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Mutanolysin, Bacteriolytic Agent for Cariogenic Streptococci: Partial Purification and Properties

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Mutanolysin partially purified from the culture filtrate of *Streptomyces globisporus* 1829 consists of two main lytic enzymes with an isoelectric point near pH 8.5 and 10, respectively, and proteolytic enzyme is associated with the latter lytic enzyme. Mutanolysin exhibited maximal lytic activity at 60 C in the pH range 6.5 to 7.0 and was stable at 50 C in the acid range. *N*-bromosuccinimide caused complete inhibition of lytic activity at 1 mM, whereas calcium and magnesium ions at the same concentration caused activation. Mutanolysin had lytic or bactericidal activity against the living cells of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguis*, *Lactobacillus acidophilus*, and *Actinomyces viscosus*, which are considered to be etiologic agents of dental caries, but had no activity against *S. aureus* and all gram-negative strains tested. The lytic activity was well retained in human saliva. Digestion of the cell walls of *S. mutans* BHT by mutanolysin was accompanied by the liberation of free amino groups and reducing sugars. Mutanolysin may be expected to be a useful agent for dental caries control.

Egg white lysozyme has been used frequently for investigation of the intracellular components of cells. Because the lytic spectrum of this enzyme was limited, other bacteriolytic enzymes from microbial sources have been used instead by numerous investigators to obtain information about the structural and immunochemical characteristics of bacterial cell walls and plasma membranes (1, 13, 17). Lysostaphin, an enzyme that rapidly lyses the cell walls of *Staphylococcus aureus*, has been found to be effective in the treatment of established staphylococci infections in experimental animals (3, 8) and to reduce the incidence of *S. aureus* in nasal carriers (9, 14). We have reported that the culture filtrate of *Streptomyces globisporus* 1829, a strain isolated from soil, is capable of rapidly lysing cells of cariogenic steptococci isolated from carious lesions in both rodents and humans (24, 25). *Streptococcus mutans*, a type of cariogenic bacteria, has been shown to form dental plaques composed of extracellular polysaccharides of a dextran type and to induce dental caries when inoculated into the oral cavities of experimental animals maintained on a high sucrose diet (5, 16, 27). Fitzgerald (4) found that in some hamsters receiving dextranase in their drinking water significantly less plaque formation occurred, and fewer dental caries developed than in animals not receiving the enzyme; an attempt has been made to control dental caries by applying dextranase (15, 18, 21). The lytic and bactericidal actions of a culture broth from *S. globisporus* 1829 against *S. mutans* also suggested its possible application for dental caries control.

In the present report, we describe some properties of the partially purified lytic enzyme (termed "mutanolysin") from *S. globisporus* 1829 and its lytic action against strains of *S. mutans*.

MATERIALS AND METHODS

Organisms. Strains of *S. mutans* used in this study were maintained in brain heart infusion agar medium (Difco). They were cultivated by stab culture at 37 C for 3 days in 1 atm of 95% nitrogen and 5% carbon dioxide. For the experiments on enzymatic lysis, the stock cultures were transferred into brain heart infusion (Difco) broth or into a medium containing 1% polypeptone (Difco), 1% meat extract (Difco), 0.5% yeast extract (Difco), 2% glucose, 1% sodium acetate, 0.5% NaCl, and 0.1 mM $MnSO_4$ (pH 7.5), and then were incubated at 37 C for 24 h in 1 atm of 95% nitrogen and 5% carbon dioxide. The culture broths were chilled in ice, and the cells were harvested by centrifugation at 10,000 rpm under cooling, washed three times with chilled deionized water, and used immediately or as lyophilized cells.

All bacteria, including *Bacillus subtilis*, *Brucella*

abortus, *Diplococcus pneumoniae*, *Escherichia coli*, *Klebsiella* species, *Lactobacillus acidophilus*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella flexneri*, *Shigella sonnei*, and *S. aureus* (all our collection), were grown on nutrient agar medium, except that brain heart infusion medium was employed for *Actinomyces viscosus*, *Streptococcus salivarius*, and *Streptococcus sanguis*, and Sabouraud medium was used for *Candida utilis*. The freshly cultured cells were harvested and then subjected to lysis to determine the lytic spectrum of mutanolysin.

Preparation of mutanolysin. *S. globisporus* 1829 was grown in a 500-ml shaking flask containing 50 ml of a medium consisting of 2% dextrin, 0.5% soybean meal (Ajinomoto), 0.2% polypeptone (Wako), 0.1% NaCl, 0.5% $Na_2HPO_4 \cdot 12H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.02% $CaCl_2$, pH 7.5. After 3 days of growth at 30 C, 1 liter of culture broth was filtered through filter paper, mixed with 40 g of Amberlite CG-50 weak cationic exchange resins (Rohm and Haas, Philadelphia) in the H^+ form, and stirred for 1 h. The resins were removed through filter paper in a Buchner funnel and washed with deionized water. The lytic components adsorbed on the resins were eluted out with 130 ml of 0.2 M Na_2HPO_4 solution (pH 7.5). The eluate was brought to 60% saturation with solid ammonium sulfate. The precipitate was allowed to settle overnight at 4 C and collected by passing through a pad of Radiolite no. 700 (Showa Chemicals) in a Buchner funnel. The lytic components in the pad were extracted with the minimal amount of deionized water, and the colored materials in the extracts were removed by treating with Duolite A-2 resins (Diamond Shamrock Chemicals) in the Cl^- form. The decolorized solution was first adjusted to pH 2.0 with hydrochloric acid and then immediately to pH 6.0 with sodium hydroxide solution. The insolubilized proteolytic components were removed by centrifugation. The clarified solution was desalted, concentrated by ultrafiltration (ULVAC, Nihonshinku), and lyophilized. At this stage, 61 mg of mutanolysin was obtained.

