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Simple and Rapid Procedure for the Selective Removal of Lysozyme from Human Saliva

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A simple and rapid method is described for the removal of lysozyme from human whole salivary supernatant. Saliva specimens were adsorbed with *Micrococcus lysodeikticus*. The saliva so treated was depleted of 95% of the lysozyme activity. Changes in total protein, lactoperoxidase, lactoferrin, immunoglobulin A, and the proportions of several anionic proteins were less than 10%. It is concluded that adsorption of saliva with *M. lysodeikticus* is a suitable procedure for the preparation of saliva that is selectively deficient in lysozyme.

Saliva plays a crucial role in the maintenance of oral health. Conditions such as radiation-induced xerostomia and severe Sjogren's syndrome characterized by markedly reduced flow of saliva are accompanied by a striking increase in oral infections of the soft and hard tissues (3, 5, 7, 13, 14, 18). The constituents of saliva that contribute to its role in the maintenance of oral health include buffers, mucins and glycoproteins (hydration and lubrication of epithelial surfaces), and antibacterial factors (3, 15, 16, 18). The antibacterial properties of saliva have long been recognized (10, 25) but had received relatively little attention until knowledge of the composition of the oral flora associated with states of health and disease (caries, periodontal diseases, mucosal infections) accumulated (3, 16).

The antibacterial substances present in saliva fall into two categories: (i) factors that exert a direct bactericidal or bacteriostatic effect on target microorganisms; and (ii) factors that may interfere with the attachment of microorganisms to oral tissues and thus eventually lead to complete clearance, or a reduction in numbers, of the target microorganisms. The principal factors in the first group include lysozyme, lactoperoxidase, and lactoferrin (15, 16). Secretory immunoglobulin A (IgA) and blood group-reactive mucinous glycoproteins are the major salivary constituents in the second group (27, 28). Several recent studies have demonstrated the antibacterial properties of lysozyme, lactoferrin, and lactoperoxidase against members of the human oral flora (1, 6, 11, 20). In general, these studies used purified substances, some from nonsalivary sources, to demonstrate antibacterial activity and to study the mechanism of antibacterial action. The actual individual contribution of the above three antibacterial substances to the total activity of natural (i.e., unaltered) saliva is not known due to the unavailability of appropriate methods. Thus, the activity of one antibacterial substance acting in a salivary secretion which contains other major antibacterial constituents presently cannot be distinguished. One approach to the resolution of this problem is to study saliva specimens which are missing single antibacterial components. Thus, a comparison of normal saliva and a selectively deficient saliva would yield an estimate of the relative contribution of the missing constituent to the total antibacterial potency (and even to other phenomena such as adherence to oral tissues) of that secretion. In this communication we report a simple method to selectively remove about 95% of the lysozyme activity from human whole salivary supernatant.

MATERIALS AND METHODS

Saliva. Unstimulated whole saliva (about 5 ml per subject) was collected by expectoration from at least four healthy adult donors and pooled. The pooled saliva was clarified by two cycles of centrifugation at 12,000 × *g* for 15 min at 4°C. The salivary supernatant was further precooled by submersion in ice for 30 min before use. All saliva used in this study was freshly collected.

Lysozyme depletion of salivary supernatant. Dried *Micrococcus lysodeikticus* cells (Sigma Chemical Co., St. Louis) were suspended to a final absorbance of 0.5 (540 nm) in 10 ml of ice-cold 10 mM phosphate-buffered saline (pH 7.0). Next, the cells were sedimented at 3,000 × *g* for 10 min at 4°C, and the pellet was placed in ice. A 10-ml amount of ice-cold whole salivary supernatant was added to the *M. lysodeikticus* pellet. A uniform suspension was obtained by thorough mixing. After 10 min of incubation in ice, the suspension was centrifuged (15,000 × *g* for 5 min at 4°C), and the supernatant solution was re-

turned to ice. The salivary supernatant so treated is referred to as having received one cycle of *M. lysodeikticus* treatment. If desired, a second cycle of *M. lysodeikticus* treatment may be performed. Thus, a unit volume of precooled single-cycled salivary supernatant is used to suspend a pellet of *M. lysodeikticus* (originally derived from a unit volume of phosphate-buffered saline-suspended cells), followed by another 10 min of incubation in the cold and centrifugation as above.

Enzyme assays. Salivary lactoperoxidase was estimated as described by Gothefors and Marklund (9). Lysozyme was determined by a spectrophotometric (21) method in phosphate-buffered saline (pH 7.0). Three quantities of salivary supernatant were always assayed. The changes in absorbance over the first minute of the reactions were determined. The absorbance changes per minute were plotted against the volume of saliva used (dose-response) to ensure that the reaction rates were directly proportional to the quantity of saliva assayed. Lactoperoxidase activity is expressed as the change of absorbance per minute per milliliter of saliva (or per milligram of salivary protein) as determined from the slope of the dose-response relationship. One unit of lysozyme will cause a change of 0.001 absorbance unit (540 nm) per min at 37°C.

