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Purification and Some Properties of Diplococcin from *Streptococcus cremoris* 346

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Eleven of 150 *Streptococcus cremoris* strains examined produced the bacteriocin diplococcin. The diplococcin activity spectrum was restricted to *S. cremoris* and *Streptococcus lactis* strains, and none of a wide range of other gram-positive or gram-negative strains were inhibited. The diplococcin produced by *S. cremoris* 346 was purified by ammonium sulfate precipitation and column chromatography. Purified diplococcin was very unstable at room temperature and lost 75% of its activity after heating at 100°C for 1 min. The proteolytic enzymes trypsin, pronase, and α-chymotrypsin completely inactivated diplococcin. The amino acid composition showed a high content of acidic and neutral acids and a correspondingly low content of basic amino acids, including one residue of ornithine per mole. From the amino acid analysis a molecular weight of 5,300 was estimated. Diplococcin was readily distinguished from the *S. lactis* bacteriocin nisin by its restricted activity spectrum, its biological properties, and by cross-reaction experiments.

Antibiotic-producing streptococci were first described by Whitehead and and Riddet (22), who isolated strains from raw milk that were capable of inhibiting growth and acid production of starter cultures during cheese making. Further studies (21) identified one such producer as *Streptococcus cremoris* and showed that the inhibitory agent was protein in nature. Mattick and Hirsch isolated inhibitor-producing strains of *Streptococcus lactis* from milk and starters. The inhibitor from these strains, which was termed nisin (11), had a wide spectrum of activity, including activity against bacilli, clostridia, and lactobacilli, and was recognized as a potential food preservative. Extensive studies established nisin in this role and provided basic data on its synthesis, chemical structure, and mode of action (6). In contrast, the earlier described inhibitor from *S. cremoris* was inactive against *Bacillus subtilis* (21) or other sporeformers and has received little attention. Oxford (12) partially purified an antibiotic-like substance termed diplococcin from *S. cremoris,* and since Oxford's work there have been no further reports on diplococcin.

S. cremoris is now the species of group N streptococci most commonly used for cheese making. It is the dominant type in many mixed-strain starters (18) and also forms the basis for both paired and multiple defined starters (9).

New single strains of *S. cremoris* have been isolated, evaluated, and used commercially in increasing numbers in recent years (5). The need to combine up to six strains when constructing multiple starters prompted a study of the extent of antibiotic production within the *S. cremoris* group. This paper also describes the purification and some properties of diplococcin isolated from producing strain *S. cremoris* 346.

MATERIALS AND METHODS

Bacterial strains. The *S. cremoris* strains were from the culture collection of the New Zealand Dairy Research Institute. These cultures from frozen stocks (−75°C) were thawed and maintained in both autoclaved reconstituted skim milk and M17 broth (17).

Detection and assay of inhibitory production. Cultures were screened for inhibitor production by both the deferred and simultaneous antagonism methods. In the deferred antagonism procedure (16) M17 agar plates were stabbed with the test culture and incubated for 48 h at 22°C. Growth which developed at the surface was killed by exposure to chloroform. The plates were then overlaid with 2.5 ml of soft agar inoculated with 0.1 ml of a 24-h broth culture of the indicator strain, *S. cremoris* 480B_1. The plates were incubated at 22°C and examined at 24 and 48 h for zones of inhibition.

Simultaneous antagonism was shown by preparing lawns of the indicator strain as described above and inoculating with 10-μl volumes of overnight cultures, grown in M17 broth, of the test organisms. Plates were incubated at 22°C and examined after 24 and 48 h.

Titers of inhibitory activity were determined by a standard surface-spotting dilution test on the appropriate indicator lawns. One arbitrary unit was defined as the highest twofold dilution of the test preparation to give definite inhibition on the indicator lawn. The titer of the preparation was the reciprocal of this dilution (14). Specific activities were expressed in terms of arbitrary units per milligram of protein after

determining the amount of protein in the samples by the method of Lowry et al. (10), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Purification of diplococcin. *S. cremoris* 346 was grown to stationary phase (1% inoculum) in M17 broth at 22°C, without agitation. Cells from this culture were removed by centrifugation, and the supernatant fluid was immersed in boiling water for 30 min. After cooling, solid ammonium sulfate was added to 60% saturation, and stirring was continued for 6 h at 4°C. The precipitate was collected by centrifugation and redissolved in a smaller volume (10 ml) of 0.01 M sodium citrate buffer, pH 5.0. This was designated "partially purified diplococcin."