Assay procedures. Lytic activity was determined as follows: a mixture of 1.9 ml of cell suspension and 2.0 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)-malate NaOH buffer (pH 7.0) was made to give an optical density of 0.6 at 600 nm in a Bausch and Lomb Spectronic 20 colorimeter and preincubated in a water bath at 37 C for 1 min. A 0.1-ml amount of adequately diluted mutanolysin solution was added to this suspension and reacted at 37 C. The decrease in optical density at 600 nm was read against a water blank at appropriate time intervals. One unit of lytic activity was defined as the amount of lytic enzyme causing a decrease in optical density of 0.01/min. For viable cell counts, the reaction mixture containing 80 μg of mutanolysin and *S. mutans* BHT cells in 4 ml of 10 mM Tris-malate NaOH buffer (pH 7.0) was incubated at 37 C for 90 min. A 0.5-ml amount of the reaction mixture was pipetted out at appropriate time intervals and added to 9.5 ml of 0.05% yeast extract solution sterilized and chilled to stop enzymatic action. A sample of the solution was admixed in mitis salivarius agar medium, poured into a petri dish, and incubated at 37 C for 3 days in 1 atm of 95% nitrogen and 5% carbon dioxide, followed by counting the colonies of viable streptococcal cells.

Proteolytic activity was determined as follows: to 2 ml of 0.6% casein (Merck) solution in 0.05 M Tris-hydrochloride buffer (pH 8.0) was added 1 ml of appropriately diluted enzyme solution. After incubation at 37 C for 10 min, reaction was stopped by addition of 3 ml of 0.01 M trichloroacetic acid, 0.02 M sodium acetate, and 0.03 M acetic acid. The precipitate was filtered off, and the absorbancy of the filtrate was determined at 275 nm in a spectrophotometer.

Protein was determined by the method of Lowry et al. (19) by using bovine serum albumin (Nutritional Biochemicals Corp.) as a standard.

Isoelectric focusing of mutanolysin was carried out by using an electrofocusing column (LKB 8100) of 110 ml capacity with a density gradient of sucrose and pH gradient of carrier ampholites ranging from 3 to 10. A 20-mg amount of mutanolysin was applied on the column and subjected to electrofocusing at 0.5 mA at 4 C for 46 h as described by Vesterberg and Svensson (26).

Free amino groups were determined according to the method of Ghuysen (7) and free reducing sugars were measured by the procedure of Park and Johnson (22).

Microscopy observation. Intact whole cells of *S. mutans* BHT were used in this investigation. The cells were statically grown at 30 C for 2 days in a medium containing 2% glucose, 1% polypeptone, 1% meat extract, 0.5% NaCl, 0.2% yeast extract, 1% sodium acetate, and 0.1 mM $MnSO_4$ (pH 7.5). The cells were harvested by centrifugation and washed twice with distilled water. To the mixture of 1.9 ml of cell suspension and 2 ml of 0.02 M Tris-hydrochloride buffer (containing 2 mM $MgCl_2$) was added 0.1 ml of mutanolysin solution (120 U/ml), and the reaction was carried out at 37 C for 60 min. The initial optical density at 600 nm gave 0.6. Samples of the solution were taken out at 0-, 5-, 10-, 15-, 20-, 30-, and 60-min intervals and diluted with a large volume of cold water to stop the enzyme reaction, followed by immediate centrifugation at 10,000 rpm at 4 C for 10 min. The precipitates were resuspended in distilled water, and a droplet of the suspension was placed on electron microscope grids. The specimens were shadowed with gold-palladium alloy at an angle of 13° and examined in an Akashi-type TRC 50 electron microscope.

Preparation of saliva. Hamster saliva was collected by stimulating the secretory ducts of salivary glands by applying 2% pilocarpine in eyes of animals anesthetized with pentobarbital. Human and hamster saliva was centrifuged at 10,000 × *g* at 5 C for 15 min and sterilized by exposure to ultraviolet irradiation for 15 min from a distance of 15 cm with a 2537-A lamp. The sterilized saliva was kept at −20 C and thawed just before use.

