Biological and Biochemical Characterization of Novel Lipid-like Antibacterial Substances (Mutilipocins) Produced by *Streptococcus mutans* Strain 32K

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Two novel antibacterial substances (designated mutilipocins) have been isolated from the culture supernatant of *Streptococcus mutans* strain 32K (serotype *c*). The mutilipocins were purified by extraction of the culture supernatant with light petroleum (b.p. range 30–60 °C), followed by Lobar column chromatography on Lichroprep RP-8. HPLC indicated that both mutilipocin preparations (ML-I and ML-II) were homogeneous. The M_r values of ML-I and ML-II were less than 1000. Both mutilipocins were unaffected by treatment over the pH range 3.0–10.0, or with phospholipase A or proteolytic enzymes, but were partially inactivated by treatment with lipase or phospholipase C. ML-II was resistant to heat treatment. TLC indicated that ML-I and ML-II contained unsaturated, aldehyde and/or ketone, and ester groups. The inhibition of *S. mutans* by ML-I and ML-II was due to bactericidal, rather than bacteriostatic, activities. The antibacterial spectra of ML-I and ML-II were narrower and more species-specific than those of bacteriocins produced by other Gram-positive bacteria.

INTRODUCTION

Streptococcus mutans is the principal etiological agent of dental caries (Hamada & Slade, 1980). Many strains of *S. mutans* produce bacteriocins which inhibit the growth of the same or closely related species (Delisle, 1975; Fukushima *et al.*, 1982; Hamada & Ooshima, 1975; Ikeda *et al.*, 1982; Paul & Slade, 1975; Rogers, 1972). Early studies on the bacteriocins of *S. mutans* suggested that their production was affected by the culture conditions (Kelstrup & Funder-Nielsen, 1977) and that their chemical and biological properties differ among strains. Several investigators (Ikeda *et al.*, 1982; Hillman *et al.*, 1984; Fukushima *et al.*, 1982) have reported that bacteriocins produced by *S. mutans* are proteins. Recently, two novel lipid-like antibacterial substances were found in the culture supernatant of *S. mutans* 32K (serotype *c*) in our laboratory, and named mutilipocins. This study reports the isolation and purification of mutilipocins, and their biological and biochemical properties.

METHODS

Bacterial strains and media. The mutilipocin producing strain *S. mutans* 32K (serotype *c*) was isolated from human dental plaque. A laboratory stock culture of *Streptococcus sobrinus* 6715 (serotype *g*) was used as an indicator strain for assaying the activity of mutilipocin. Other bacteria used in this study are listed in Table 1. Stock cultures of each strain were maintained at 4 °C on Brain Heart Infusion (BHI; Difco) agar containing excess CaCO₃. The partially defined medium M4 (Fukushima *et al.*, 1981) containing 0.05% (v/v) Tween 80 was used for the production of mutilipocin.

Screening strains of the *S. mutans* group for mutilipocin production. Mutilipocin production was assayed by the overlay procedure of Fredericq (1957). Strains of *S. mutans* were grown in BHI broth for 18 h at 37 °C and 0.1 ml of an appropriate dilution of this culture was seeded onto mitis-salivarius (MS) agar (Difco). After anaerobic incubation for 18 h at 37 °C, the plates were overlaid with the same agar containing the indicator strain *S. sobrinus* 6715. Following incubation of the plates for an additional 24 h, the zone of inhibition surrounding the indicator strain was measured.

Mutalipocin assay. Mutalipocin activity was determined by using a modification of the method of Hayes *et al.* (1983). A BHI agar plate was overlaid with 100 μ l of a suspension of exponential-phase cells of *S. sobrinus* 6715 ($OD_{540} = 1.0$). Test culture supernatants and mutalipocin preparations were serially diluted in 0.01 M-sodium phosphate buffer (pH 7.2) and light petroleum (b.p. range 30–60 °C), respectively, and samples (10 μ l) were placed in duplicate into wells in the agar plates. After 18 h incubation at 37 °C, the diameter of the zone of inhibition of the indicator organism around each well was measured. The log of mutalipocin concentration was proportional to the diameter of the inhibitory zone. Mutalipocin activity was expressed as arbitrary units (U) ml⁻¹. The number of mutalipocin U ml⁻¹ was defined as the reciprocal of the highest dilution of supernatant that gave a clear zone of inhibition of *S. sobrinus* 6715 cell growth according to the method of Dajani *et al.* (1976).

