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Growth Inhibition of Oral Streptococci in Saliva by Anionic Proteins from Two Caries-Free Individuals

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Mixed saliva from two caries-free individuals possessed antimicrobial activity toward *Streptococcus mutans* and *S. sanguis*. This inhibitory activity was attributed to the presence of a group of four anionic proteins each of which strongly inhibited the growth of the oral streptococci in a saliva protein-based medium but not in a medium containing amino acids as a nitrogen source. These proteins, with isoelectric points of 4.70, 4.90, 4.98, and 5.05, respectively, neither reacted with antisera to immunoglobulin A, G, or M nor appeared to be functionally related to a number of salivary peroxidases, lactoferrin, or lysozyme. On this basis, they may represent a previously unreported group of growth-inhibitory antimicrobial factors occurring in the saliva of some individuals.

Saliva contains a number of proteins which may play a contributory role in the regulation of oral microbial ecology. Salivary protein factors are known to be important in the selective adherence and accumulation of certain microorganisms on the dentition (19, 23), whereas other proteins such as lysozyme (15), lactoferrin (1, 2), lactoperoxidase (21, 27), and secretory immunoglobulin A (IgA) (3, 18) contribute to the antibacterial properties associated with saliva. In addition, we have shown that certain proteins in saliva are metabolized in vitro as nitrogenous growth substrates by the oral streptococci, whereas the protein-free fraction of saliva is not growth supportive for these microorganisms (8, 9). In a subsequent study (12) saliva from caries-active (CA) individuals was found to be considerably more growth supportive for cariogenic *Streptococcus mutans* than was saliva from caries-free (CF) individuals. Isoelectric focusing studies revealed that the saliva from CF individuals was relatively free of the types of proteins which promote the growth of *S. mutans* whereas such proteins were consistently present in the saliva of CA individuals. Under the conditions of that study, no evidence of growth-inhibitory activity for *S. mutans* was seen in saliva from the CF subjects. However, it has since been found that saliva from two other CF individuals, who consistently had low to negative *S. mutans* levels in dental plaque samples, is inhibitory to the growth of *S. mutans* in a saliva protein medium which normally supports growth. This paper describes the isolation of proteins from the saliva of these individuals which account for this inhibitory activity and their comparative antibacterial activity against *S. mutans* and another prominent member of the dental plaque microflora, *S. sanguis*.

MATERIALS AND METHODS

In this study saliva was obtained from young adult males 17 to 22 years of age who were not on any type of restrictive diet. An individual was considered to be CF if he had a decayed, missing, filled-surface index of 0 and to be CA if he had a minimum decayed, missing, filled-surface index of 20 and at least one active carious lesion or a history of restored lesions within the past year.

Collection and treatment of saliva. Mixed saliva collections were made from two 17-year-old CF subjects who consistently had low or negative *S. mutans* levels in dental plaque samples. To minimize the contribution of proteins of bacterial origin, the subjects rinsed their mouths with three changes of sterilized distilled water. Salivary flow was stimulated by chewing on sterile, washed rubber bands, and the saliva was expectorated into a chilled graduated cylinder. The saliva was clarified by centrifugation (15,000 × *g*, 20 min), after which the supernatant was concentrated to 5 ml by membrane ultrafiltration (8) to obtain a protein fraction of constituents of >10,000 daltons. These saliva protein concentrate (SPC) preparations were used immediately in growth studies or for purification of proteins.

Assay of inhibitory activity. Inhibitory activity present in the protein fractions was monitored in a saliva protein-based medium (SBM) consisting of the chemically defined basal medium of Reiter and Oram (22) which was supplemented with 190 μg of protein per 300 μl of medium from an established growth-supportive SPC of a reference, formerly CA (decayed, missing, filled-surface index > 20), donor who has been CF for the past 11 years. The basal medium contained glucose (1%, wt/vol), mono- and dibasic potassium phosphate, ascorbic acid, purine and pyrimidine bases, minerals, vitamins, and 50 μg of cysteine per

ml. The medium also contained 800 μg of streptomycin sulfate per ml to inhibit the growth of contaminating organisms. This basal medium does not support the growth of *S. mutans* or *S. sanguis*. Test protein fractions were added to 300-μl quantities of SBM to give a final volume of 400 μl, and the medium was inoculated with 5 μl of washed cell suspensions (approximately 8×10^6 colony-forming units) of streptomycin-resistant indicator strains of *S. mutans* VA-29R (type *c*) or *S. sanguis* 903-1600 derived from a fresh 18-h culture in Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-glucose broth (10).Inoculated media were incubated under 90% N_2–10% CO_2 for 18 h at 35°C, after which the absorbance of the growth cultures at 600 nm was determined in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with semimicro-quartz cuvettes (0.4 by 1.0 cm). The inability of any test system to support more than scant microbial growth was verified by reincubating the growth cultures for 72 h.