RESULTS

Purification. The lytic enzymes in the culture filtrate of *S. globisporus* 1829 were found to

be basic proteins and, therefore, they were adsorbed on a weak cationic exchange resin, Amberlite CG-50, directly from culture filtrate, and easily eluted out with 0.2 M Na_2HPO_4 solution. On the other hand, proteolytic activity remained in the filtrate unadsorbed on the resins, and nearly 75% of proteolytic activity was removed. The precipitate obtained by bringing the eluate to 60% saturation with solid ammonium sulfate was dissolved in distilled water. The solution, with considerable brownish color, was treated successfully with a weak basic resin, Duolite A-2, to remove the color. Preliminary experiments by using isoelectric focusing resulted in the eluate from Duolite A-2 resins containing, in addition to lytic enzymes, two different proteolytic enzymes with isoelectric points near pH 5 and 10, respectively. The activity of the former proteolytic enzyme was destroyed immediately when pH was brought to 2.0 and precipitated when the pH of the solution was readjusted to its isoelectric point of 5.0. On the other hand, the lytic activity did not change during that treatment. After this treatment, 96% of the original proteolytic activity was removed as shown in Table 1. Ultrafiltration was conducted to separate enzyme proteins from the acid-treated solution containing salts. The recovery of the lytic activity by ultrafiltration was compared with the three membranes of different pore sizes: HF-35, HFA-180, and G-20T. The HF-35 membrane (Eastman Organic Chemicals) is reported to retain molecules with a molecular weight of 5,000 or above. The HFA-180 (Abcor) and G-20T (Nihonshinku) membranes are reported to have pore sizes which cut off molecules with molecular weights of 15,000 and 20,000, rspectively. The recovery of lytic or proteolytic activities after treatment with the HF-35, HFA-180, and G-20T membranes were 100, 86, and 47%, or 100, 85, and 43%, respectively. Although an accurate recovery could not be obtained because of a slight inactivation of enzyme during ultrafiltration, leakages of proteolytic enzyme from the membranes were entirely parallel to that of the lytic enzyme(s). Then the HF-35 membrane was used to remove the excess salts from the decolorized solution and to concentrate the solution, and 93% of the initial activity was recovered from the concentrate obtained by ultrafiltration. At this purification stage, about 32% of lytic activity was recovered with an increase only twice in specific lytic activity in comparison with that of the starting material, whereas the specific proteolytic activity decreased to about 0.25 (Table 1). Mutanolysin was subjected to electrofocusing (Fig. 1). When the optical density of the eluted fractions were measured at 280 nm, two main protein peaks were observed. The one with an isoelectric point near pH 8.5 had lytic activity, but the other peak with an isoelectric point near pH 10 had proteolytic activity in addition to the lytic activity.

Enzymatic properties. Figure 2 shows the activities of the lytic and proteolytic enzymes in mutanolysin at different pHs. The lytic activity was affected greatly by pH. The optimal pH for the lytic activity ranged from 6.5 to 7.0 in phosphate buffer. The curve of proteolytic activity clearly differed from that of lytic activity, and the optimal pH was 12. The proteolytic activity decreased remarkably as pH was lowered below 7. The lytic and proteolytic activities determined by changing the incubation temperature from 37 to 70 C are shown in Fig. 3. The lytic activity was highest at 60 C, whereas the maximal proteolytic activity was obtained at 65 C. Upon heating for 10 min, a slight decrease in lytic activity at 55 C, a rapid decrease at 55 to 65 C, and inactivation at over 70 C were observed (Fig. 4). The stability curve of proteolytic enzyme was similar to that of lytic enzymes. When mutanolysin was incubated at 37 C for 24 h in the various buffers with different pH, the lytic activity was found to be retained in the acidic pH side, but the activity decreased with increase in pH over 6.0. Approximately 50% of the proteolytic activity still

TABLE 1. *Summary of partial purification data for the lytic enzyme from Streptomyces globisporus 1829*

Step	Vol (ml)	Total protein (mg)	Lytic activity			Proteolytic activity	
			U × 10^2	Recovery (%)	Sp act (U/mg)	U × 10^2	Recovery (%)
Culture filtrate	1,000	750	1,140	(100)	152	924	(100)
Amberlite CG-50 eluate	171	319	730	64	229	240	27
$(NH_4)_2SO_4$ precipitate	22	257	547	48	212	223	24
Duolite A-2 eluate	26	192	516	45	269	153	17
pH treatment	26	122	394	35	323	36	4
Ultrafiltration	3	122	368	32	302	38	4
Lyophilized material	61mg	110	367	32	335	33	3

