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Selective Antibacterial Properties of Lysozyme for Oral Microorganisms

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The antibacterial properties of lysozyme were investigated with oral microorganisms representing the seven serotypes (*a* through *g*) of *Streptococcus mutans*, *Veillonella alcalescens*, and the virulent (V) and avirulent (AV) strains of *Actinomyces viscosus* T14. Growth of bacteria in defined medium was monitored spectrophotometrically after the addition of various amounts (25 μg to 5 mg/ml) of enzyme. No growth inhibition of *V. alcalescens* was observed. Inhibition of *A. viscosus* T14(V) and *A. viscosus* T14(AV) occurred with 160 μg of lysozyme per ml. Of the *S. mutans* cultures tested, the serotype *a* and *b* strains were inhibited with as little as 25 μg of enzyme per ml, whereas *e* and *f* strains were most resistant to the bacteriostatic activity of lysozyme. The presence of DL-threonine or sucrose in growth medium did not significantly affect the results. A lysoplate assay was developed to rapidly survey the bacterial cultures for their susceptibility to the lytic ability of the enzyme. Lysis, as a measure of a zone of clearing in agarose plates, occurred for all microorganisms in the presence of lysozyme after the subsequent addition of NaCl or detergent. The bactericidal activity of lysozyme was determined on *S. mutans* BHT and *S. mutans* LM-7 by the pour plate technique. Preincubation of *S. mutans* LM-7 with as much as 1 mg of enzyme for 90 min did not affect viability or growth, whereas preincubation of *S. mutans* BHT with 1 mg of lysozyme resulted in no recoverable colony-forming units. An antigen containing extract of *S. mutans* LM-7 blocked the growth inhibitory property of lysozyme. Human lysozyme was a more effective antibacterial factor than hen egg white lysozyme. Total growth inhibition of *S. mutans* BHT was effected with 40 μg of human enzyme, and as little as 10 μg of human enzyme inhibited growth for greater than 20 h. The data presented indicate that different mechanisms may be responsible for the bacteriostatic, lytic, and bactericidal properties of the enzyme and that lysozyme is a selective but effective antibacterial factor for oral microorganisms.

Lysozyme (EC 3.2.1.17, β-1,4-glycan hydrolase) hydrolyzes glycosidic linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the cell wall peptidoglycan (31). Many of the assays for this enzyme are based on the hydrolysis of the microorganism *Micrococcus lysodeikticus* (26, 29) and are thus a function of muramidase activity of the enzyme. Adsorption of this cationic molecule to the bacterial cell wall is strongly dependent on pH and ionic strength (10), and lysis does not always result from a reaction between the molecule and the cell wall substrate (8). Thus, modification or differences in the structure and three-dimensional configuration of the cell walls of bacteria and in environmental conditions could determine the susceptibility to lysis by this enzyme. Unfortunately, the degree of susceptibility of microorganisms to the lytic ability of the enzyme under a single set of conditions has generally been used as an index of the lysozyme's potential as an antibacterial agent. In addition, due to commercial availability, hen egg white lysozyme (HEWL) has usually been used to evaluate the role of the enzyme as a bactericidal factor. However, recent studies have indicated that the results obtained from assays with HEWL as an enzyme source should not necessarily be extrapolated to include the human enzyme (5, 32; V. J. Iacono, B. J. MacKay, S. DiRienzo, and J. J. Pollock, J. Dent. Res. **58A**:157, 1979). It may be for these reasons that some investigators have reported that most oral microorganisms are not susceptible to lysozyme (14, 18, 22), whereas others have suggested that lysozyme may be an effective host defense factor for oral streptococci (7).

The studies reported in this communication were designed to evaluate the role of lysozyme as an antibacterial factor against oral microor-

ganisms and to begin to elucidate potential mechanisms whereby lysozyme could exert its antibacterial properties. A lysoplate assay system was developed by using oral bacteria as the substrate to effectively monitor the lytic ability of the enzyme. The results presented indicate that (i) the antibacterial role of lysozyme should not be equated only with its ability to lyse microorganisms; (ii) different mechanisms may be responsible for the enzyme's bactericidal, bacteriostatic, and lytic properties; (iii) cell surface characteristics, other than the peptidoglycan, may determine the susceptibility of microorganisms to lysozyme and; (iv) human lysozyme is an effective but selective antibacterial factor, which is suggestive of an important role in the regulation of the oral flora.

MATERIALS AND METHODS

Lysozyme. HEWL (crystallized three times) was obtained from Sigma Chemical Co. The concentration of HEWL in stock solutions was determined spectrophotometrically at 280 nm with an extinction coefficient of $E^{1\%}_{1cm}$ of 26.4 (35). Purified human lysozyme (HuL) isolated from the urine of patients with monocytic and myelomonocytic leukemia (26) was kindly provided by Elliott F. Osserman. The concentration of HuL in stock solutions was determined spectrophotometrically at 280 nm with an extinction coefficient of $E^{1\%}_{1cm}$ of 25.5 (4).

Bacterial strains and growth conditions. *Streptococcus mutans* strains AHT, BHT, GS-5, LM-7, and 6715 (serotypes *a*, *b*, *c*, *e*, and *g*, respectively) (27) were obtained from Harold Jordan. *S. mutans* strains FA-1, B13, and OMZ-175 (serotypes *b*, *d*, and *f*, respectively) (27) were provided by Rosemary Linzer. *Actinomyces viscosus* T14(V) (virulent) and *A. viscosus* T14(AV) (avirulent) were obtained from Sigmund Socransky. Cultures of *Veillonella alcalescens* were provided by Daniel Fine. All cultures were maintained in the lyophilized state in the Stony Brook culture collection before use. *A. viscosus* strains were grown aerobically in chemically defined medium (DM) (34). *S. mutans* cultures were grown in DM, DM supplemented with 20 mM DL-threonine or DM supplemented with 0.5% sucrose. *V. alcalescens* was grown in DM in the absence of glucose but supplemented with 0.5% Tween 80 and 1% sodium lactate and incubated under anaerobic conditions (GasPak, BBL Microbiology Systems). Growth of all cultures was measured spectrophotometrically at 700 nm (Spectronic 70, Bausch & Lomb, Inc.).