Purification. The crude mutalipocin was initially extracted from 3 litres of culture medium by the Folch method (Folch *et al.*, 1951). *S. mutans* 32K was grown aerobically for 18 h at 30 °C in M4 medium containing 0.05% (v/v) Tween 80. The culture supernatant, obtained after centrifugation (12000 g, 10 min, 4 °C), was evaporated to dryness at room temperature under reduced pressure and the extract homogenized with 10 parts chloroform/methanol (2:1, v/v). The homogenate was centrifuged at 1500 g for 10 min, and the extract was collected, mixed with 0.5% (v/v) NaCl in the ratio 1:10 and stored overnight at 4 °C. After gentle removal of the upper aqueous layer, the clear chloroform phase was recovered. The remaining chloroform extract at the interface and in the aqueous layer was recovered by mixing with an equal volume of chloroform. The chloroform extracts were combined and concentrated under reduced pressure. The residue was treated with distilled water/n-hexane (1:1, v/v), and then the same volume of light petroleum was added to the aqueous layer. The extraction procedure was repeated three times. Following removal of the gelatinous Tween 80 from the light petroleum layer, the remaining light petroleum layer was evaporated to a small volume. Of this material, 5 ml (crude mutalipocin) was then applied to a Lobar column containing Lichroprep RP-8 prepared in methanol/water (90:10, v/v). The column was eluted with the same solvent under low pressure and the eluate was monitored for absorbance at 254 nm. The position of the peak with mutalipocin activity coincided with that of the UV absorbance peak. The active fractions were pooled, lyophilized and then reappplied to a Lobar column containing Lichroprep RP-8 prepared in methanol/water (80:20, v/v). The column was eluted using the same solvent under low pressure. Two active fractions, which were designated ML-I and ML-II, were obtained by the above process.

Analytical methods. Analytical reverse-phase HPLC (RP-HPLC) was done with a model 510 liquid chromatograph fitted with a 254 nm wavelength detector (Waters). The column used was a Lichrosorb RP-8 (4 $\times$ 200 mm, Merck). Analytical TLC was performed on Kiesel gel 60F₂₅₄ (Merck) with chloroform/methanol/ammonia (60:35:8, by vol.) and on RP-8 F₂₅₄ with methanol/water (85:15, v/v). Standard lipids were used for comparison. ML-I and ML-II were also recognized by detecting the spots under UV light (254 nm). Lipids were detected on TLC plates using the following reagents: non-specific lipids – I₂ (Barrett, 1962); phospholipids – Dittmer (Dittmer, 1964); choline – Dragendorff (Beiss, 1964); glycolipids – α -naphthol/H₂SO₄ (Jacin & Mishkin, 1965); amino phosphatides – ninhydrin (Fahmy *et al.*, 1961); steroids – Zactis (Lowry, 1968); unsaturated groups – OsO₄; carbonyl groups – 2,4-dinitrophenylhydrazine (Skipski & Barclay, 1969); esters – hydroxylamine hydrochloride (Skidmore & Entenman, 1962). The *M_r* range of ML-I and ML-II was determined by the method of Ikeda *et al.* (1982). ML-I and ML-II solutions (1 ml) were placed onto sterile ultrafiltration membranes (Toyo; *M_r* cut off at 10000, 5000, 1000 and 500) laid on M4 semisolid medium seeded with *S. sobrinus* 6715. After 18 h of incubation at 37 °C, the diameter of the zone of inhibition was measured.

Bactericidal effect. *S. sobrinus* 6715 was grown for 18 h at 37 °C in M4 medium. The cells were collected, washed three times with NaCl and suspended to an OD_{540} of 1.0. Samples (5 μ l) of the cell suspension were added to 5 ml 0.05 M-Tris/HCl, pH 7.0, containing 100 μ g ML-I ml⁻¹ or 50 μ g ML-II ml⁻¹. The mixtures were incubated for 15, 30, 45 and 60 min at 37 °C, and then samples of the mixtures were immediately diluted and spread on BHI agar plates. The number of c.f.u. was counted after overnight incubation at 37 °C.

Method for assessing antibacterial activity. Sterile solutions containing various concentrations of ML-I and ML-II were prepared in light petroleum. From each solution, 10 μ l of material was taken and carefully spotted onto a BHI agar plate seeded with 5×10^5 c.f.u. of the test strain as described for the mutalipocin assay. After 18 h incubation at 37 °C, the diameter of the zone of inhibition was measured. Mutalipocin activity was expressed as the minimum inhibitory concentration (MIC) for test strains.