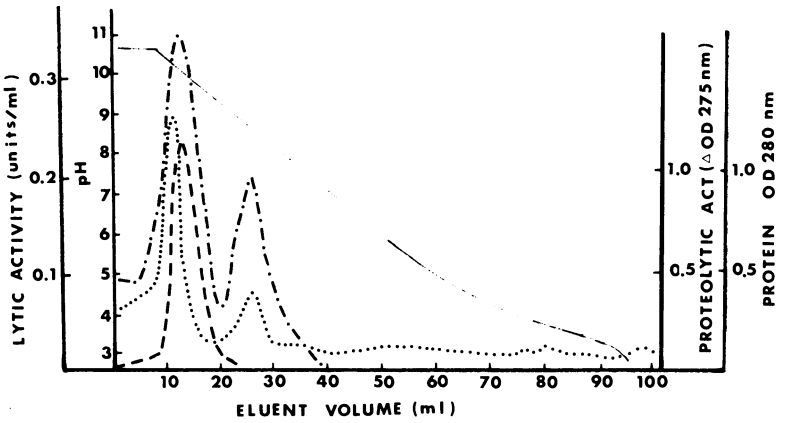

FIG. 1. *Results of mutanolysin subjected to electrofocusing.*

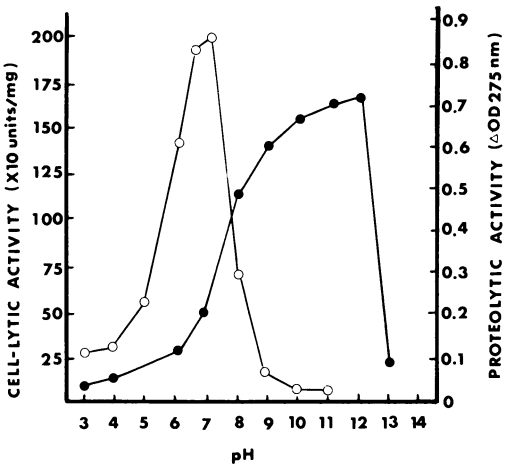

FIG. 2. *Activities of lytic and proteolytic enzymes in mutanolysin at different pHs.*

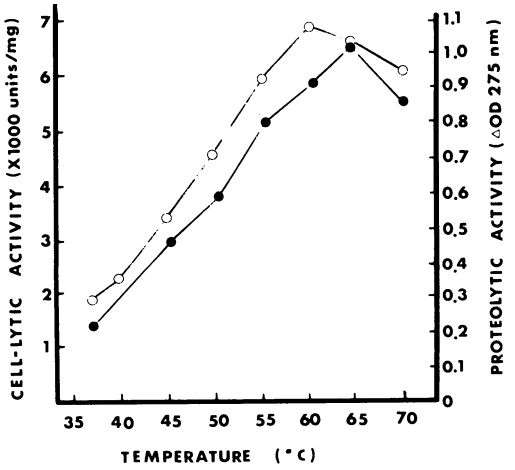

FIG. 3. *Lytic and proteolytic activities as determined by change in incubation temperature.*

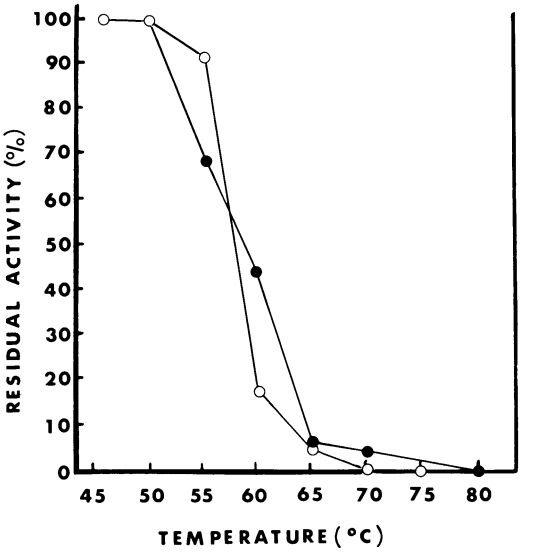

FIG. 4. *Effect of heat on lytic and proteolytic activities.*

remained even in the alkaline side at pH 11 (Fig. 5).

Effect of metal ions and various inhibitors. The lytic activity increased by 2.5- to 3.5-fold in 1 mM of Mg^{2+}, Mn^{2+}, Ca^{2+}, Ba^{2+}, and Co^{2+}, decreased by 80 to 90% in 1 mM of Ag^{+} and Cu^{2+}, and was lost completely in 1 mM of Fe^{3+} and Hg^{2+}. No metal ions activated proteolytic activity, and 50 or 80% inhibition was observed with Zn^{2+} and Pb^{2+} or with Hg^{2+} and Cd^{2+}, respectively (Table 2). Decrease in proteolyic activity with Cu^{2+}, Ag^{+}, and Fe^{3+} was not so strong as that in the lytic activity. Data on the effect of molarity of Ca^{2+} and Mg^{2+} upon the lytic activity are shown in Table 3. The lytic activity was maximal at a concentration of 1

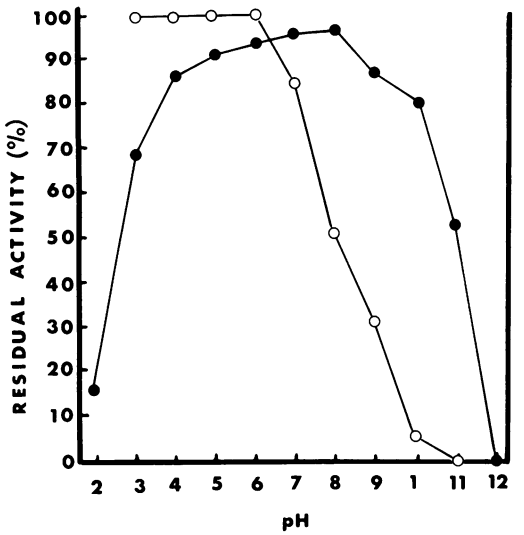

FIG. 5. *Effect of pH on the stability of lytic and proteolytic enzymes.*

TABLE 2. *Effect of various metal ions on lytic and proteolytic activities in mutanolysin*