Bacterial extracts. An antigen preparation containing the serotype *b* antigen of *S. mutans* BHT was obtained from cold 10% trichloroacetic acid extracts of lyophilized whole cells as described by Mukasa and Slade (23). The serotype *e* antigen of *S. mutans* LM-7 was extracted from lyophilized whole cells by autoclaving in saline by the method of Hamada and Slade (16). Both antigen preparations were stored in the lyophilized state before assay for inhibition of lysozyme activity.

Effects of lysozyme on bacterial growth. The growth-inhibiting potential of lysozyme for oral microorganisms was determined by monitoring growth as change in optical density at 700 nm (OD_{700}). An inoculum of 10^9 cells (0.2 ml) from 18-h cultures was added to 9.8 ml of fresh medium (16- by 125-mm tubes) containing various amounts of lysozyme. Growth was observed until cultures lacking the enzyme reached stationary phase and, in certain instances, up to 3 days after inoculation for those cultures which contained enzyme.

In some studies 18-h cultures were centrifuged (2,000 × *g*; 10 min; 4°C), washed (three times) with cold distilled water, and suspended to a concentration of 10^9 cells per ml of distilled water in 0.5 ml containing various amounts of lysozyme. The suspensions were incubated with stirring for up to 90 min at 37°C after which was added 9.5 ml of fresh medium, and growth was monitored as described above.

Lysis assay. The following lysoplate method was developed to rapidly screen microorganisms for their susceptibility to lysis effected by lysozyme alone, or upon subsequent addition of salts or detergents. Cells from late-log-phase cultures were washed twice and resuspended in 0.01 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 8.2) (OD_{700} of 0.8), and 10 ml was added to an equal volume of agarose (Seaplaque, Marine Colloids, Inc., Rockland, Maine; final concentration, 1%) containing various amounts of lysozyme. After incubation with stirring for 1 h at 37°C, 5.4 ml was poured into petri dishes (60 by 15 mm) and allowed to harden at room temperature. Wells (0.2 mm in diameter) were made with a gel punch (Gelman Instrument Co., Ann Arbor, Mich.), and 10 μl of either Tris, Sodium dodecyl sulfate (SDS, 2%) or sodium chloride (NaCl, 2 N) was applied. Plates were incubated for 6 h at 37°C, and the diameters of clear zones of lysis were measured with a caliper.

Bactericidal assay. The effect of lysozyme on the viability of *S. mutans* BHT and *S. mutans* LM-7 was investigated. Overnight cultures were diluted with fresh DM to an OD_{700} of 0.9, and aliquots (200 μl) were added to various amounts of lysozyme in 500 μl of distilled water. After preincubation with stirring for 30, 60, and 90 min at 37°C, 0.1 ml of the cell suspensions was removed and serially diluted in Tris, and appropriate dilutions were plated in triplicate onto mitis salivarius agar (Difco) by the pour plate technique. Plates were incubated for 48 h at 37°C, and the resulting colony-forming units (CFU) were counted.

Inhibition of lysozyme. Dextran 2000 (Pharmacia Fine Chemicals) and soluble antigen preparations of *S. mutans* BHT and *S. mutans* LM-7 were preincubated with lysozyme in Tris for 1 h at 37°C before it was added to DM. The medium was then inoculated with *S. mutans* BHT and growth monitored as described above.

RESULTS

Effect of lysozyme on microbial growth. In the first set of experiments the effect of HEWL on four oral microorganisms was examined by monitoring growth as change in OD_{700}. As shown in Fig. 1, the inhibitory effects of

HEWL were not only concentration dependent but they were also dependent on the microbial strain tested. Total growth inhibition of *S. mutans* BHT was effected by as little as 75 μg of enzyme per ml at that incubation period (8 h) when cultures lacking enzyme reached stationary phase (Fig. 1). A significant percent inhibition (57%) was noted with as little as 25 μg of HEWL. When allowed to incubate for at least 24 h, cultures with less than 75 μg of HEWL per ml grew to the same extent as controls. When *S. mutans* LM-7 was grown in the presence of HEWL, little inhibition was noted. Greater than 1 mg of HEWL was necessary to effect significant inhibition (Fig. 1). When *S. mutans* LM-7 was grown in the presence of 5 mg of HEWL per ml of culture medium, complete growth occurred after 24 h of incubation (Fig. 1). Growth of *A. viscosus* T14(V) was affected by HEWL. However, greater than 1 mg of HEWL was necessary to effect a significant degree of inhibition after 24 h of incubation (Fig. 1). Even with 5 mg of HEWL, complete inhibition was never observed. HEWL did not effect the growth of *V. alcalescens* (Fig. 1).

