Factors Influencing Measurement of Human Salivary Lysozyme in Lysoplate and Turbidimetric Assays

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The use of different assay conditions has complicated the evaluation of studies relating salivary lysozyme levels to oral or systemic disease. The purpose of this study was to compare values obtained for lysozyme activity in mixed saliva of 104 healthy subjects by using two assay techniques and four variations in sample preparation. Lysozyme activity was assayed by the turbidimetric and lysoplate methods with human colostrum lysozyme as the standard. Lysozyme activity in saliva samples made 0.5 M with respect to NaCl was compared with that in untreated samples with and without centrifugation. Mean values for lysozyme concentration in centrifuged saliva were 2.2 µg/ml with the turbidimetric assay and 5.9 µg/ml with the lysoplate assay. In samples which were salt treated before centrifugation, mean concentrations increased to 17.3 and 72.9 µg/ml, respectively. The results for uncentrifuged saliva were four to five times higher than the results for centrifuged saliva in each of the assay systems. Salt treatment without centrifugation produced values comparable to those obtained with centrifugation. The addition of salt to human colostrum or hen egg white lysozyme generally resulted in a 20 to 25% increase in expressed activity. These results indicate that (i) the measurement of lysozyme in the supernatant of centrifuged saliva is of questionable value, (ii) most of the lysozyme in whole saliva is inactive and may be activated by markedly increasing the ionic strength, and (iii) values for lysozyme activity in whole saliva are much greater in the lysoplate assay than in the turbidimetric assay when the same standard is used.

It has long been suggested that the presence of lysozyme in biological fluids such as saliva is an important factor in the nonspecific defense mechanisms of the body against microbial invasion. However, the precise role of lysozyme in these defense mechanisms and, indeed, the importance of lysozyme have remained elusive (4). There has been a significant amount of effort on the part of various investigators to relate differences in the concentration of lysozyme in human saliva to various oral or systemic disease states (1, 2). Attempts to evaluate the results from any one study in terms of those obtained from another study on the same pathophysiology have been frustrated by the lack of use of a single assay system and by the use of different standard lysozyme preparations.

In general, two assays, a turbidimetric assay (10, 11) and the lysoplate technique (7), have been used. They have in common the use of the same biological event, the lysis of "*Micrococcus lysodeikticus*." Studies on blood serum which have compared the two assays for the measurement of lysozyme concentration in the same serum samples have yielded divergent results (3, 9), reflecting differences in the techniques. Similar comparisons are not available for saliva.

The interaction of lysozyme with components such as bacteria, bacterial cell wall fragments, and proteins, such as mucins, which are found in saliva, has been suggested to present a problem in the accurate determination of lysozyme concentration in saliva (13). Several studies have suggested the use of a mild acid treatment of whole saliva or parotid saliva to dissociate such complexes before assay (8, 14). Other investigators have used an immunologically based assay which is not dependent on the expression of biological activity to determine the concentration of lysozyme in saliva (12). Because the level of active lysozyme may be more

relevant for elucidating a defensive role for this protein, an assay that differentiates between active and inactive lysozyme in saliva would provide meaningful results.

The purpose of this study was to directly compare the levels of lysozyme measured with either a turbidimetric assay or the lysoplate assay in human saliva and to evaluate a method not involving the relatively slow and drastic change in pH suggested by other investigators to be necessary for the accurate measurement of total lysozyme activity in saliva.

MATERIALS AND METHODS

Saliva collection and sample preparation. Whole saliva was collected from 104 subjects by paraffin stimulation. A 1-ml portion of each sample was frozen and stored at -20°C for subsequent lysozyme assays. Before the assays, the sample was divided in half, and one portion was diluted with an equal portion of 1.0 M NaCl. The portions were further divided, and half of each (250 µl of saliva) was centrifuged at 5,000 × g in a Fisher Microfuge for 4 min at 4°C. Thus, for each subject four separate assay samples were obtained: (i) the original whole saliva, (ii) whole saliva after salt treatment, (iii) the supernatant fraction from centrifuged whole saliva, and (iv) the supernatant fraction from centrifuged salt-treated whole saliva.