Metal ion (nM)	Relative activity (%)	
	Cell lysis	Proteolysis
None	100	100
Li^{+}	92	97
Mg^{2+}	350	95
Ca^{2+}	345	82
Mn^{2+}	335	65
Cu^{2+}	21	90
Zn^{2+}	54	59
Fe^{2+}	53	104
Fe^{3+}	0	74
Co^{2+}	244	67
Ni^{2+}	89	63
Ag^{+}	13	88
Cd^{2+}	53	23
Ba^{2+}	358	91
Hg^{2+}	0	16
Pb^{2+}	71	43
Ethylenediaminetetraacetic acid	76	87

mM and decreased as ionic strengths were increased.

Table 4 shows the effect of some inhibitors on the lytic and proteolytic activities. Mutanolysin was preincubated with various enzyme inhibitors at 37 C for 5 min, and the residual activities were assayed. Both lytic and proteolytic activities were not affected by chelating agents, sulfhydryl inhibitors, carbonyl reagents, sulfhydryl compounds, soybean trypsin inhibitor (Sigma, type 1-S), and diisopropyl phosphofluoridate, but were inhibited nearly completely at a concentration of 0.1 mM *N*-bromosuccinimide. The lytic activity decreased to 13%, even at the low concentration of 0.01 mM of the inhibitor.

Lytic and bactericidal action of mutanolysin. Figure 6 represents the typical time course curves of lysis and viable cell counts when mutanolysin is allowed to act on *S. mutans* AHT and BHT. The optical density at 600 nm, due to whole cell suspensions of *S. mutans* AHT or BHT, decreased to 40 or 20% in 90 min in the presence of 20 μg of mutanolysin per ml. However, the rate of decrease in viable cell counts with *S. mutans* AHT was, on the contrary, about 1,000-fold greater than that with *S. mutans* BHT. For example, the viable cell counts with *S. mutans* AHT decreased from 2×10^7 to 2×10^2 cells/ml after 90 min of incubation (equivalent to 99.999% killing of the initial cells), whereas the decrease in those with *S. mutans* BHT was from 5×10^6 to 3×10^4 cells (99.0% were killed). Mutanolysin causes lysis of *S. mutans* BHT in human and hamster saliva (Fig. 7). Although the lytic activity in mutanolysin was suppressed in the golden hamster's natural saliva with pH 9.5, the activity was recovered when the pH of the saliva was adjusted to neutral. In human natural saliva, streptococcal cells were well lysed, even though a complete decrease in optical density did not occur because of turbidity attributed to the opaque saliva.

Spectrum of lytic activity. Mutanolysin was tested against cell suspensions of various living microorganisms to obtain its lytic spectrum. The results (Table 5) show the relative lysis of microorganisms tested by mutanolysin when lysis with *S. mutans* BHT was expressed as 100%. All gram-negative organisms were resistant to lysis. The genus *Streptococcus* was found to have a specific susceptibility to lysis by

TABLE 3. *Effect of concentration of calcium and magnesium ions on lytic activity in mutanolysin*

Concn (mM)	Relative activity (%)	
	Ca^{2+}	Mg^{2+}
None	100	100
0.05	174	186
0.10	200	193
0.25	208	198
0.50	213	208
1.00	219	211
2.50	195	198
5.00	178	148
10.00	87	81
25.00	22	37

TABLE 4. *Effect of various inhibitors on lytic and proteolytic activities in mutanolysin*

Inhibitor	Relative activity (%)	
	Cell lysis	Proteolysis
None	100	100
Chelating agent		
α-α Dipyridyl	101	116[a]
8-Hydroxyquinoline	105	83
Potassium oxalate	97	83
Succinic acid	98	90
O-phenanthroline	101	
Sodium diethyldithiocarbamate	94	93
Thiourea	102	90
Sodium pyrophosphate	85	77
Sulfhydryl inhibitor		
Iodoacetic acid	96	93
Sodium arsenite	90	119
Sodium arsenate	87	104
Sodium monofluoroacetate	90	105
Carbonyl reagent		
Hydroxylamine hydrochloride	97	98
Semicarbazid hydrochloride	112	88
Phenylhydrazine hydrochloride	100	110
Thiosemicarbazid hydrochloride	109	98
Hydrazine sulfate	107	85
Sulfhydryl comound		
L-Ascorbic acid	106	99
2-Mercaptoethanol	94	110
Glutathione	105	98
L-Cysteine hydrochloride	104	102
Dimercaprol	86	74
Other inhibitor		
NaF	91	110
Sodium azide	90	102
Soybean trypsin inhibitor	75[b]	85[b]
Diisopropyl fluorophosphate		
1.0 mM	69	76
0.1 mM	80	82
N-Bromosuccinimide		
1.0 mM	0	0
0.1 mM	8	0
0.01 mM	13	73
0.001 mM	61	96