The growth absorbance values were transformed to their arithmetic equivalents by a conversion table and then analyzed by the one-way analysis of variance classification (25). Where the *F* statistic was significant (5% level of probability), the Dunnett procedure (25) for multiple comparisons with a control was applied to determine which treatment means were different.

Purification of proteins. Initial separation of SPC into separate fractions of proteins was accomplished as follows. A 5-ml amount of SPC was mixed with a 95-ml volume of an aqueous slurry containing 5 g of a Bio-Lyte (Bio-Rad Laboratories, Richmond, Calif.) electrofocusing gel and 2.5 ml of LKB ampholines (LKB, Bromma, Sweden) in the pH range of 3.5 to 10.0. This mixture was transferred to an electrofocusing tray (LKB), and the total weight was reduced 25% by evaporating excess moisture under an air stream. Electrophoresis was conducted at 8-W constant power for 19 h at 10°C in an LKB 2117 Multiphor system. The beginning and final voltages of electrophoresis were 200 and 1,200, respectively. After electrofocusing, a presized sheet of absorbent paper was layered on the gel surface and left in place for 10 min to allow for protein adsorption. The paper was then removed and placed in a solution of 11.5% trichloroacetic acid and 3.5% sulfosalicylic acid for 1 h and then dried at 65°C. The print was stained with 0.1% Coomassie brilliant blue R-250 to develop protein zones. The pH gradient formed during electrofocusing was determined by pH measurement, and based on location in this gradient, protein zones identifiable on the developed print were isolated into one of the following grouped fractions: A-2, pH range, 4.30 to 4.70; B-1, pH range, 4.70 to 5.40; B-2, pH range, 5.50 to 6.60; C-1, pH range, 6.65 to 6.80; C-2, pH range, 6.84 to 7.20; or D, pH range, 7.30 to 9.5. A seventh fraction, A-1, derived from a section of the gel in the pH range of less than 4.30 and which did not contain protein, served as a gel control.

Gel sections corresponding to each of the above areas were transferred to small elution columns, and the protein constituents were eluted using 2 gel column volumes of 0.067 M potassium phosphate buffer, pH 8.0. The protein-containing eluates were placed in Spectraphor (Spectrum Medical Industries, Los Angeles, Calif.) selective membrane (3,500-dalton cutoff) dialysis tubes, dialyzed against 3 liters of distilled water at 4°C for 24 h, and then stored at 20°C.

The fraction of proteins exhibiting the strongest antimicrobial activity (B-1) was refocused as described above, except ampholines in the pH range of 4.0 to 6.0 were used. Individual protein zones identifiable from a protein print were eluted from the respective gel section, and after dialysis, the electrophoretic homogeneity of the preparations was determined by analytical isoelectric focusing (9). Purity of the individual protein zones was assessed by treating samples with dimethylaminoazobenzene isothiocyanate (5) to determine whether a single N-terminus residue is produced after two-dimensional chromatography on polyamide sheets (6).

Comparisons with known salivary antimicrobial factors. Reaction with antisera to IgA, IgG, or IgM was determined using low-level Endoplate (Kallestad Laboratories, Inc., Chaska, Minn.) immunodiffusion test kits. Salivary lactoperoxidase was determined by measuring formation of tetraguaiacol from guaiacol in the presence of hydrogen peroxide (4). Salivary peroxidase catalyzing the oxidation of potassium iodide was determined by the method of Iwamoto et al. (16), whereas peroxidse in saliva catalyzing the oxidation of *p*-phenylenediamine was determined as described by Pilz et al. (20). Preliminary tests for lytic properties were performed by adding inhibitor proteins to potassium phosphate-buffered (pH 7.0) cell suspensions of *S. mutans* or *S. sanguis* (initial absorbance = 0.6), after which the change in absorbance at 600 nm was monitored at 30-s intervals for 4 min. Treated cell suspensions also were examined microscopically for evidence of protein-mediated cellular aggregation.

