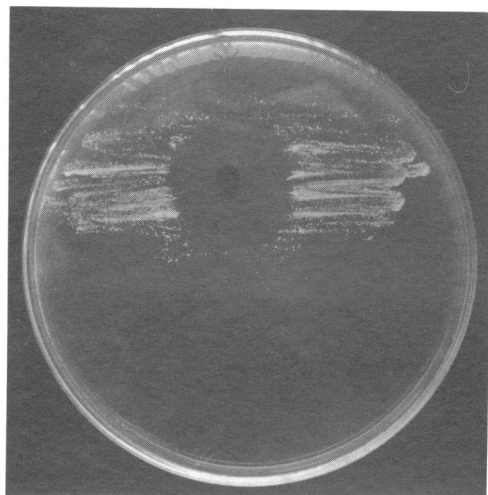


FIG. 4. Effect of acnecin on the indicator strain. Acnecin (60 U) and *P. acnes* EXC-1 (1.1×10^8 colony-forming units) were mixed, and a drop of the mixture was placed on a brain heart infusion-yeast extract agar plate. After incubation for 3 days, the upper spot was streaked out with a sterile loop and incubated for a further 3 days.

precipitated from the ultrasonic extract by centrifugation at $100,000 \times g$ in the purification step. (ii) The molecular weight of native acnecin (60,000) is too small to be a phage particle. (iii) In the course of experiments of viable count of the indicator cells treated with acnecin, not only the viability of the cells, but also the turbidity of the mixture was not reduced.

Most bacteriocins are extracellular and found in culture supernatants, whereas others are surface bound or intracellular. Acnecin could not be detected in the culture supernatant, even if it was concentrated up to 10-fold, and attempts to liberate bacteriocins from the cell surface with high concentrations of salt (8, 11) were not effective. Therefore, acnecin probably occurs intracellularly, although the possibility that acnecin could be liberated from the cell surface by mechanical treatment such as the ultrasonication employed here, similar to the case of the bacteriocin of *S. mutans* (6, 21), cannot be excluded.

Although most bacteriocins of gram-positive bacteria exhibit activity against a wide range of species (31), the inhibitory effect of acnecin was restricted to members of the same species or to *C. parvum*, a closely related species (18). The fact that the producer strains were resistant to acnecin leads us to an expectation that immune mechanisms to endogenous bacteriocin exist in this case as documented for immunity to colicin (13, 29) and cloacin (4, 20).

The acnecin that we describe here is rather peculiar in that its effects are bacteriostatic, since a number of kinetic studies of bacteriocins from various species have revealed that these others act as bactericidal agents. Although the action of acnecin seems to be different in this respect, it has a notable feature in common with other bacteriocins. As Jetten and Vogels pointed out (14), there are many similarities in amino acid composition among bacteriocins from different species of *S. aureus* (14), *S. marcescens* (8), *Lactobacillus fermenti* (5), and *Proteus morgani* (30) and colicin (11). The most striking similarity is high content of aspartic acid, glutamic acid, glycine, and alanine. The contents of these four amino acids were calculated to be 41, 49, 44, 42, and 46%, respectively. The corresponding value of acnecin was 43%.

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