[a] The agent was used at a concentration of 0.1 mM.
[b] To 10 μg of mutanolysin, 400 and 300 μg of inhibitor for lysis and proteolysis were added, respectively.

mutanolysin. *A. viscosus* which causes periodontal disease and fissure caries in hamsters was found to be highly susceptible to lysis just as was *S. mutans* BHT. Among the gram-positive strains tested, only *S. aureus* was resistant to mutanolysin. *Candida albicans*, a species of yeast, was not lysed.

Electron microscopy observation on lysis. The course of lysis of *S. mutans* BHT was followed by electron microscopy. Concentration of mutanolysin was adjusted so that lysis with the whole-cell suspension initiated by an optical density of 0.6 at 600 nm would be completed in 1 h. Figure 8 shows the time course of the cell lysis. The oval masses of uniform thickness in Fig. 8a are untreated streptococcal cells. When mutanolysin was added to a cell suspension, it was observed that a cell was spurting the protoplasm out of one side of the central cell by bursting in 5 min as shown in Fig. 8b. The edges of the cells lost some thickness. The cell seen in Fig. 8b left only a semicircular shell. Figure 8c

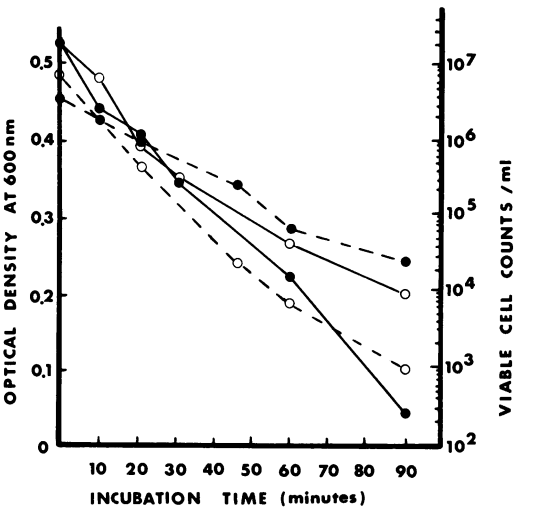

FIG. 6. *Time course curves of lysis and viable cell counts by mutanolysis on S. mutans.*

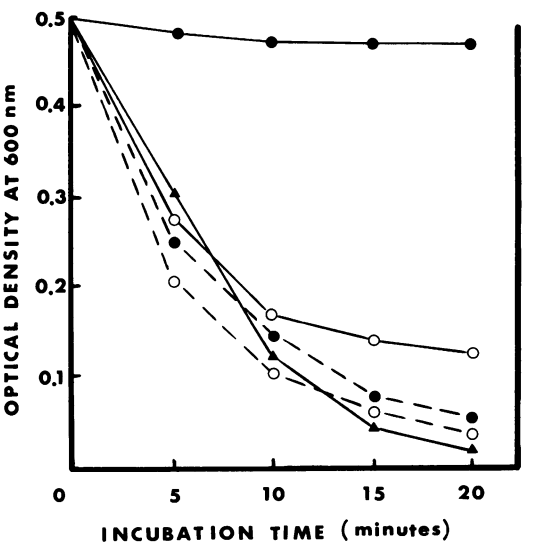

FIG. 7. *Lysis of S. mutans by mutanolysin in human and hamster saliva.*

TABLE 5. *Susceptibility of various bacteria to mutanolysin*

Organism	Lytic activity relative value (%)[a]
Actinomyces (Odontomyces) viscosus 15987	112
Bacillus subtilis PCI 219	28
Brucella abortus Kusayanagi	0
Diplococcus pneumoniae I	38
Escherichia coli K-12 p-512	0
Klebsiella sp. no. 13	0
Lactobacillus acidophilus IFO 1110	82
Listeria monocytogenes LI-2402	32
Proteus vulgaris OX_{19}	0
Pseudomonas aeruginosa Tsuchijima	0
Salmonella typhimurium S-9	0
Shigella flexneri 2a EW 10	0
Shigella sonnei EW 33	0
Staphylococcus aureus Terajima	0
Streptococcus mutans AHT	113
S. mutans BHT	100
S. mutans FA-1	73
S. mutans HS-1	117
S. mutans HS-6	101
S. mutans Ingbritt	53
S. mutans K1-R	114
Streptococcus salivarius IFO 3350	42
S. salivarius HHT	33
Streptococcus sanguis OMZ 9	21
S. sanguis M5	96
S. sanguis ATCC 10556	8
Streptococcus CHT (unidentified)	116
Candida albicans ATCC 10257	0

[a] Relative ratio of lysis for the tested organisms when the lysis on *S. mutans* BHT was expressed as 100%.