Protein content. The protein content of the salivary protein preparations used in these studies was estimated from the amino acid content determined on a Beckman 118H amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) as described previously (9).

RESULTS

Initial tests indicated that the SPC fractions from the two CF donors were not readily used as an amino nitrogen source for the growth of *S. mutans* VA-29R or *S. sanguis* 903-1600. These SPCs were then tested for inhibitory properties by adding them to the control growth medium, SBM, which contained SPC of established growth-supportive activity from a separate formerly CA donor source. Whereas both indicator organisms grew in SBM alone, growth was inhibited in the presence of SPC from either CF donor source (Table 1). Although SPC from donor B appeared to be more inhibitory to the growth of *S. mutans* VA-29R than the growth of *S. sanguis* 903-1600, the growth response difference between organisms was not statistically significant.

After electrophoretic separation of the CF SPCs into fractions of proteins comprising different pH ranges, inhibitory activity toward both organisms was principally associated with proteins having isoelectric points (pIs) in the pH ranges of 4.30 to 4.70 (fraction A-2) and 4.70 to

TABLE 1. Effect of SPC from CF donors on growth of *S. mutans* VA-29R or *S. sanguis* 903-1600 in SBM

Source of SPC	μg of protein added to SBM[a]	Absorbance at 600 nm[b]	
		S. mutans VA-29R	*S. sanguis* 903-1600
None		0.02	0.00
SBM (control)		0.90 ± 0.06	0.84 ± 0.03
Donor A	330	0.59 ± 0.11[c]	0.51 ± 0.08[c]
Donor B	370	0.32 ± 0.07[c]	0.50 ± 0.07[c]

[a] Amount of protein from SPC of indicated donor which was added to SBM. SBM contains 190 μg of protein per 400 μl of medium from SPC of a separate donor source as the nitrogen source for growth.

[b] Each value represents the mean ± the standard error of the mean of absorbance at 600 nm for four independent experiments after the second transfer. For CF tests, growth cultures were incubated for 48 h at 35°C; for controls, growth cultures were incubated for 18 h at 35°C.

[c] Significantly different ($P = 0.05$) from control cultures.

5.40 (fraction B-1). Fractions of proteins having higher pIs showed little evidence of inhibitory activity. Based on analytical isoelectric focusing, fraction A-2 from each donor contained two protein zones having pIs of 4.60 and 4.70. Fraction B-1 from donor A consisted of six proteins having pIs of 4.70, 4.90, 4.98, 5.05, 5.12, and 5.16, whereas that from donor B contained protein zones with pIs of 4.70, 4.90, 4.98, 5.05, 5.20, and 5.25. The occurrence of the pI 4.70 protein zone in fractions A-2 and B-1 was caused by intersection of this zone in the fixed-position grid used for gel sectioning. The protein constituents of the B-1 fractions were further separated by preparative electrofocusing in the pH range of 4.0 to 6.0. For comparative purposes, proteins with pIs of 4.70, 4.90, and 5.05 also were purified from SPC of the formerly CA donor which was used as the control nitrogen source in SBM. The purity of the individual zones was evaluated by analytical isoelectric focusing and by reaction with dimethylaminoazobenzene isothiocyanate followed by two-dimensional chromatography. The pI 4.70, 4.90, 4.98, and 5.12 proteins from donor A each gave a single but different N-terminus residue (Table 2), but multiple N-terminus residues were produced by the pI 5.05 protein zone. Proteins with pIs of 4.70, 4.90, 4.98, and 5.05 from donor B gave N-terminus residues identical to those listed for donor A. In comparison, the electrophoretically identical pI 4.70 and 4.90 proteins purified from SPC of the formerly CA donor gave different N-terminus residues (Table 2), but the pI 5.05 protein zone gave the same multiplicity of N-terminus residues. This zone, as purified from SPCs of these three donor sources, may represent either an aggregate of different proteins or a protein having nonidentical subunits.

The relative inhibitory activity of the individual purified protein preparations from the CF donors toward growth of *S. mutans* VA-29R or *S. sanguis* 903-1600 was tested by adding comparable amounts of each test protein separately to SBM. Although SBM alone supported good growth of both indicator organisms (Table 3), microbial growth was almost completely inhibited by the proteins with a pI of 4.70, 4.90, or 4.98, regardless of donor origin. In contrast, the pI 5.20 protein from donor B was not inhibitory to the growth of either organism.