These preliminary observations prompted us to investigate the effects of HEWL on strains representing the seven serotypes of *S. mutans*, and both the virulent and avirulent strains of *A. viscosus* T14. The results of these experiments are shown in Table 1. Serotype *a* (AHT) and *b* (BHT, strains of *S. mutans* FA-1) were the most susceptible to lysozyme. Significant growth inhibition was effected by as little as 25 μg of HEWL per ml for all three strains tested (47% for AHT, 57% for BHT, and 56% for FA-1) (Table 1). The serotype *e* and *f* strains (LM-7 and 0M7-175, respectively) were the most resistant of the *S. mutans* cultures tested. Greater than 1 mg of HEWL per ml was necessary to effect significant inhibition of these strains (Table 1). *S. mutans* GS-5 and *S. mutans* B13 were intermediate in their susceptibility to growth inhibition by HEWL. Slight inhibition was effected by low levels of enzyme (25 μg), which became significant as the enzyme concentration

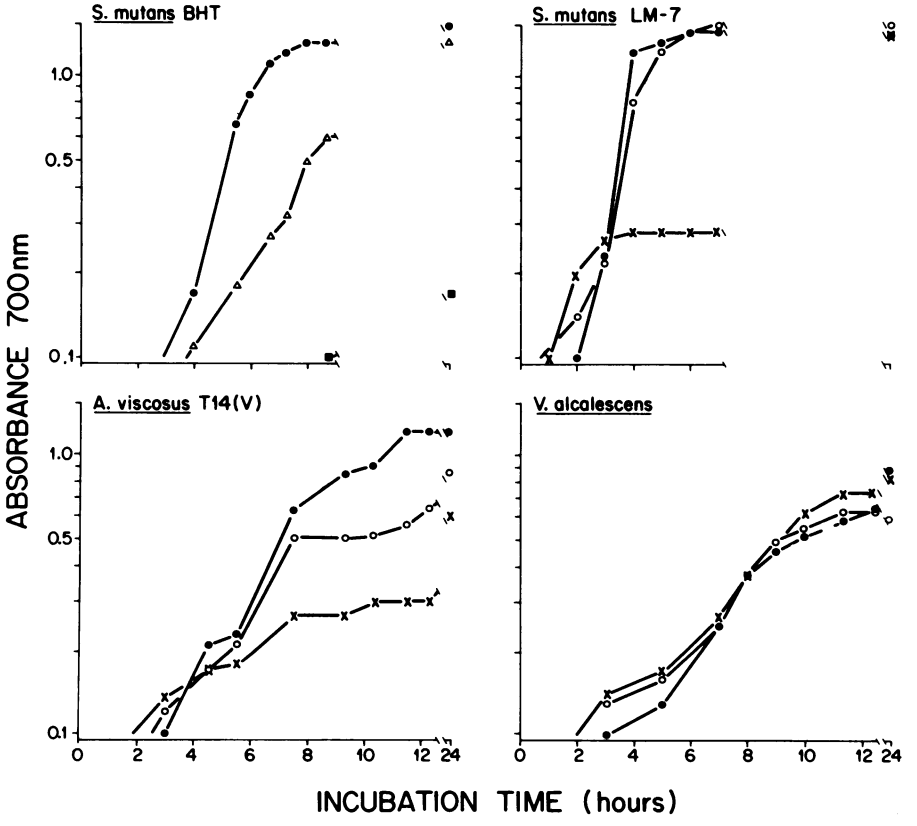

FIG. 1. *Comparison of growth of S. mutans BHT, S. mutans LM-7, A. viscosus T14(V), and V. alcalescens in DM and DM containing various amounts of HEWL.* ●, *DM;* △, *DM plus 25 μg of HEWL per ml;* ■, *DM plus 75 μg of HEWL per ml;* ○, *DM plus 1 mg of HEWL per ml;* ×, *DM plus 5 mg of HEWL per ml.*

increased (Table 1).

Growth inhibition of *A. viscosus* T14(V) and *A. viscosus* T14(AV) occurred with 160 μg of HEWL (Table 1). However, the virulent strain was less susceptible to the effects of the enzyme (Table 1).

S. mutans BHT and *S. mutans* LM-7 were then grown in DM supplemented with 0.5% sucrose to determine the effect of glucan synthesis on HEWL activity. As shown in Fig. 2, the presence of sucrose in growth medium did not hinder the growth inhibitory effects of HEWL on *S. mutans* BHT. Alternatively, sucrose, and presumably glucan synthesis, did not enhance the ability of HEWL to inhibit the growth of the relatively resistant *S. mutans* LM-7 (Fig. 2).

The development of the cell wall is determined by conditions of growth (6, 12, 19). It has been reported that the presence of threonine in growth medium may interfere with the cross-linking of the peptidoglycan in *S. mutans* BHT (R. M. McCarron and Y. F. Chang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K116, p. 166) which could make it more susceptible to lysozyme. *S. mutans* BHT and *S. mutans* LM-7 were therefore grown in DM supplemented with 20 mM DL-threonine, and the effects of HEWL on growth were examined. As shown in Fig. 2, *S. mutans* LM-7 was still almost totally resistant to the effects of HEWL. However, when the strain was grown in the presence of 5 mg of HEWL per ml, a slight bacteriostatic effect was observed (Fig. 2). This effect was not unlike that observed when this microorganism was grown in DM alone (Fig. 1 and Table 1). In addition the growth of *S. mutans* BHT was inhibited with as little as 25 μg of enzyme, similar to that observed with DM (Fig. 1 and Table 1).

Lytic ability of lysozyme. Figure 3 is of representative lysoplates of *S. mutans* BHT, *S. mutans* LM-7, *A. viscosus* T14(V), and *V. alcalescens* after incubation with 160 μg of HEWL per ml. Clearing was not detected for any strains after the addition of Tris to the wells (Fig. 3). Nor was any clearing observed if the bacteria were plated in the absence of HEWL. Only slight but incomplete clearing was observed after the addition of 2 N NaCl to the wells of those plates containing *S. mutans* strains and *A. viscosus* T14(V) (Fig. 3). However, no clearing was seen in *V. alcalescens* plates upon the addition of salt. When 2% SDS was added to the wells, lytic zones were observed only in the presence of the enzyme for all strains tested, including *V. alcalescens* and *S. mutans* LM-7 (Fig. 3).