Further purification was by ion-exchange chromatography on carboxymethyl cellulose (Whatman Biochemicals Ltd., Maidstone, Kent, England) at 4°C. Samples (4 ml) were applied to a column (1.2 by 12 cm) and washed with 0.01 M sodium citrate buffer, pH 5.0, and elution was carried out with a linear gradient of 0 to 0.5 M NaCl. The fractions were read at 280 nm and assayed, and fractions containing inhibitory activity were pooled, dialyzed, and concentrated with polyethylene glycol. This purified material was then sterilized by membrane filtration (Millipore Corp., Bedford, Mass.) and stored at −75°C.

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried out at pH 8.9 (2), pH 2.2 (7), and pH 7.0 on 10% polyacrylamide gels in the presence of 0.1% (wt/vol) sodium dodecyl sulfate (20). After electrophoresis at 5 mA/gel, the gels were removed from the tubes, fixed in 20% sulfosalicylic acid, stained in 0.25% Coomassie blue for 2 to 3 h, and destained electrophoretically or by washing in repeated changes of 7% acetic acid.

Effect of enzymes. Trypsin (BDH, Pode, England), pronase (Koch Light Laboratories, Colnbrook, Bucks, England), and α-chymotrypsin (Sigma Chemical Co.) were each dissolved in 0.01 M phosphate buffer, pH 7.0, to give solutions containing 0.1 mg of enzyme per ml. Diplococcin was mixed with equal volumes of the enzyme solutions and incubated at 37°C for 1 h; as a control, diplococcin without added enzyme was treated in the same manner as the test preparations.

Molecular weight estimation. Partially purified diplococcin (3 ml) was applied to a Sephadex G-100 column (1.6 by 78 cm) equilibrated with 0.01 M sodium citrate (pH 5.0) buffer and 0.1 M NaCl at 4°C. Blue dextran 2000 (1 mg) and 2,4-dinitrophenylalanine (40 μg) were used to calibrate the column. Ovalbumin (molecular weight, 45,000), ribonuclease (molecular weight, 13,700), nisin (molecular weight, 7,000), and lima bean trypsin inhibitor (molecular weight, 6,500) were used as the reference proteins. The flow rate was 12 ml/h, and 2.4-ml fractions were collected and assayed for absorbance at 230 nm and for diplococcin activity. The titer of each active fraction was determined.

Amino acid analysis. Aliquots (1 ml) of pure diplococcin were hydrolyzed in vacuo for 24 and 72 h in 6 M HCl at 110°C, dried, and redissolved in 5 ml of sample buffer (pH 2.2). The samples were analyzed on a Locarte Mk IV analyzer using the normal program (13). Nisin (Koch Light Laboratories) hydrolyzed for 24 h with 6 M HCl at 110°C, DL-lanthionine (BDH), L-ornithine, and a 25-nm/ml basic physiological mixture (Beckman Instruments, Inc., Fullerton, Calif.) were also analyzed.

RESULTS

Screening of cultures. Eleven of 150 *S. cremoris* strains were found to produce inhibitor(s) after simultaneous and deferred antagonism tests on M17 agar plates. Inhibition was not due to bacteriophage, hydrogen peroxide, or a low pH. Dilution to extinction of the inhibitor preparation gave diminished zones but no bacteriophage plaques. Incorporation of varying concentrations of catalase into M17 agar did not interfere with the inhibitory effect. When the pH of the cultures after growth was readjusted to 6.0 to 6.5, there was no reduction in inhibition.

The activity spectrum of these producers was restricted to other *S. cremoris* and *S. lactis* cultures. Inhibition was more pronounced in the former strains than in the latter. No inhibition was observed with a variety of other gram-positive or gram-negative organisms. These included strains (number given in parentheses) of *Bacillus* (2), *Micrococcus* (1), *Staphylococcus* (6), *Streptococcus* (4), *Enterobacter* (2), *Escherichia* (4), *Pseudomonas* (3), *Flavobacterium* (1), and *Salmonella* (4). As expected, none of the producer cultures appears active when tested against itself by the stab procedure, indicating that the producers are immune to at least nominal levels of their own inhibitors. It was not possible to differentiate the inhibitors from one another on the basis of their activity spectrum, biological properties (heat, sensitivity to enzymes and chloroform), or cross-reaction experiments. These strains were designated as diplococcin producers, and *S. cremoris* 346 was selected for further investigation.