We previously reported (12) that the limited growth response of *S. mutans* VA-29R on SPCs from a number of other CF individuals (age range, 18 to 22 years) was not attributable to the presence of inhibitory substances. In this former study, inhibitory activity was tested for by adding SPC to the chemically defined basal medium supplemented with amino acids as the nitrogen source for growth (complete defined medium [10]). Because the present tests were conducted using SBM, SPCs from four of the previously tested CF individuals were compared with the B-1 fraction of proteins derived from SPCs of donors A and B for their potential to inhibit the growth of *S. mutans* VA-29R in both the complete defined medium and SBM. In these tests, *S. mutans* VA-29R grew in the control medium (Table 4), and, as expected, growth in SBM was strongly inhibited by the addition of the B-1 fraction of proteins from either donor A or donor B. However, neither of these fractions, or unfractionated SPC from which they were obtained (data not shown), inhibited the growth of *S. mutans* VA-29R in the complete defined medium. Conversely, SPCs from the four previously tested CF donors did not inhibit the growth of *S.*

TABLE 2. Comparison of N-terminus residue of proteins purified from SPC of CF donor A with electrophoretically similar proteins purified from SPC of a formerly CA donor

pI of protein	N-terminus residue[a]	
	Donor A	Formerly CA donor
4.70	Valine	Histidine
4.90	Tryptophan	Lysine
4.98	Arginine	ND[b]
5.05	Valine, methionine, and leucine or isoleucine	Valine, methionine, and leucine or isoleucine
5.12	Histidine	ND[b]

[a] Identification based on three separate determinations.

[b] ND, Not determined; protein zones at the indicated pI have not been purified from the saliva of this donor source.

TABLE 3. Influence of individual purified protein zones from the B-1 fractions of CF donors on growth of *S. mutans* VA-29R or *S. sanguis* 903-1600 in SMB

Test system	Donor source	μg of protein added to SBM[a]	Absorbance at 600 nm[b]	
			S. mutans VA-29R	*S. sanguis* 903-1600
SBM (control)			0.78	0.90
+pI 4.70	A, B	35	0.08[c]	0.06[c]
+pI 4.90	A, B	39	0.09[c]	0.06[c]
+pI 4.98	A, B	30	0.09[c]	0.06[c]
+pI 5.05	A, B	35	0.33[c]	0.18[c]
+pI 5.12	A	30	0.22[c]	0.10[c]
+pI 5.20	B	35	0.60	0.70

[a] Amount of protein from indicated source added to SBM which already contains 190 μg of protein from growth-supportive SPC in a final volume of 400 μl.

[b] Each value represents the mean of three replicates. Growth absorbance was determined after the second transfer into growth culture, which was incubated for 48 h at 35°C; control medium was incubated for 18 h.

[c] Significantly different ($P = 0.05$) from control cultures.

mutans VA-29R in either test system.

Based on pIs alone, the inhibitory proteins do not correspond with any of the inhibitory factors normally associated with saliva, such as lysozyme, secretory IgA, lactoferrin, or lactoperoxidase, which have pIs above pH 7.00. The purified inhibitory proteins were tested for a functional relationship to these cationic inhibitor proteins. The results of these tests indicated that the purified proteins did not react with antisera to IgA, IgG, or IgM, and none displayed enzymatic properties consistent for salivary lactoperoxidase or for peroxidases catalyzing the oxidation of potassium iodide or *p*-phenylenediamine. Also, none of the proteins exhibited lytic properties toward either *S. mutans* VA-29R or *S. sanguis* 903-1600, and none of them appeared to promote cellular aggregation of these organisms.

TABLE 4. Comparison of growth inhibition of *S. mutans* VA-29R in complete defined amino acid medium or SMB by protein fraction B-1 from SPC of CF donors A and B or by SPC from other CF donors

Donor source	Saliva fraction[a]	Absorbance of second transfer[b]	
		SBM	Complete defined medium
Control		0.98	1.07
Donor A	B-1	0.06[c]	0.97
Donor B	B-1	0.08[c]	1.05
CF-040	SPC	1.32[c]	1.35
CF-043	SPC	1.19	1.20
CF-016	SPC	1.19	1.18
CF-029	SPC	1.22	1.22

[a] Test media contained 35 μg of protein from the B-1 fraction of CF donor A or B or 310 μg of protein from SPC of other CF donors; SBM also contained 190 μg of protein from growth-supportive SPC.