IgA, lactoferrin, protein, and PAGE. Salivary IgA levels were estimated by radial immunodiffusion by using commerically available plates (LC-Partigen Plates, Behring Diagnostics, Somerville, N.J.). Serum (Behring Diagnostics) that contained a standardized IgA concentration served as the primary standard. All salivary IgA (mainly secretory IgA) values are thus expressed as serum IgA equivalents. Saliva specimens were assayed in duplicate or triplicate. Precipitin zones were allowed to develop over 2 to 3 days at room temperature. The salivary IgA values reported here are lower than the actual values since secretory IgA (unavailable to us) was not used as the primary standard (4, 24). Lactoferrin was estimated by a rocket immunoelectrophoresis procedure as described by Tabak et al. (23). Human colostral lactoferrin (Calbiochem-Behring Corp., La Jolla, Calif.) served as the primary standard. Rabbit anti-human lactoferrin antisera (Behring Diagnostics) were added to the agarose to give a final concentration of 0.2% (vol/vol). Single rocket patterns were obtained with both standard lactoferrin and saliva specimens. Protein was estimated by the Folin phenol procedure (12) with bovine serum albumin as the standard. Polyacrylamide gel electrophoresis (PAGE) of salivary proteins employed a modification (8) of a published technique (22). Gels stained with Coomassie brilliant blue were scanned with a Joyce-Loeble MK III CS microdensitometer.

RESULTS

Depletion of lysozyme. Lysozyme depletion of whole salivary supernatant by incubation with *M. lysodeikticus* is shown in Fig. 1. Maximal removal of lysozyme activity occurred at *M. lysodeikticus* concentrations of 0.5 to 1.0 (absorbancy at 540 nm). Since increasing the concentration of the organism from an absorbancy of 0.5 to 1.0 gave only a minor increase in additional lysozyme removal, we adopted the lower cell concentration (absorbancy of 0.5) for routine use. The adsorption temperature (0°C) and time (10 min) were chosen to reduce the risk of lysis of the organism and also to decrease nonselective adsorption of salivary proteins other than lysozyme.

The results of several independent experiments are collected in Table 1. In this case lysozyme is expressed as units per milliliter of whole salivary supernatant. One cycle of treatment resulted in a mean (± standard error) of 85.3 ± 3.0% depletion of lysozyme. Two cycles of treatment averaged 95.3 ± 1.9% depletion of lysozyme. In two cases shown (control levels of 112 and 226 U/ml), one can see the incremental lysozyme depletion for one and two cycles of treatment (112 U/ml, 80 and 90%; 226 U/ml, 82 and 70%). Thus, two cycles of treatment under the conditions specified in Materials and Meth-

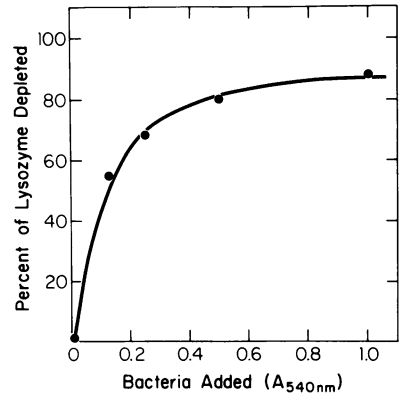

FIG. 1. *Adsorption of salivary lysozyme by M. lysodeikticus. Data shown are derived from one cycle of treatment with the microorganism.*

TABLE 1. *Lysozyme depletion of whole salivary supernatant by cold adsorption with M. lysodeikticus*

Adsorption	Lysozyme (U/ml)		
	Control	Treated	% Depleted
One cycle	112	22	80.4
	264	52	80.3
	264	32	87.9
	226	41	81.9
	104	4	96.2
			Avg, 85.3 ± 3.0[a]
Two cycles	112	2	98.2
	240	4	98.3
	226	13	94.2
	139	13	90.6
			Avg, 95.3 ± 1.9[a]

[a] Value ± standard error.

ods result in ca. 95% removal of lysozyme activity.

Specificity of *M. lysodeikticus* treatment. The effect of two treatment cycles of whole salivary supernatant on other major antibacterial substances is given in Table 2. In general, treatment of saliva resulted in a loss of ca. 10% of the total protein and lactoperoxidase. Over 90% of the lysozyme was removed. Lactoferrin decreased about 10% (micrograms per milligram), and IgA increased about 20% (micrograms per milligram).