Purification of diplococcin. Production of diplococcin by *S. cremoris* 346 in the supernatant of M17 broth at 22°C could be detected 6 to 8 h after growth began and paralleled growth for the most part (Fig. 1). The highest titer was reached as the cells entered the stationary phase and the pH of the broth was 4.8 to 5.0. Titers of diplococcin from cells grown in reconstituted skim milk at 22°C were comparable to those of broth. Attempts to induce diplococcin production with mitomycin C (0.5 to 2 μg/ml) or ultraviolet light (5 to 30 s) did not enhance the production of diplococcin when the cells were subsequently grown in liquid media.

The purification procedure is summarized in Table 1. The crude supernatant was heated before precipitation with ammonium sulfate to sterilize the supernatant and to inactivate any

heat-labile enzymes that might reduce the recovery of antibiotic during purification.

Ammonium sulfate precipitation eliminated most of the contaminating protein. Further purification was achieved by chromatography on carboxymethyl cellulose CM 32 (Fig. 2). Fractions 18 through 22 contained the antibiotic when assayed as described previously.

When the purified diplococcin from CM 32 chromatography was run on sodium dodecyl sulfate-polyacrylamide gels, a single, diffuse band was observed that migrated near the bromophenol blue dye band. No bands could be observed when the polyacrylamide gels were run at low or high pH in the absence of 0.1% sodium dodecyl sulfate. In a further experiment, purified material eluted from a Sephadex G-100 column (1.5 by 100 cm) as a single symmetrical peak of activity, indicating that the preparation was homogeneous.

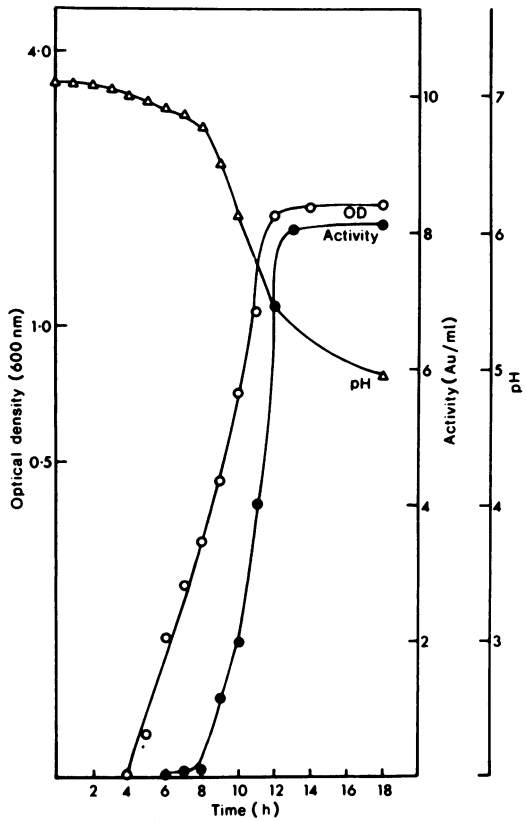

FIG. 1. *Measurement of growth and diplococcin production in M17 broth with S. cremoris 346 culture. Au, Arbitrary units.*

Properties of diplococcin. Samples of both partially purified and purified diplococcin stored at −75°C showed no loss in activity for up to 3 months. By comparison, at 4°C and at room temperature purified diplococcin was unstable, being completely inactivated after 1 week and 5 h, respectively. Attempts to stabilize purified diplococcin with Mg^{2+} and Ca^{2+} (10^{-2} M) and protein (bovine serum albumin, 0.5% [wt/vol]) were unsuccessful, but full protection was achieved by the addition of complete M17 broth to the pure samples.

Other properties investigated were the sensitivity to temperature, changes in pH, and the susceptibility to proteolytic enzymes. The partially purified diplococcin (pH 5.0) showed no detectable loss in activity when heated at 100°C for 1 h. However, with increasing alkalinity up

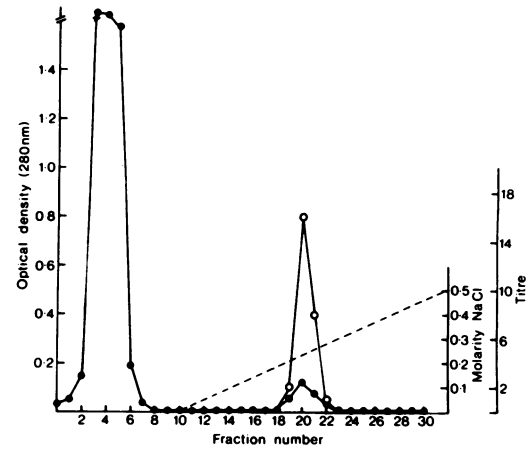

FIG. 2. *Carboxymethyl cellulose chromatography of diplococcin 346 after ammonium sulfate precipitation. Elution was with a continuous gradient 0 to 0.5 M NaCl in 0.01 M sodium citrate buffer, pH 5.0. Each 3-ml fraction was assayed for diplococcin activity* (○) *and protein* (●).