Chemicals. Lipase, phospholipase A, phospholipase C and phospholipase D were obtained from Sigma. Lichroprep RP-8 and Lichrosorb RP-8 were from Merck.

RESULTS

Mutalipocin production

Out of 192 human clinical isolates of *S. mutans*, 50 (26%) demonstrated antibacterial activity by the overlay procedure and the supernatant of *S. mutans* 32K formed the clearest and widest zone of inhibition against *S. sobrinus* 6715.

Fig. 1. Growth curve of *S. mutans* 32K and the secretion of mutalipocin activity. ○, Cell growth (OD_{540}) at 30 °C in M4 medium; the minimum generation time was 80 min. ●, Mutalipocin activity, determined as described in Methods.

Fig. 2. Lobar column chromatography of crude mutalipocin. Crude mutalipocin was applied on a column (10 × 240 mm) of Lichroprep RP-8 (size A) at room temperature, a flow rate of 2.0 ml min⁻¹ and a pressure of 1.2 kg cm⁻². The eluate was monitored at 254 nm. The mobile phase consisted of methanol/water (90:10, v/v) (a) or methanol/water (80:20, v/v) (b). Mutalipocin activity (●) is expressed as U ml⁻¹.

Optimal production of mutalipocin was obtained when *S. mutans* 32K was grown aerobically in M4 medium at 30 °C; the addition of 0.05% Tween 80 to the culture medium gave 10-fold higher amounts of mutalipocin (data not shown). The secretion of mutalipocin into M4 medium was dependent upon the phase of bacterial growth (Fig. 1). Mutalipocin continued to accumulate in the culture medium during the exponential phase, and the greatest amount of mutalipocin was present during the late-exponential phase of growth. Thus, for optimal production of mutalipocin, the cultures were grown to the late exponential phase ($OD_{540} = 1.3$). Although a small amount of mutalipocin was found in the cell fractions after disruption of the cells, its activity was very weak.

Purification of mutalipocin

Crude mutalipocin extracted from the culture supernatant of *S. mutans* 32K was passed through a Lichroprep RP-8 Lobar column using methanol/water (90:10, v/v) as eluant (Fig. 2a). Highly polar materials were not retained in the column, while the antibacterial agent eluted after

Fig. 3

Fig. 3. Separation of ML-I and ML-II by HPLC. Active fractions that were pooled after Lobar column chromatography were applied on a column (4×200 mm) of Lichrosorb RP-8 (size A) at room temperature and at a flow rate of 1.0 ml min^{-1} and a pressure of 150 kg cm^{-2} . The eluate was monitored at 254 nm. The mobile phase consisted of methanol/3% (v/v) aqueous triethylamine, pH 3.0 (75:25, v/v).

Fig. 4

Fig. 4. Bactericidal effect of purified ML-I and ML-II on cells of *S. sobrinus* 6715. ○, Control (no mutalipocin); ●, $100 \mu\text{g ML-I ml}^{-1}$; △, $50 \mu\text{g ML-II ml}^{-1}$.

20 min as a broad-shouldered peak. The active fractions were pooled, concentrated and rechromatographed on the same column using a different methanol/water ratio (80:20, v/v) for elution (Fig. 2*b*). Two peaks were obtained, designated ML-I and ML-II; both exhibited mutalipocin activity, with ML-II showing twofold higher activity than ML-I. Further analysis of the fractions on a Lichrosorb HPLC using methanol/3% (v/v) aqueous triethylamine, pH 3.0 (75:25, v/v) also revealed two distinct peaks (Fig. 3).

The purified ML-I was stable, but ML-II lost antibacterial activity during storage in light petroleum at 4°C for several months. The addition of an antioxidizing agent (butylated hydroxytoluene) in light petroleum did not prevent the loss of activity. The yields of purified ML-I and ML-II from 3 l of culture were $500 \mu\text{g}$ and $100 \mu\text{g}$, respectively. The specific activities of ML-I and ML-II were 2000 and 8000 U mg^{-1} respectively.

The M_r range of the mutalipocins was estimated by membrane exclusion since it was shown that the activity as determined by the diameter of the clear zone was proportional to the pore size of the membranes. More than 80% of the activity of ML-I and ML-II was retained by using membranes that excluded molecules of M_r larger than 1000, but the activity was not retained by using membranes that excluded molecules of M_r larger than 500. These results indicated that the M_r range of these mutalipocins was between 500 and 1000.