shows some loss in thickness of the cells at the terminal positions and a large, flattened shell which occurred by rupturing at the equatorial ring of the cell. This effect became more marked by digesting to 10 min, and many cells changed to circular or semicircular shells (Fig. 8d). A pair of semicircular shells split into two parts was observed after 20 min of incubation (Fig. 8e), and the lysis was most drastic on the cross wall at the equator. In the semicircular shells, a visible inner line which appears to be a cross wall at next cell division, appeared in parallel with a diametrical outer edge. A cell in Fig. 8f, which loses thickness by leaking intracellular materials, shows three faded lines in it. Two of them are situated symmetrically about a diametrical line. After 60 min, a number of cells disappeared against background, and some thin, indefinite sheets remained (Fig. 8g). However, the enlarged photograph (Fig. 8f) shows that the sheets consist of semicircular fragments or secondarily torn off fragments.

Products of cell walls degraded by mutanolysin. A reaction mixture containing 250 mg of cell walls of *S. mutans* BHT, 400 mg of NaN_3, 1,100 U of mutanolysin, and 1 mM $MgSO_4$ in 50 ml of 0.02 M phosphate buffer (pH 6.5) was incubated at 37 C for 72 h. The samples were collected at different time intervals and heated at 100 C for 5 min. After centrifugation, the supernatant was analyzed for amino groups and reducing sugars. The results are shown in Fig. 9. Maximal lysis of cell walls was obtained in 7 h with the liberation of 1 mol of reducing sugars and 2 mol of free amino groups per 1 mol of total glutamic acid in the digests.

DISCUSSION

Mutanolysin from *S. globisporus* 1829 contains mainly one proteolytic and two lytic enzymes. The lytic enzymes found were basic proteins with an isoelectric point near pH 8.5 and 10 by electrofocusing. The latter lytic enzyme possessed the same isoelectric point as the proteolytic enzyme, and they seemed to have a similar molecular size from the fact that both enzymatic activities leaked out in nearly equal ratios into the filtrates through three kinds of membranes. Furthermore, they seemed to have some specific tryptophan residues on the active sites, as postulated from the fact that they were inactivated by oxidation with *N*-bromosuccinimide. Final proof of the identity of both enzymes must await complete purification data. However, it seems more probable that both of the lytic and proteolytic enzymes are different upon further purification by diethylaminoethylcellulose and *O*-(carboxymethyl)-Sephadex column chromatography.

In the purification process, removal of cations such as calcium and magnesium derived from the culture filtrate results in apparent loss of lytic activity. Hence, the assay of lytic activity in each of the purification processes was made in the presence of 1 mM $MgCl_2$ to obtain a true yield. Nevertheless, the specific lytic activity did not increase as we expected in the purification procedure. The main reason for a little increase in specific lytic activity probably lies in the removal of proteolytic enzymes, which enhances an apparent lytic activity by its clearing action against cell debris during the purification steps. Sudo and Dworkin (23) have made a similar interpretation in studies on lytic enzyme from *Myxococcus xanthus* that the degree of purification could not be calculated in such a standard assay to measure decrease in turbidity of a suspension of whole cells, because the