TABLE 1. *Yield and specific activity of diplococcin 346 at various stages in the purification procedure*

Stage	Vol (ml)	AU[a]/ml	Protein (mg)	Sp act (AU/mg)	Recovery (%)	Purification (fold)
Culture supernatant fluid	1,000	8,000	6,300	1.26	100	1
Ammonium sulfate precipitate	40	5,120	392	13.0	64	11
CM 32 chromatography	720	4,320	3.6	1,200	54	952
Pure diplococcin	90	3,600	2.8	1,285	45	1,020

[a] AU, Arbitrary units.

to pH 11.0, diplococcin became less heat stable. In contrast, the pure preparation was unstable even at acid pH and lost 75% of its activity at pH 5.0 after 1 min at 100°C. In another experiment, two 3-ml samples of purified material, one heated for 3 min at 100°C and the other unheated, were passed through a Sephadex G-50 column. Both samples gave identical peaks, suggesting that inactivation of diplococcin did not result in hydrolysis of the protein but that it remained as an intact molecule. Both partially purified and purified preparations were completely inactivated by the enzymes trypsin, pronase, and α-chymotrypsin.

Molecular weight estimation. Partially purified diplococcin was used for molecular weight estimations, since a considerable loss of activity occurred when purified diplococcin was chromatographed on Sephadex G-100. The broad asymmetrical peak of antibiotic activity observed for diplococcin was considered to consist of two overlapping peaks corresponding to components with molecular weights of about 6,000 and 9,100 (Fig. 3).

Amino acid composition. The amino acid composition of purified diplococcin is presented in Table 2. An unidentified peak, which eluted between histidine and lysine, was found to be ornithine. This was based on a comparison of its elution position with that of an ornithine standard, cochromatography of ornithine and the hydrolysate of diplococcin, and comparison with the amino acids from a basic physiological calibration mixture. When a hydrolysate of nisin and a lanthionine standard were subjected to amino acid analysis, it was found that the elution positions of β-methyl-lanthionine and lanthionine did not correspond to those of the amino acids present in a diplococcin hydrolysate. This was also clearly demonstrated by cochromatography of the hydrolysates of nisin and diplococcin.

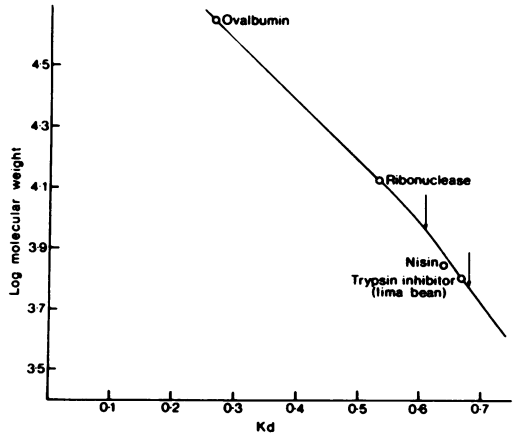

FIG. 3. *Estimation of the molecular weight of diplococcin from Sephadex G-100 chromatography. The arrows indicate the elution positions of the peaks of diplococcin activity.*

DISCUSSION

The antibacterial inhibition shown by 15 of 150 strains of *S. cremoris* is caused by the antibiotic diplococcin. Although any substance produced by one strain active against another has been classically termed "antibiotic" (19), this overall grouping is now so broad that subdivision is desirable. Hence, although diplococcin has been categorized as an antibiotic, it shows characteristics consistent with those of the class of bacterial inhibitors called bacteriocins. That is, diplococcin has a protein nature, an inhibitory spectrum centered about the homologous species, and plasmid-borne genetic determinants (15; unpublished data).