The diameters of the lytic zones measured for each of the microbial strains after the addition of Tris, NaCl, and SDS are shown in Table 2. The ability of HEWL to lyse cultures grown in medium containing 20 mM DL-threonine was also determined. Lysis occurred for all strains after the addition of SDS. Compared with the relative differences in susceptibility of each of the strains to the growth inhibitory effects of lysozyme, marked differences were not observed for cell lysis (Table 2). However, in contrast to that observed for *S. mutans* strains and *A. viscosus* T14, no lysis of *V. alcalescens* was observed after the addition of 2 N NaCl to the center well (Table 2). The presence of DL-thre-

TABLE 1. *Effect of HEWL on microbial growth*

Strain tested	Percent inhibition[a] by HEWL								
	25[b]	50	75	100	160	320	500	1,000	5,000
S. mutans[c]									
Strain AHT(a)	47	97	NG[d]						
Strain BHT(b)	57	99	NG						
Strain FA-1(b)	56	93	NG						
Strain GS-5(c)	7	7	14	44	89	91	93	91	91
Strain B13(d)	15	15	15	15	42	54	64	78	78
Strain LM-7(e)								CG[e]	85
Strain OMZ-175(f)								CG	53
Strain 6715(g)							CG	30	77
A. viscosus T14(V)				CG	28	28	35	56	79
A. viscosus T14(AV)				CG	35	44	68	85	91
V. alcalescens									CG

[a] Inhibition is expressed as the percent reduction in absorbance of cultures containing HEWL compared with the non-enzyme-containing cultures at that incubation time when the latter reached stationary phase levels.

[b] HEWL amounts listed represent the final concentration of enzyme in micrograms per milliliter of DM.

[c] Strains of *S. mutans* representing each of the known seven serotypes (*a* through *g*).

[d] NG, No growth.

[e] CG, Complete growth.

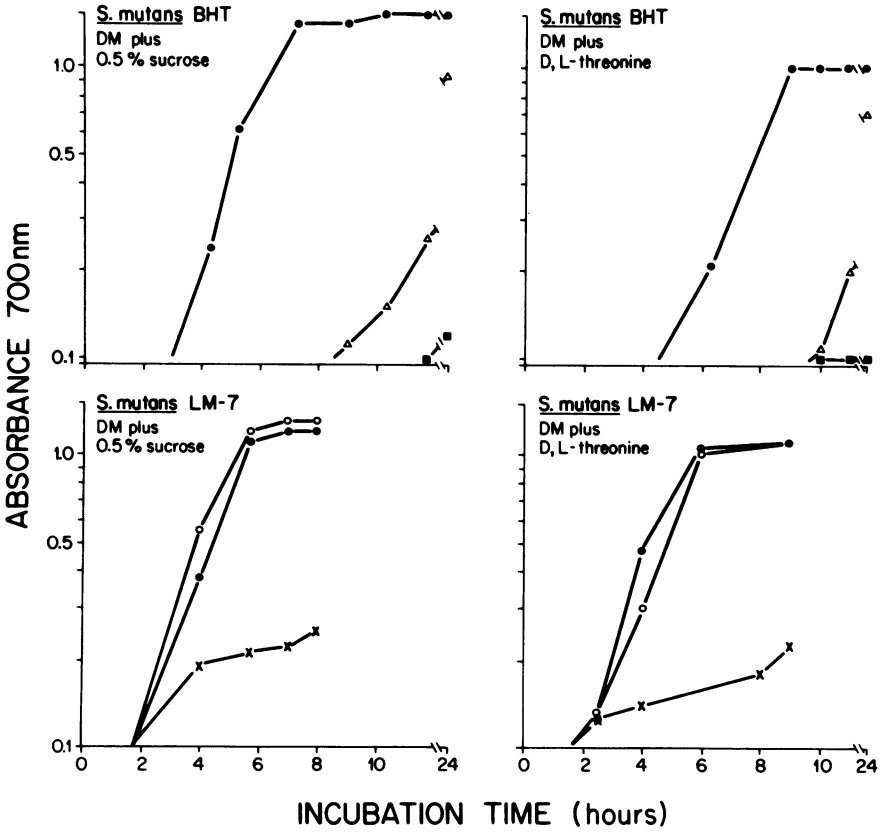

FIG. 2. *Comparison of growth of S. mutans BHT and S. mutans LM-7 in DM supplemented with 0.5% sucrose and in DM supplemented with 20 mM* DL*-threonine, each medium containing various amounts of HEWL.* ●, *Medium alone;* △, *medium plus 25 μg of HEWL per ml;* ■, *medium plus 75 μg of HEWL per ml;* ○, *medium plus 1 mg of HEWL per ml;* ×, *medium plus 5 mg of HEWL per ml.*

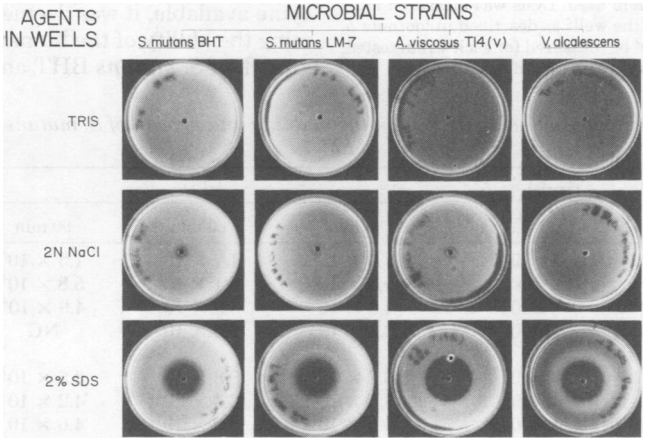

FIG. 3. *Representative lysoplates of S. mutans BHT, S. mutans LM-7, A. viscosus T14(V), and V. alcalescens in the presence of 160 μg of HEWL and after the application of 10 μl of Tris, 2 N NaCl, and 2% SDS to the wells as indicated.*

onine in the growth medium did not contribute to the lytic ability of the enzyme, since 2% SDS had to be applied to achieve a zone of clearing in the lysoplates.