Lysozyme assays. Each sample was assayed in duplicate by both the lysoplate and the turbidimetric technique. The results were expressed in micrograms of lysozyme per milliliter of starting whole saliva based on comparison with the activity of human colostrum lysozyme (Sigma Chemical Company, St. Louis, Mo.). Standard solutions were prepared by dissolving the respective lysozyme preparation in deionized water. The protein concentration was established by spectroscopy. Stock solutions of standards of 200 to 250 µg/ml were prepared and stored at 4°C. Independent dilu-

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FIG. 1. Activity of human colostrum lysozyme and hen egg white lysozyme. (a) Lysoplate assay; (b) turbidimetric assay. Δ , Colostrum lysozyme; $\circ$, hen egg white lysozyme. For the turbidimetric assay, one unit of activity was defined in terms of the amount of lysozyme which caused a decrease of 0.001 A_{490} U/min at 25°C. Points represent means of triplicate assays from 11 series of experiments. Lines were derived by linear regression ($r^2 > 0.98$).

tions ranging from 8 to 84 $\mu\text{g/ml}$ were made for each series of assays, and the same dilutions were used for both the lysoplate and the turbidimetric assay. Four dilutions, ranging from 12.5 to 100 $\mu\text{g/ml}$, of hen egg white lysozyme (Worthington Diagnostics, Freehold, N.J.) were also included in each assay series. These standards were prepared in the same manner as the human colostrum lysozyme standards. Concentrations of standards were selected to accommodate the range of lysozyme activity expected for the whole saliva samples. In both assays, reference curves established with these concentrations exhibited strong linearity (Fig. 1). Hen egg white lysozyme activity was approximately 40% that of human colostrum lysozyme, and this relationship was consistent in both assays.

Lysoplate assay. Lysoplates were prepared as described by Osserman and Lawlor (7) by using 1% agarose (type I, low EEO; Sigma) and "*M. lysodeikticus*" cells (Worthington). Portions (5 μl) of the samples or standards were placed in the 2-mm wells, and the lysoplates were incubated for 18 h at room temperature (23 to 25°C). Diameters of lysis zones were determined directly with a dial caliper (Mitutoya; obtained from Laboratory Supplies Co., Hicksville, N.Y.) accurate to 0.02 mm. The results for standards were plotted on a semilogarithmic graph, and the values for samples were extrapolated from this reference curve.

Turbidimetric assay. The turbidimetric assays were performed by using a Kontron 810/820 UV-VIS Spectrophotometer essentially as described by Shugar (10), except that the wavelength of measurement was 490 nm. This wavelength was selected to provide a useful compromise between sensitivity and accuracy, as discussed by Morsky (6). A 10- μl portion of the samples or standards was added to a 990- μl portion of a suspension of "*M. lysodeikticus*" cells (30 mg of dried cells suspended in 100 ml of 0.1 M sodium phosphate [pH 7.0] by stirring for 1 h at room temperature). In situations, such as with centrifuged saliva, in which low activity was observed, the assay was repeated with a 25- μl portion of the sample with 975 μl of substrate. The decrease in optical density was monitored at 490 nm as a function of

time by using a chart recorder. The slope of the line was measured from the first 2 to 5 min of reaction. One unit was defined as the amount of lysozyme activity required to cause a decrease of 0.001 A_{490} U/min at 25°C. Lysozyme values for samples were converted to micrograms per milliliter by using the reference curve established with standard lysozyme preparations.

Control experiments. A separate control experiment was conducted to determine the effect of increased ionic strength on the lysozyme activity of the standard preparations of human colostrum lysozyme and hen egg white lysozyme. In this experiment, 0.5 M NaCl was used as the diluent in place of deionized water, making the solutions equivalent to the salt-treated saliva samples in ionic strength. Activity was compared with that of the regular standard preparations over the same range of concentrations in deionized water. There was generally somewhat greater activity (20 to 25%) in both assays with the salt-treated standards than with the same standards in deionized water. The addition of 0.5 M NaCl alone to either assay system in the absence of sample or standard did not result in activity.

Because diluted saliva samples were being compared with undiluted samples, several saliva samples were diluted with water or were made 0.5 M with respect to NaCl by the addition of solid salt. The results obtained were equivalent to those with undiluted saliva and with saliva samples diluted with 1.0 M NaCl, respectively.

To compare the salt treatment method with the acid treatment method described by Virella and Goudswaard (13), additional saliva samples were treated in the following manner. A portion of whole saliva was taken into the whole, salt-treated whole, centrifuged, and centrifuged salt-treated fractions as described above. A second portion was placed in dialysis against 0.1 M Tris acetate (pH 4.5; Spectrapor 8,000- to 10,000-molecular-weight membranes) for 1 h at room temperature. A portion of this material was removed, and the pH was raised to 7.5 by the addition of solid Tris base. The remaining material was subjected to centrifugation at $10,000 \times g$ for 10 min at 4°C, and the supernatant fraction

TABLE 1. Lysozyme activity in mixed saliva according to sample preparation and assay technique^a