The stability of bacteriocin preparations has previously been shown to decrease substantially with increased purification (3). Whereas the pu-

TABLE 2. *Amino acid composition of diplococcin 346*

Amino acid	Residues per mol	Most probable residues per mol		
		Diplococcin	Nisin[a]	Subtilin[a]
Aspartic acid	4.1	4	1	1
Threonine	2.2	2		
Serine	4.7	5	1	
Glutamic acid	5.9	6		3
Proline	2.6	3	1	1
Glycine	9.0	9	3	2
Alanine	3.7	4	2	1
Cysteine				
Valine	2.5	3	1	1
Methionine	0.5	1	2	
Isoleucine	1.4	1	3	1
Leucine	2.5	3	2	4
Tyrosine	1.2	1		
Phenylalanine	1.3	1		1
Histidine	1.4	1	2	
Lysine	3.0	3	3	3
Arginine	2.0	2		
Tryptophan	1.0	1		1
Ornithine	1.3	1		
Lanthionine			1	1
β-Methyl lanthionine			4	4
Dehydrobutyrine			1	1
Dehydroalanine			2	2
Σ residues		51	29	27
Mol wt		5,300	3,350	3,320

[a] Data from Gross (4).

rification procedure did not result in a large loss of activity, the purified diplococcin was unstable, and the inability of Ca^{2+}, Mg^{2+}, and bovine serum albumin to stabilize this material made further experiments difficult. However, M17 broth, which is a complex medium, was able to stabilize purified diplococcin. Although no study of the effects of individual components in M17 broth was undertaken, presumably the loss of certain protective molecules during purification may have resulted in the instability of diplococcin.

In the partially purified form, diplococcin, like some of the bacteriocins from other gram-positive species, e.g., *Staphylococcus epidermidis, Lactobacillus,* and *Streptococcus agalactiae,* was resistant to heat at low pH. The loss of activity upon heating the purified form is likely to be due to denaturation of the protein or to the loss of a cofactor. It is unlikely to be due to the effects of proteolysis, since low-molecular-weight products were not observed upon subsequent gel chromatography of the heated material.

The shape of the peak of diplococcin activity by chromatography of the ammonium sulfate-precipitated fraction on Sephadex G-100 indicated the presence of two components having molecular weights of 6,000 and 9,100. Although the molecular weight values are only approximate since polypeptides with molecular weights of <10,000 do not always behave as ideal globular proteins on Sephadex G-100 (1), the presence of monomer-dimer forms of diplococcin cannot be discounted. These values for the molecular weight are, however, consistent with the minimum molecular weight of 5,300 obtained from the amino acid composition (Table 2).

The observation that diplococcin is a protein (12) was confirmed. Biological activity is rapidly destroyed by several proteolytic enzymes, the bacteriocin could be stained by Coomassie brilliant blue R250 and gave a positive Folin reaction, and amino acid analysis revealed the presence of amino acids expected in a normal protein hydrolysate. The presence, however, of small amounts of carbohydrate or lipid could not be discounted.

The observation by Hirsch (Proc. Soc. Appl. Bacteriol., p. 26, 1946) that diplococcin is different from nisin produced by *S. lactis* was also confirmed in this study. Diplococcin has an activity spectrum confined to activity against other group N streptococci and is sensitive to proteolytic enzymes. In comparison, nisin has a much wider activity spectrum that includes activity against many gram-positive, but not gram-negative, organisms. Although it is not sensitive to most proteolytic enzymes, it is inactivated by α-chymotrypsin (8). It is apparent that the amino acid composition of diplococcin (Table 2), and therefore the structure, is considerably different from those of nisin and subtilin, both of which are pentacyclic peptides (4). Both nisin and subtilin contain three residues of α,β-unsaturated amino acids (two residues of dehydroalanine and one residue of dehydrobutyrine) as well as one residue of lanthionine and four residues of β-methyl-lanthionine. The unusual sulfur-containing acids lanthionine and β-methyl lanthionine were absent in diplococcin. The α,β-unsaturated acids cannot be identified directly in acid hydrolysates as they are subject to degradation during acid hydrolysis (4). Diplococcin is unusual, however, in that it appears to contain one residue of ornithine per mole of protein. Ornithine is known to be produced from arginine by alkali treatment of proteins (4). However, this is unlikely since diplococcin was not subjected to alkaline conditions during purification.

Starters for cheese making are chosen on the basis of properties which include reliable acid production and differing sensitivities to virulent bacteriophages. Strains of *S. cremoris* that are diplococcin producers cannot be included as components of either paired or multiple starters, without rapidly becoming dominant and consequently affecting acid production. The property of immunity is the ability of a bacterium which produces a bacteriocin to survive the action of a similar bacteriocin which would otherwise kill it. This property could be utilized by constructing multiple starters composed entirely of such starter strains. At present, however, this approach has not been possible due to the relatedness between the bacteriophage patterns of many of the producers. An alternative approach has been to "cure" such strains of their ability to produce diplococcin without affecting any other starter properties. This aspect is presently under investigation.

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