Bactericidal effect

The kinetics of cell killing by ML-I and ML-II are shown in Fig. 4. Treatment of *S. sobrinus* 6715 grown to exponential phase with either ML-I or ML-II decreased the number of c.f.u. The data show that ML-I and ML-II have bactericidal rather than merely bacteriostatic effects. The shape of the plots in Fig. 4 is consistent with single-hit kinetics for the killing of *S. sobrinus* 6715 by ML-I and ML-II.

Sensitivities of various bacteria to ML-I and ML-II

Various genera were tested for their sensitivities to purified ML-I and ML-II (Table 1). The antibacterial activities of ML-I and ML-II were greater against most strains of streptococci

Table 1. Relative antibacterial activity of mutalipocins ML-I and ML-II extracted from *S. mutans* strain 32K

Indicator strain	MIC ($\mu\text{g ml}^{-1}$)	
	ML-I	ML-II
<i>Streptococcus cricetus</i> OMZ-61 (a)*	1.0	0.1
<i>Streptococcus rattus</i> BHT (b)*	1.0	0.1
<i>Streptococcus mutans</i> PS-14 (c)*	2.0	0.1
<i>Streptococcus mutans</i> LM-7 (e)*	2.0	0.5
<i>Streptococcus mutans</i> OMZ175 (f)*	2.0	0.2
<i>Streptococcus sobrinus</i> B-13 (d)*	4.0	1.0
<i>Streptococcus sobrinus</i> 6715 (g)*	1.0	0.2
<i>Streptococcus salivarius</i> HHT	0.5	0.1
<i>Streptococcus sanguis</i> ATCC 10556	0.8	0.1
<i>Streptococcus mitis</i> ATCC 9811	2.0	0.2
<i>Streptococcus faecium</i> ATCC 6057	2.0	0.2
<i>Streptococcus bovis</i> ATCC 9809	2.0	0.2
<i>Streptococcus lactis</i> ATCC 19435	1.0	0.1
<i>Streptococcus pyogenes</i> 703	8.0	4.0
<i>Lactobacillus casei</i> ATCC 7469	8.0	4.0
<i>Actinomyces viscosus</i> ATCC 19246	8.0	4.0
<i>Actinomyces naeslundii</i> ATCC 12104	8.0	2.0
<i>Streptococcus aureus</i> ATCC 13709	> 100	8.0
<i>Escherichia coli</i> K12	> 100	> 50
<i>Klebsiella pneumoniae</i> NUD	> 100	> 50

* Serotype of the *S. mutans* group.

(except for *S. pyogenes*), including seven serotypes of the *S. mutans* group, than against strains of *Lactobacillus casei*, *Actinomyces viscosus* and *Actinomyces naeslundii*, and that they were ineffective against the strains of *Staphylococcus aureus* (ML-I only), *Escherichia coli* and *Klebsiella pneumoniae*.

Properties of ML-I and ML-II

The characteristics of ML-I and ML-II are summarized in Table 2. The activities of ML-I and ML-II were not affected by pH over the range 3.0–10.0 at 30 °C for 1 h, but both activities were lost during storage at pH 2.0. ML-I, unlike ML-II, was partially inactivated by storage at pH 11.0. The activity of ML-I was reduced by heating at 100 °C for 30 min and at 121 °C for 30 min, but ML-II was not affected by these treatments. The activities of ML-I and ML-II were not influenced by phospholipase A, but were slightly affected by lipase and phospholipase C. ML-II, but not ML-I, was partially affected by phospholipase D. The activities of ML-I and ML-II were not affected by proteolytic enzymes such as trypsin and pronase (data not shown). These results suggested that ML-I and ML-II might be phospholipids, but they differed from any of the commercial phospholipids tested. Table 3 shows the R_F values of ML-I and ML-II on TLC in comparison with other lipid standards. Under the conditions used, ML-I and ML-II were differentiated from other lipid standards including phospholipids. The spots of ML-I and ML-II on TLC were positive with I_2 , OsO_4 , 2,4-dinitrophenylhydrazine and hydroxylamine hydrochloride, but were negative with the Dittmer, Dragendorff, α -naphthol/ H_2SO_4 , ninhydrin and Zactis reagents. These results indicate that ML-I and ML-II contain unsaturated, carbonyl and ester groups. ML-I and ML-II were freely soluble in methanol, acetone and light petroleum, but insoluble in chloroform and water.

DISCUSSION

It is well known that many strains of *S. mutans* produce antibacterial substances, so-called bacteriocins, which are also produced by a variety of Gram-positive and Gram-negative bacteria. For example, Ikeda *et al.* (1982) have purified a bacteriocin from *S. mutans* C3603

Table 2. *Properties of the mutalipocins ML-I and ML-II extracted from S. mutans strain 32K*

The percentages in parentheses represent the residual activity compared to the control.