As noted above, two cycles of treatment resulted in a loss of about 10% of the total protein. The quantities of lysozyme protein normally present in saliva (2) are too low to account for this much protein loss. Therefore, control and treated saliva specimens were subjected to PAGE in an effort to determine whether other proteins were selectively removed by *M. lysodeikticus* (Fig. 2). The data shown are microdensitometer scans of stained gels. The microdensitometer deflection marked by the heavy arrow is the junction between the 6 and 10% gels (see reference 8 for details of the gel system). No obvious difference in the PAGE protein profiles was noted except for the presence of a sharp, faint protein band in the saliva specimens exposed to *M. lysodeikticus* (area between peak 6 and the 6/10 gel junction on the upper scan). This sharp protein band was superimposed on a broad saliva protein band and was never noted in untreated saliva specimens. Thus, visual inspection of both the original stained gels and the microdensitometer scans indicated that there was no selective removal of those proteins that were visualized by this PAGE system. This conclusion is supported by the data in Table 3. The areas of the numbered protein peaks (Fig. 2) were estimated by a triangulation method. The proportion of the total peak area present in the individual peaks was then calculated. The means (±standard error) of two independent experiments are given in the table. A considerable degree of similarity exists between control and treated saliva specimens with respect to the proportional distribution of protein (i.e., peak area).

TABLE 2. *Effect of M. lysodeikticus treatment[a] of whole salivary supernatant on selected salivary constituents*

Salivary constituent	Control	Treated
Protein (mg/ml)	1.76	1.55
Lysozyme (U/mg)	128	8
Lactoperoxidase (A[b]/min per mg)	0.642	0.548
Lactoferrin		
(μg/mg)	6.19	5.55
(mg%)	1.09	0.86
IgA		
(μg/mg)	36.4	45.2
(mg%)	6.4	7.0

[a] Two cycles of treatment.
[b] A, Absorbancy.

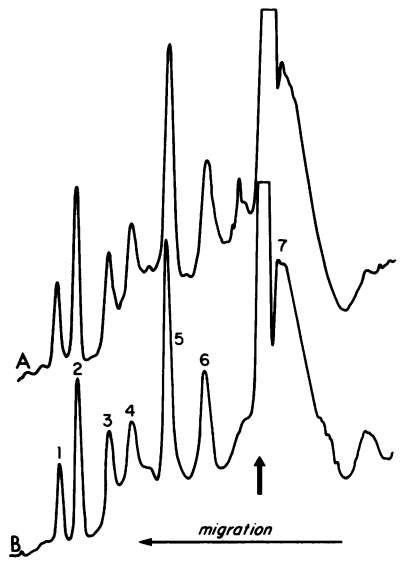

FIG. 2. *Polyacrylamide gel electrophoresis of control and treated saliva. Data shown are microdensitometer scans of stained gel lanes. Heavy arrow indicates the junction between 6 and 10% acrylamide slabs. Direction of electrophoretic migration is indicated by the horizontal arrow. (A) Treated saliva; (B) control saliva.*

TABLE 3. *Proportional distribution of peak area among the major saliva proteins (PAGE)*

Protein band	% of total protein (SE)[a]	
	Control	Treated
1	2.9 (0.99)	2.4 (0.64)
2	6.1 (1.27)	5.7 (0.92)
3	5.1 (0.78)	5.3 (0.57)
4	8.2 (1.49)	6.7 (2.40)
5	15.8 (0.64)	15.6 (0.64)
6	11.2 (0.42)	13.5 (2.62)
7	50.9 (1.06)	51.1 (7.92)

[a] SE, Standard error.

The results from several experiments are summarized in Fig. 3. The original values used to calculate the percentages shown were based on milligrams of salivary protein. Data from both one and two cycles of treatment are included. The accumulated results (Fig. 3) demonstrate the reasonably selective nature of the *M. lysodeikticus* treatment procedure.