[b] Each value represents the mean of duplicate replicates. Growth cultures were incubated for 18 h at 35°C.

[c] Significantly different ($P = 0.05$) from control cultures.

DISCUSSION

The limited growth response of *S. mutans* on the protein fraction of saliva from CF individuals has been previously attributed to the inability of *S. mutans* to use specific salivary proteins as nitrogenous growth substrates (12). It now appears that the growth-limiting properties of saliva from some CF individuals may be due also to the presence of certain growth inhibitory proteins. In this study individual anionic proteins having pIs of 4.70, 4.90, 4.98, and 5.05 from the saliva of two CF individuals were shown to be inhibitory to the growth of both *S. mutans* VA-29R and *S. sanguis* 903-1600 under conditions which required the utilization of saliva proteins as nitrogenous sources. Proteins having pIs identical to the pI 4.70 and 4.90 inhibitory proteins also appear as normal constituents in mixed and submandibular saliva of other CF and CA individuals as well (12). In CA subjects these proteins have been shown to serve as specific nitrogenous growth substrates for *S. mutans* or *S. sanguis*, whereas in other CF subjects examined they are refractory to degradation and therefore are not used as growth substrates by these streptococci (7, 9, 11, 12). Thus, saliva from CA and CF individuals may contain proteins having similar physicochemical properties but which, nonetheless, may exhibit contrasting biological properties toward the plaque-forming oral streptococci. The specific glandular source of these inhibitory proteins is under investigation.

Despite the wide discrepancy in pI between the anionic inhibitory proteins and the known cationic inhibitor proteins of saliva, they could nonetheless represent degradation products of one of these cationic proteins in which the

inhibitory properties of the parent protein were conserved. Whereas we have not established whether our proteins are related immunologically to any of the established antimicrobial factors of saliva, no evidence was obtained in this study which indicates that our proteins were functionally related to any of the known cationic inhibitor proteins. A peroxidase with a pI of 4.3 has been identified in mixed saliva but is not present in parotid saliva (20, 26). This enzyme, possibly of leukocytic origin, apparently can use either guaiacol or *p*-phenylenediamine as the hydrogen donor, however, none of our proteins reacted with either substrate. Based on the evidence obtained thus far, it is possible that our inhibitory proteins may comprise a previously unreported group of antimicrobial factors which can occur in saliva of at least some CF individuals. Further confirmation of this finding would be important because CA and CF individuals apparently do not differ substantially in salivary levels of the cationic inhibitory protein constituents (17).

Additional supportive evidence that the inhibitory anionic proteins may be different from previously described salivary antimicrobial factors was obtained from studies which showed that the inhibitory activity toward *S. mutans* was markedly influenced by the nitrogen source available for growth, as indicated by inhibition in SBM but not in the amino acid medium. These proteins also have been found to inhibit growth of *S. mutans* or *S. sanguis* in the basal medium supplemented with Trypticase (data not shown). It has been shown that the metabolism of protein substrates by *S. lactis* (13, 14, 24, 28) requires both a functional proteolytic enzyme system and peptide transport mechanisms but that growth on amino acids requires only functional amino acid transport mechanisms. Since these physiological parameters also are applicable to the oral streptococci, it is possible that the anionic proteins inhibit growth by interfering with the normal metabolism of protein or peptide substrates. The chemical composition and the mechanism of inhibition of these proteins are being examined.

The microbial spectrum of sensitivity to the proteins has not been established. However, preliminary results have shown that these proteins are inhibitory to the growth of *S. mutans* isolates but not to the growth of *S. salivarius* isolates purified from dental plaque samples of donor B (*S. mutans* has not been found in dental plaque samples of donor A).

The results of this study now indicate that limited growth of *S. mutans* on saliva from CF individuals may be relatable to more than one factor. In some of the individuals, the proteins present in saliva cannot be metabolized by the oral streptococci and hence cannot support growth, but in others, certain anionic proteins can actually prevent the oral streptococci from growing on normally growth-supportive salivary proteins. Thus, the protein composition of saliva may be a determinant of the oral microbial ecology of an individual and, by extension, of his inherent susceptibility or resistance to dental caries.

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