Effect of lysozyme on bacterial viability. The effect of HEWL on viability was investigated with *S. mutans* BHT and *S. mutans* LM-7, as the former was the most susceptible and the latter was the most resistant of the *S. mutans* strains to growth inhibition. Lysozyme had no effect on the viability or growth of *S. mutans* LM-7 (Table 3). Preincubation of the serotype *e* strain with as much as 1 mg of HEWL for 90 min did not affect bacterial growth (Table 3). *S. mutans* BHT was quite susceptible to killing by HEWL (Table 3). Preincubation of *S. mutans* BHT with 1 mg of HEWL for 90 min resulted in no recoverable viable CFU in pour plates. After 90 min of incubation with 100 and 320 μg of HEWL per ml, less than 0.04% of the *S. mutans* BHT cultures were viable. The bactericidal effects of HEWL on this serotype *b* strain were reflected by the lack of detectable growth in 18-h cultures after 90 min of preincubation with the enzyme (Table 3).

TABLE 2. *Lytic ability of HEWL[a] on selected oral microorganisms in the lysoplates*

Strain tested	Diameter of lytic zone (mm)			
	Tris[b]	2 N NaCl	2% SDS	DL-Threonine[c]
S. mutans				
Strain AHT(a)	0[d]	5.6 ± 0.2[e]	16.6 ± 0.9	13.7
Strain BHT(b)		5.9 ± 0.4	15.7 ± 0.8	13.4
Strain GS-5(c)		6.0 ± 0.5	17.8 ± 1.9	16.0
Strain LM-7(e)		7.4 ± 0.6	14.9 ± 0.6	13.8
Strain 6715(g)		7.4 ± 0.4	15.3 ± 2.8	13.5
A. viscosus T14(V)		5.8 ± 0.3	18.4 ± 3.4	18.5
A. viscosus T14(AV)		6.0 ± 0.7	16.6 ± 3.1	19.2
V. alcalescens		0	18.8 ± 2.9	17.7

[a] HEWL was incorporated into 1% agarose to a final concentration of 160 μg/ml.

[b] Tris, 2 N NaCl, or 2% SDS, each at a volume of 10 μl, was added to wells and diameters of lytic zones measured after 6 h of incubation at 37°C.

[c] Cells from late-log-phase cultures grown in the presence of 20 mM DL-threonine were used. Lysis was measured after application of 2% SDS to the wells as described in footnote *b*.

[d] 0, No lytic zones could be measured for each strain tested.

[e] Mean of triplicate assays ± standard deviation.

Inhibition of lysozyme activity. The selectivity in the antibacterial properties of HEWL for the serotypes of *S. mutans* led us to investigate whether the observed effects were due in part to differences in microbial surface components. Experiments were designed to assay for blockage of the effects of HEWL on the susceptible strain *S. mutans* BHT. The potential inhibitors selected in preliminary studies included dextran and antigen-containing extracts of *S. mutans* BHT and *S. mutans* LM-7. Dextran had no effect on the growth inhibitory properties of HEWL (Table 4). A concentration of 500 μg of BHT extract per ml permitted 20% growth in the presence of 50 μg of HEWL (Table 4). However, total growth of *S. mutans* BHT occurred when the enzyme was preincubated in as little as 125 μg of *S. mutans* LM-7 extract (Table 4). HEWL was observed to form an opalescent suspension when incubated with the amounts of LM-7 extract tested. When these suspensions were centrifuged and the supernatants were added to DM, total growth of *S. mutans* BHT occurred with even the lowest amount (12.5 μg) of LM-7 extract, i.e., total inhibition of enzyme activity.

Growth inhibition by the human enzyme. The antibacterial properties of HuL were then investigated. Due to the limited amount of enzyme available, it was deemed feasible to determine the ability of the human enzyme to inhibit growth of *S. mutans* BHT and *S. mutans* LM-7,

TABLE 3. *Effect of preincubation with HEWL on the viability and growth of S. mutans BHT and S. mutans LM-7*

Microbial strain	HEWL (μg/ml)	CFU[a]			Culture growth[c]
		30 min[b]	60 min	90 min	
S. mutans BHT	0	1.6×10^8	1.7×10^8	1.5×10^8	CG
	100	5.3×10^6	8.1×10^5	5.8×10^4	NG
	320	4.0×10^6	6.9×10^5	4.9×10^4	NG
	1000	2.4×10^6	2.5×10^5	NG	NG
S. mutans LM-7	0	6.7×10^7	8.8×10^7	3.5×10^7	CG
	100	9.5×10^7	7.4×10^7	4.2×10^7	CG
	320	5.6×10^7	6.0×10^7	4.6×10^7	CG
	1000	9.5×10^7	1.1×10^8	1.0×10^8	CG

[a] CFU, Colony-forming units per milliliter of reaction mixture were measured after 48 h of incubation.

[b] Incubation periods with HEWL.

[c] Preincubation for 90 min with HEWL followed by 18 h of growth in DM. CG, Complete growth; NG, no growth.

an HEWL-susceptible and -resistant strain, respectively. As shown in Table 5, *S. mutans* LM-7 was found to be equally resistant to HuL and HEWL at the concentrations studied. HuL was found to be a more effective antibacterial factor than HEWL for *S. mutans* BHT. Total inhibition of growth was effected with 40 μg of HuL, and as little as 10 μg of HuL inhibited the growth of *S. mutans* BHT for greater than 20 h.