DISCUSSION

As mentioned earlier, the availability of saliva specimens that are selectively deficient in anti-

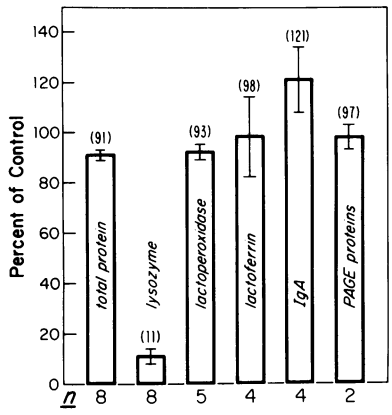

FIG. 3. *Summary of the effect of M. lysodeikticus treatment of saliva on the concentrations of several salivary constituents. The numbers in parentheses are the means. Vertical lines represent standard errors. Number of independent experiments is given by n. Control refers to saliva not treated with the organism.*

bacterial substances would greatly aid in the determination of the proportional contribution of individual substances to the total antibacterial activity of saliva. There are two potential sources of selectively depleted saliva specimens: (i) human subjects naturally deficient in a single antibacterial substance, and (ii) experimental depletion of normal saliva specimens of single antibacterial constituents. Saliva obtained from a subject exhibiting a selective deficiency must, of necessity, be compared with saliva from another (control) individual. In consideration of the large inter- and intraindividual variability in the levels of salivary constituents, it is unlikely that a truly suitable control saliva could be found to be matched to a naturally deficient saliva for experimental comparisons. In our opinion, it seemed more reasonable to attempt to selectively remove antibacterial substances from normal saliva specimens. If the method of removal of the desired antibacterial substance were truly selective for that substance, then an exquisitely matched control and selectively depleted saliva pair would be available for comparative analyses.

The results of this study demonstrate that it is possible to selectively remove 95% of the lysozyme from human whole salivary supernatant. Lactoperoxidase, lactoferrin, and total protein exhibited, on the average, only a 5 to 10% loss. Similarly, no selective depletion of the major anionic proteins displayed by PAGE was noted. It must be recognized that the results summarized in Fig. 3 represent means of several experiments. For any given saliva specimen that is subjected to the *M. lysodeikticus* treatment, one must determine the pre- and post-treatment concentrations of those salivary constituents relevant to the particular experimental use planned for the lysozyme-depleted specimen.

It is of interest to note that IgA values were increased (121 ± 13%) in lysozyme-depleted specimens. It has recently been reported that IgA and secretory IgA standards yield nonparallel standard curves in radial immunodiffusion assays (17). Since the secretory IgA standard curve had a greater slope than the serum IgA standard curve (17), the percent change reported here in salivary IgA (Fig. 3) after exposure to *M. lysodeikticus* may be underestimated. It has been reported (26) that lysozyme may associate with immunoglobulins. Thus, it is possible that lysozyme may interfere with the determination of IgA by the radial immunodiffusion technique used here. Clearly, a larger number of observations are required to establish whether an actual relationship exists between lysozyme depletion and increased IgA values (by radial immunoassay). It has been reported that salivary lysozyme levels were elevated in subjects with salivary immunodeficiencies (2). The lysozyme assay used in the study was an agar diffusion method. Perhaps diffusion-based IgA and lysozyme assays are influenced by salivary lysozyme-IgA associations that either exist initially or develop during the course of the determinations.

The average loss of 10% of total protein after *M. lysodeikticus* treatment cannot be due solely to lysozyme removal. Lysozyme represents less than 1% of the total salivary protein. The 10% protein loss may be due to removal of other cationic salivary proteins, nonselective (i.e., universal) removal of small quantities of the anionic proteins displayed by the PAGE system used here, removal of salivary mucins, or a combination of the above. Release of significant quantities of proteins by *M. lysodeikticus* during the saliva treatment was not evident as judged by PAGE. Except for the faint sharp band noted earlier, no new proteins were observed. The origin of the faint sharp band is actually unknown but is most likely from the bacterium.

It should be recognized that the general approach adopted here, i.e., that of using a bacterium as an absorbant of a salivary antibacterial substance, was explored almost 40 years ago (25). Among other studies, the investigators reported that incubation of *M. lysodeikticus* with saliva in the cold for 24 h resulted in the removal of an anti-staphylococcal factor. Based on the results presented here, it is reasonable to suppose that the anti-staphylococcal factor(s) included salivary lysozyme.

Finally, the procedure described here appears

to be suitable for the preparation of saliva that is greatly deficient in only one major antibacterial substance. Since the remaining antibacterial substances and total protein differ in concentration by less than 10% from the untreated saliva, a potentially useful system is available to query the participation of salivary lysozyme in various biological phenomena. Thus, it is now possible to determine the effect of lysozyme depletion on the (i) antibacterial activity of saliva, (ii) salivary enhancement/inhibition of adherence of bacteria to oral epithelial cells, and (iii) biological and biochemical properties of salivary pellicle formed on enamel surfaces or hydroxyapatite. For example, a recent investigation (19) pointed out the possibility that salivary lysozyme may be involved in the attachment of a *Streptococcus sanguis* isolate to hydroxyapatite surfaces. An investigation of the adherence-promoting capacity of lysozyme-depleted saliva compared to the untreated control saliva would aid in the assessment of the role of lysozyme in this model system.

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