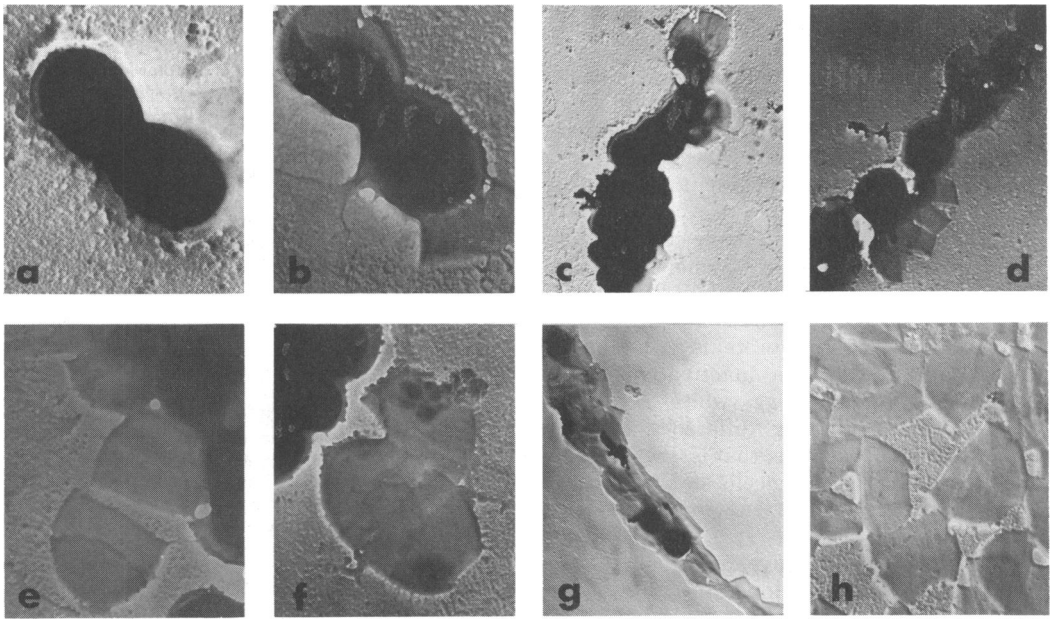

FIG. 8. *Time course of cell lysis.*

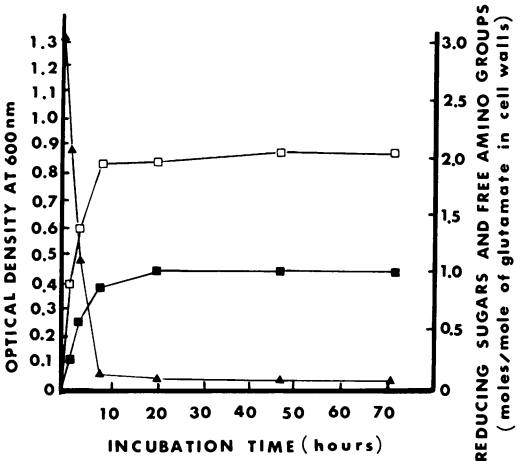

FIG. 9. *Analysis of cell wall digests for amino groups and reducing groups.*

standard assay did not differentiate some lytic from proteolytic enzymes and, therefore, the total activity of the crude preparations might be overestimated.

To investigate oral application of mutanolysin for dental caries control, its lytic action against *S. mutans* BHT cells suspended in human and hamster saliva was compared with that in buffer solution. Cariogenic streptococci were well lysed in human natural saliva, but the lytic action was completely suppressed in hamster natural saliva. The pH of saliva was 8.5 in human and 9.5 in hamster, and it was obvious that the suppressed lytic activity in the latter saliva was due to its high pH; when the saliva was neutralized to pH 7, the lytic activity of mutanolysin was restored to normal. No great difference was made in either rate or extent of lysis of the streptococcal cells between human saliva and buffer control. A shift of pH to alkali of harvested saliva agrees closely with the results of Charlton et al. (2). They observed that the pH of hamster saliva ranged between 6.3 and 9.0, depending on the time of exposure to air; e.g., the resting saliva around the parotid duct where the area is not directly in contact with air remained near pH 6.3, whereas sites which are more directly accessible to air gave readings as high as pH 9.0. They suggested that loss of carbon dioxide to the air was responsible for the shift to alkali. We also observed that a pH value of harvested saliva changed to alkaline in several minutes after spitting out of an oral cavity. Microbial plaques in the sulci of caries-active teeth actually ferment sucrose and produce strong acid. Accordingly, it is possible to conclude that the effect of mutanolysin in hamster saliva in vitro is not identical to that in vivo, and mutanolysin will be efficacious in experimental dental caries control in hamsters because the pH of saliva in the hamster is rather acidic.

The rapid solubilization of cell walls by mutanolysin was accompanied by the liberation of reducing sugars and free amino groups. The results show that mutanolysin contained glyco-

sidase and peptidase or amidase. We have obtained some evidence that the two main lytic enzymes seemed to be *N*-acetyl muramidase. However, the lytic mechanisms must be elucidated on pure cell walls by using perfectly purified enzymes to explain whether peptidase or amidase in mutanolysin is identical with the proteolytic enzyme or not.

Mutanolysin was found to have certain properties similar to egg white lysozyme; e.g., mutanolysin is inert to *S. aureus* and all gram-negative bacteria tested, exhibits some glucosidase activity, consists of basic proteins with alkaline isoelectric points and is inactivated by *N*-bromosuccinimide-like egg white lysozyme. However, there is a big difference between mutanolysin and lysozyme; that is, the former is capable of lysing the living cells and cell walls of *S. mutans*, whereas the latter is inactive against these strains.

Fitzgerald et al. (4) have reported that the active *Lactobacillus* developed in dental caries in weanling rats appeared to be a variety of *Lactobacillus acidophilus*. Recently, Ikeda et al. (11) have observed that *S. mutans* and *Lactobacillus* in plaque related to the initiation of human dental caries, and the latter bacteria became a sizable plaque microflora only after the appearance of caries. *Odontomyces viscosus* (renamed *Actinomyces viscosus*) isolated from subgingival plaque in hamsters has been demonstrated to have an ability to produce experimental periodontal disease in hamsters. Mutanolysin was easily capable of lysing cariogenic streptococci with four morphological categories conveniently classified by Jablon and Zinner (12). In addition, mutanolysin was also capable of lysing the living cells of *A. viscosus* and *L. acidophilus*. Mutanolysin may be expected to be a potentially useful agent for dental caries control because it (i) has lytic action against *S. mutans*, *S. sanguis L. acidophilus*, and *A. viscosus*, (ii) has optimal pH with acidity, (iii) has lytic activity in human saliva, and (v) is activated by calcium ions at concentrations generally found in human saliva (20).

In a subsequent paper, we will describe mutanolysin efficacy in vitro on the elimination of microbial plaques.

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