TABLE 4. *Effects of microbial components on the growth inhibitory properties of HEWL for S. mutans BHT*[a]

Microbial component	Amt (μg/ml)	HEWL (μg/ml)	% Growth[b]
None		0	100
None		50	0
Dextran	500	50	0
BHT extract	500	50	20
LM-7 extract	500	50	100
	250		100
	125		100
	62.5		25
	25		25
	12.5		10

[a] Dextran or antigen extracts of *S. mutans* BHT and *S. mutans* LM-7 were preincubated with HEWL (50 μg) for 1 h before they were added to DM.

[b] Growth was monitored spectrophotometrically at 700 nm for 20 h.

TABLE 5. *Comparison of the effects of HuL and HEWL on the growth of S. mutans LM-7 and S. mutans BHT*[a]

Strain tested	HEWL (μg/ml)	Hul (μg/ml)	Incubation time for complete growth (h)
S. mutans LM-7	—[b]	—	6
	40	—	6
	—	40	6
S. mutans BHT	—	—	7
	40	—	36
	—	40	NG[c]
	20	—	24
	—	20	46
	10	—	9
	—	10	27
	5	—	7
	—	5	8
	2.5	—	7
	—	2.5	7

[a] *S. mutans* LM-7 and *S. mutans* BHT were inoculated into DM, DM supplemented with HEWL, or DM supplemented with HuL. Growth was monitored spectrophotometrically at 700 nm for greater than 3 days.

[b] —, No HEWL or Hul was added.

[c] NG, No growth.

DISCUSSION

It has been reported that most oral microorganisms are not lysed or killed by lysozyme (14). In this communication we present evidence that lysozyme is a selective and effective antibacterial agent and that the bactericidal or bacteriostatic properties of the enzyme are not always equated to its lytic ability. When strains representing the seven serotypes of *S. mutans* were used in the assays for growth inhibition by HEWL, marked differences in the enzyme's effectiveness were observed. *S. mutans* BHT, a serotype *b* strain (24), was the most susceptible of all strains to the growth inhibitory properties of the enzyme. And in support of that noted by other investigators (7), this strain was found to be readily killed by HEWL (Table 3). These findings, however, cannot be generalized for all serotypes of *S. mutans*. Significant growth inhibition did not occur with *S. mutans* LM-7 and *S. mutans* OMZ-175 serotype *e* (16) and *f* strains (17), respectively. Nor was *S. mutans* LM-7 killed in the presence of as much as 1 mg of HEWL (Table 3). *S. mutans* AHT reacted very similarly to the serotype *b* strains, whereas *S. mutans* GS-5 and *S. mutans* B13 were intermediate in their susceptibility to lysozyme. A possible explanation for these results could be related to the enzyme's ability to gain access to the peptidoglycan of the cell wall or to other cell structures, such as the cytoplasmic membrane and components of an autolytic enzyme system. Among all serotypes, *S. mutans* BHT is known to possess the highest negative ζ-potential (25) and the most negatively charged surface antigen (2). It is therefore conceivable that relatively more of the cationic lysozyme molecules could bind to this microorganism and subsequently penetrate the cell to reach its substrate. In support of this concept we previously found that after exposure of *S. mutans* strains to HEWL, more of the enzyme bound to the *b* serotype than to any of the other serotypes tested (28). It is of interest that the most enzyme resistant of the *S. mutans* strains tested were the serotype *e* and *f* strains. The predominant antigenic determinant for the *e* serotype is a β-linked glucose-glucose dimer (16), and that for the *f* serotype is a terminal α-1,6-diglucose (17). Both of these terminal disaccharides may fit into the active site of lysozyme and possibly prevent the penetration of the enzyme through the cell surface. We observed that an antigen extract of *S. mutans* LM-7 was highly effective in blocking the growth inhibitory properties of lysozyme (Table 4). However, this extract did not consist solely of the type-specific polysaccharide. Other cell components could be involved in the observed inhibition. These might

include nucleoprotein, lipoteichoic acid, and possibly membrane glycolipid. Our laboratory is currently in the process of isolating the active fraction (V. J. Iacono, B. J. MacKay, S. DiRienzo, J. J. Pollock, and J. M. Zuckerman, J. Dent. Res. vol. **59B,** Abstr. no. 89, 1980). Cell components which block lysozyme activity may also be found in *A. viscosus* T14. The virulent strain of *A. viscosus* T14 was found to be less susceptible than the avirulent strain to the antibacterial effect of HEWL (Table 1). The extracellular polysaccharide slime, synthesized by the avirulent strain but not the virulent strain (36), may be important in the explanation of our results. Our laboratory immunochemically characterized the slime and isolated a predominant antigenic component which consisted primarily of *N*-acetylglucosamine (20). Such a molecule may fit into the active site of the enzyme. However, whether the slime interacts with lysozyme and modifies its antibacterial properties in vivo is not known.

The question of lysozyme sensitivity, capsulation, and the presence of extracellular slimes is an interesting one. It is well known that when *S. mutans* is grown in the presence of added sucrose, it forms glucan capsules. The presence of a glucan capsule could modify lysozyme action, should there be a reaction between the capsular substance and lysozyme. However, our observations suggest that this may not occur. *S. mutans* BHT was found to be equally susceptible to the antibacterial properties of HEWL when grown in either glucose- (Fig. 1) or sucrose-enriched (Fig. 2) media. These results are similar to those obtained from studies with *Bacillus anthracis* in that the presence of a capsule does not necessarily reflect the sensitivity of the microorganism to lysozyme (15).