Property	Response by:	
	ML-I	ML-II
pH stability (aqueous solution)		
pH 2.0	Labile (25%)	Labile (25%)
pH 3.0-10.0	Stable (100%)	Stable (100%)
pH 11.0	Labile (50%)	Stable (100%)
Heat stability (aqueous solution)		
100 °C, 30 min pH 7.0	Labile (50%)	Stable (100%)
121 °C, 30 min pH 7.0	Labile (37%)	Stable (100%)
Enzyme treatment		
Lipase (pH 7.0)	Labile (80%)	Labile (84%)
Phospholipase A (pH 8.0)	Stable (100%)	Stable (100%)
Phospholipase C (pH 6.6)	Labile (90%)	Labile (50%)
Phospholipase D (pH 6.0)	Stable (100%)	Labile (50%)

Table 3. *R_F values of the mutalipocins ML-I and ML-II extracted from S. mutans strain 32K and of lipid standards on TLC plates*

Substance	R _F *	R _F †
ML-I	0.76	0.40
ML-II	0.73	0.30
Sphingomyelin	0.85	0
Phosphatidylcholine	0.82	0
Ganglioside	0.02	0
Phosphatidylethanolamine	0.88	0
Cholesterol	0.95	0.45
Oleic acid	0.80	0.08
Linoleic acid	0.87	0.08
Linolenic acid	0.93	0.08

* Developed in chloroform/methanol/ammonia (60:35:8, by vol.) on Kiesel gel 60F₂₅₄.

† Developed in methanol/water (85:15, v/v) on RP-8 F₂₅₄.

(serotype *c*) culture supernatant which has an M_r of approximately 4800 and which exhibits a narrow antibacterial spectrum. Fukushima *et al.* (1982) have also reported an extracellular bacteriocin from *S. mutans* RM-10 of unknown serotype with an M_r of approximately 973 000. Hillman *et al.* (1984) have isolated a low- M_r cell-free bacteriocin from *S. mutans* JH 1000 (serotype *c*) with a broad antibiotic spectrum.

In this paper, we have reported the purification of a new type of antibacterial substance (mutalipocin) from the culture supernatant of *S. mutans* 32K (serotype *c*), which has different characteristics when compared to bacteriocins. Mutalipocins are lipid-like, low- M_r substances which are soluble in organic solvents. No protein, carbohydrate or phosphorus could be detected in the purified mutalipocins.

Mutalipocins were optimally secreted from cells during the late exponential phase of growth, and the amount present in the medium decreased during the stationary phase. It is possible that mutalipocins could be adsorbed onto cells during the late stationary phase of cell growth. Although mutalipocins are stable physico-chemically, their purification was difficult because of the very low amounts excreted. Mutalipocins have a narrower species-specific spectrum of antibacterial activity than do the bacteriocins produced by *S. mutans* and other Gram-positive bacteria (Tagg *et al.*, 1976), but they act bactericidally on the susceptible indicator cells in a manner similar to the bacteriocins reported by Delisle (1975) and Paul & Slade (1975).

Some unsaturated fatty acids, such as linoleic acid and linolenic acid, are known to inhibit the growth of *S. aureus* and *S. mutans*, and their effectiveness is related both to the degree of unsaturation and to the configuration of the molecule about double bonds (Butcher *et al.*, 1976; Fukui *et al.*, 1980). Carson & Daneo-Moore (1980) also demonstrated that the unsaturated fatty acids oleic and linoleic acid induce lysis of *Streptococcus faecalis*, while saturated fatty acids have little or no effect. Although none of the commercial unsaturated fatty acids or phospholipids that we examined were identical to mutalipocin, it is possible that unsaturated groups in mutalipocin may contribute to antibacterial activity as a result of their auto-oxidation.

Mutalipocins have promising possibilities for the prevention of dental caries induced by *S. mutans*, since they have antibacterial activity. In addition, neither mutalipocin is inactivated by proteolytic enzymes or pH, suggesting that mutalipocins may retain their activities in the complex environment of the oral cavity, with contains many different proteolytic enzymes (Nord *et al.*, 1969; Yoshimura *et al.*, 1984). We are currently investigating the mutalipocin producing *S. mutans* strain 32K *in vivo* and *in vitro* for its ability to invade dental plaque and to replace the indigenous flora.

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