The gram-negative oral microorganism we tested for lysozyme sensitivity was *V. alcalescens.* It was totally resistant to the growth inhibitory properties of HEWL. As compared with gram-positive bacteria, the peptidoglycan of gram-negative bacteria is much thinner, existing usually as a monolayer. The outer membrane of gram-negative microorganisms is external to and covalently linked to the peptidoglycan and serves as a permeability barrier (3, 9). Because of this diffusion barrier, lysozyme would not be able to gain access to the peptidoglycan unless damage to the outer membrane occurred (37). Lysozyme has been shown to bind lipopolysaccharide of *Pseudomonas aeruginosa* (11), and this might also occur with *Veillonella* lipopolysaccharide (1). Binding to lipopolysaccharide or other structures might therefore result in damage to the outer membrane such that addition of 2% SDS to the bacterial lysozyme complex resulted in lysis in the lysoplate assay (Table 2 and Fig. 3).

The fact that some of the microorganisms which were not inhibited in growth or were not killed by lysozyme were still lysed by the enzyme suggested perhaps that mechanisms for bacteriostatic, bactericidal, and lytic activities were not necessarily identical. Hydrolysis of peptidoglycan by lysozyme is one explanation for the enzyme's antibacterial mechanism; however, effects may also depend upon properties of lysozyme distinct from its muramidase function. We grew *S. mutans* BHT and *S. mutans* LM-7 in a medium containing DL-threonine which has been reported to inhibit lysine incorporation into cell wall cross-links and thus would make the cell more susceptible to the lytic activity of lysozyme (6; R. M. McCarron and Y. F. Chang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K116, p. 166). However, neither growth inhibition nor lysis was enhanced by this procedure (Fig. 2 and Table 2). The cell wall is a dynamic layer in growing cells and there are differences in the quantitative or qualitative composition of the cell walls, or both, of *S. mutans* serotypes (21). Such differences may make it possible for lysozyme killing or lysis, or both, of *S. mutans* to be a function of the relative number of exposed sensitive sites in the wall at any one time during growth. It is also possible that lysozyme activates a well-regulated autolytic peptidoglycan hydrolase of low activity (33) in some microorganisms. In this regard we observed a dechaining phenomenon during growth of *S. mutans* LM-7 in lysozyme containing medium in the absence of both growth inhibition and cell lysis (data not shown). Although gross visible lysis of *S. mutans* BHT is not evident in the presence of lysozyme alone, cell wall damage probably does occur since enzyme-treated serotype *b* strains are not only killed but are dissolved when the pH of the incubation mixture is raised to 12 (33) or after the addition of sodium chloride or SDS (Fig. 3 and Table 2).

Our data revealed that the human leukemic enzyme was a more effective inhibitor of growth of *S. mutans* BHT than HEWL (Table 5). Should human salivary lysozyme exhibit comparable or even greater antibacterial activity for sensitive oral microorganisms, it could be of significance in terms of regulating the oral microflora. Several investigators have attempted to define the role of lysozyme in the oral cavity (7, 13, 14). However, much of this work has been done with HEWL and not HuL. It is now known that there are distinct variations in activity which exist between the lysozymes of different organisms (30). Similar to our observations with *S. mutans* BHT, Carroll and Martinez (5) found

that HuL was a more effective antibacterial factor for *B. subtilis* than either HEWL or rabbit enzyme. As suggested by these same investigators (5), one should not generalize the role(s) of lysozyme from one animal to the next or from one microbial species to the next. We can add to these concerns in that one should not generalize the role of the enzyme from one environment to the next. This would be especially true for the oral environment where there are extremely complex microflora and "growth" conditions characterized by continuous variations in pH, ionic strength, and available nutrients.

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LITERATURE CITED

1. **Bladen, H. A., and S. E. Mergenhagen.** 1964. Ultrastructure of *Veillonella* and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. **88**:1482-1492.
2. **Bratthall, D.** 1970. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. Odontol. Revy **21**:143-152.
3. **Braun, V., and Rehn, K.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. **10**:426-438.
4. **Canfield, R. E., J. C. Collins, and J. H. Sobel.** 1974. Human leukemia lysozyme, p. 63-70. In E. F. Osserman, R. E. Canfield, and S. Beychok (ed.), Lysozyme. Academic Press, Inc., New York.
5. **Carroll, S. F., and R. J. Martinez.** 1979. Role of rabbit lysozyme in in vitro serum and plasma serum bactericidal reactions against *Bacillus subtilis*. Infect. Immun. **25**:810-819.
6. **Chassy, B. M.** 1976. A gentle method for the lysis of oral streptococci. Biochem. Biophys. Res. Commun. **68**:603-608.
7. **Coleman, S. E., I. Van de Rijn, and A. S. Bleiweis.** 1971. Lysis of cariogenic and noncariogenic oral streptococci with lysozyme. J. Dent. Res. **50**:939-943.
8. **Colobert, L., and J. Lenoir.** 1956. Etude du mécanisme de la lyse de Coccus P (sp. *Sarcena flava*) par le lysozyme. Ann. Inst. Pasteur (Paris) **91**(*Suppl*):74-88.
9. **Costerton, J. W., J. M. Ingram, and K. J. Cheng.** 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. **38**:87-110.
10. **Davies, R. C., A. Neuberger, and B. M. Wilson.** 1969. The dependence of lysozyme activity on pH and ionic strength. Biochim. Biophys. Acta **178**:306-317.
11. **Day, D. F., M. L. Marceau-Day, and J. M. Ingram.** 1978. Protein-lipopolysaccharide interactions. I. The reaction of lysozyme with *Pseudomonas aeruginosa* LPS. Can. J. Microbiol. **24**:196-199.
12. **De Boer, W. R., F. J. Kruyssen, and J. T. M. Wouters.** 1979. Cell wall metabolism of *Bacillus subtilis*. Antonie van Leeuwenhoek. J. Microbiol. Serol. **45**:315-317.
13. **Eisenberg, R. J., and K. Lillmars.** 1975. A method for gentle lysis of *Streptococcus sanguis* and *Streptococcus* mutans. Biochem. Biophys. Res. Commun. **65**:378-384.
14. **Gibbons, R. J., J. D. de Stoppelar, and L. Harden.** 1966. Lysozyme insensitivity of bacteria indigenous to the oral cavity of man. J. Dent. Res. **45**:877-881.
15. **Gladstone, G. P., and H. H. Johnston.** 1955. The effect of cultural conditions on the susceptibility of *Bacillus anthracis* to lysozyme. Br. J. Exp. Pathol. **36**:363-372.
16. **Hamada, S., and H. D. Slade.** 1976. Purification and immunochemical characterization of type *e* polysaccharide antigen of *Streptococcus mutans*. Infect. Immun. **14**:68-76.
17. **Hamada, S., K. Gill, and H. D. Slade.** 1976. Chemical and immunological properties of the type *f* polysaccharide antigen of *Streptococcus mutans*. Infect. Immun. **14**:203-211.
18. **Hamada, S., M. Torii, S. Kotani, N. Masuda, T. Ooshima, K. Yokogawa, and S. Kawata.** 1978. Lysis of *Streptococcus mutans* cells with mutanolysin, a lytic enzyme prepared from a culture liquor of *Streptomyces globisporus* 1829. Arch. Oral Biol. **23**:543-549.
19. **Holden, J. T., W. Grant, and J. Degroot.** 1979. Lysozyme sensitivity of pantothenate-deficient *Lactobacillus plantarum* growth with exogenous fatty acids. Biochim. Biophys. Acta **574**:173-176.
20. **Iacono, V. J., M. J. Levine, and G. J. Shemaka.** 1977. Immunochemical characterization of the extracellular slime of *Actinomyces viscosus* T14. J. Dent. Res. **56B**: 154.
21. **Inoue, M., S. Hamada, T. Ooshima, S. Kotani, and K. Kato.** 1979. Chemical composition of *Streptococcus mutans* cell walls and their susceptibility to *Flavobacterium* L-11 enzyme. Microbiol. Immunol. **23**:319-328.
22. **Iwamoto, Y., T. Watanabe, A. Tsunemitsu, K. Fukui, and T. Moriyama.** 1971. Lysis of streptococci by lysozyme from human parotid saliva and sodium lauryl sulfate. J. Dent. Res. **50**:1688.
23. **Mukasa, H., and H. D. Slade.** 1972. Chemical composition and immunological specificity of the streptococcal group O cell wall polysaccharide antigen. Infect. Immun. **5**:707-714.
24. **Mukasa, H., and H. D. Slade.** 1973. Structure and immunological specificity of the *Streptococcus mutans* group b cell wall antigen. Infect. Immun. **7**:578-585.
25. **Olsson, J., P. O. Glantz, and B. Krasse.** 1976. Electrophoretic mobility of oral streptococci. Arch. Oral Biol. **21**:605-609.
26. **Osserman, E. F., and D. P. Lawlor.** 1966. Serum and urinary lysozyme (muramidase) in monocytic and myelomonocytic leukemia. J. Exp. Med. **124**:921-955.
27. **Perch, B. J. Kjems, and T. Ravn.** 1974. Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. Acta Pathol. Microbiol. Scand. **82**:357-370.
28. **Pollock, J. J., V. J. Iacono, H. Goodman-Bicker, B. J. MacKay, L. I. Katona, and E. Thomas.** 1976. The binding, aggregation, and lytic properties of lysozyme, p. 325-352. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings, Microbial Aspects of Dental Caries. Special Supplement to Microbiology Abstracts, Vol. III. Information Retrieval, Inc., Washington, D.C.
29. **Prasad, A. L. N., and G. Litwack.** 1963. Measurement of the lytic activity of lysozymes. Anal. Biochem. **6**:328-334.
30. **Saint-Blancard, J., P. Chuzel, Y. Mathieu, J. Perrot, and P. Jolles.** 1970. The influence of pH and ionic strength in the lysis of *M. lysodeikticus* cells by six human and four avian lysozymes. Biochim. Biophys. Acta **220**:300-306.
31. **Salton, M. R. J.** 1961. The anatomy of the bacterial surface. Bacteriol. Rev. **25**:77-99.
32. **Selsted, M. E., and R. J. Martinez.** 1978. Lysozyme: primary bactericidin in human plasma serum active against *Bacillus subtilis*. Infect. Immun. **20**:782-791.
33. **Shockman, G. D., L. Daneo-Moore, J. B. Cornett, and M. Mychajonka.** 1979. Does penicillin kill bacteria? Rev. Infect. Dis. **1**:787-796.
34. **Socransky, S. S., C. Smith, and A. D. Manganiello.** 1973. Defined media for the cultivation of oral gram

positive rods. J. Dent. Res. **52**:120.

35. **Sophianopoulos, A. J., C. K. Rhodes, D. N. Holcomb, and K. E. Van Holde.** 1962. Physical studies of lysozyme. I. Characterization. J. Biol. Chem. **237**:1107–1112.
36. **Taichman, N. S., B. F. Hammod, C.-C. Tsai, P. C. Baehni, and W. P. McArthur.** 1978. Interaction of inflammatory cells and oral microorganisms. VII. In vitro polymorphonuclear responses to viable bacteria and to subcellular components of avirulent and virulent strains of *Actinomyces viscosus* T14. Infect. Immun. **21**:594–604.
37. **Witholt, B., and M. Boekhout.** 1978. The effect of osmotic shock on the accessibility of the murein layer of exponentially growing *Escherichia coli* to lysozyme. Biochim. Biophys. Acta **508**:296–305.