Sample prepn ^b	Amt of lysozyme ($\mu\text{g/ml}$ of saliva) ^c by indicated assay technique					
	Lysoplate			Turbidimetric		
	Mean $\pm$ SD	Median (range)	Modal value	Mean $\pm$ SD	Median (range)	Modal value
W	22.7 $\pm$ 23.7	14.0 (1.2–130.0)	8.5	11.1 $\pm$ 6.3	10.5 (1.3–34.2)	10.5
WS	79.9 $\pm$ 86.9	54.0 (1.3–505.0)	11.5	17.9 $\pm$ 11.1	16.3 (1.5–50.0)	6.5
C	5.9 $\pm$ 9.3	2.7 (0.5–64.0)	0.8	2.2 $\pm$ 1.6	1.7 (0.7–10.8)	1.0
CS	72.9 $\pm$ 84.2	43.0 (1.6–580.0)	36.0	17.3 $\pm$ 11.2	15.5 (1.5–46.6)	6.7

^a Human colostrum lysozyme was used as the standard in both assays.^b W, Uncentrifuged whole saliva; WS, uncentrifuged salt (0.5 M)-treated saliva; C, supernatant of centrifuged whole saliva; CS, supernatant of centrifuged salt-treated whole saliva.^c Values were obtained from the assay of saliva from 104 subjects. All assays were performed in duplicate.

was neutralized as described above. These various samples were compared in the same assay series by both the turbidimetric and the lysoplate method.

Analysis of results. Means and standard deviations of lysozyme activity in the saliva from the 104 subjects were calculated for each sample preparation and assay method. Modal values were also determined.

The distribution of values was tested for normality by using the Kalmogorov-Smirnov procedure. The values obtained in the lysoplate assay for all of the variations of sample preparation were skewed to the right, with their modes over a lower value. Transformation to logarithms resulted in a normal distribution for all sets of values. Turbidimetric results, although distributed too uniformly and too flatly to be exactly normal, were close enough to normality to use untransformed. Pearson product-moment correlation coefficients were determined between sample preparations and between the two assay methods. Because some distributions were not exactly normal, nonparametric Spearman rank correlation coefficients were also determined.

RESULTS

The results obtained for the individual saliva samples are shown in Table 1, and the Pearson correlation coefficients for the different saliva sample preparations are shown in Table 2.

By both assays, lysozyme activity was reduced by 75 to 80% in saliva samples directly centrifuged under our conditions. Salt treatment of whole saliva increased detectable lysozyme activity by more than threefold in the lysoplate

assay and by approximately half this proportion (1.6) in the turbidimetric assay. In both assays, significant correlations were observed between values obtained for uncentrifuged whole, uncentrifuged salt-treated, and centrifuged salt-treated samples. In the lysoplate assay, correlation coefficients ranged from 0.860 to 0.952 for these samples. Coefficients were comparable for turbidimetric results. In all cases, *P* values were 0.0001. Centrifuged samples exhibited significant (*P* = 0.0001) correlation with other samples in the lysoplate assay, but the coefficients were lower than between the other samples. In the turbidimetric assay, the centrifuged samples correlated significantly only with the uncentrifuged whole saliva samples, but the coefficient was quite low.

The results obtained with the lysoplate assay were consistently higher than those obtained with the turbidimetric assay. This difference was two- to threefold for the whole and centrifuged fractions but increased to four- to fivefold for the salt-treated fractions. The Pearson product-moment coefficients of correlation between logarithms of lysoplate results and initial units of turbidimetric results were 0.567 for uncentrifuged whole saliva, 0.721 for uncentrifuged salt-treated saliva, 0.527 for centrifuged whole saliva supernatant, and 0.776 for centrifuged salt-treated whole saliva supernatant. The values obtained from the two assays were significantly correlated for all of the sample preparations (*P* = 0.0001).

Spearman rank correlation coefficients are not shown. These values were within ± 0.05 of the Pearson scores, except for correlation of the centrifuged whole saliva supernatant values in the turbidimetric assay. These coefficients

TABLE 2. Pearson product-moment correlation coefficients between variations in saliva sample preparation

Samples compared ^a	Correlation coefficient ^b by indicated assay method	
	Lysoplate	Turbidimetric
W vs WS	0.860 ^c	0.900 ^c
W vs C	0.607 ^c	0.193 ^d
W vs CS	0.861 ^c	0.904 ^c
C vs WS	0.469 ^c	0.163 ^c
C vs CS	0.460 ^c	0.160 ^c
CS vs WS	0.952 ^c	0.904 ^c

^a W, Uncentrifuged whole saliva; WS, uncentrifuged salt (0.5 M)-treated saliva; C, supernatant of centrifuged whole saliva; CS, supernatant of centrifuged salt-treated whole saliva.^b Correlation coefficient between logarithms of lysoplate results and initial units of turbidimetric results.^c *P* = 0.0001.^d *P* = 0.0395.^e Not significant.TABLE 3. Lysozyme activity in human mixed saliva after acid or salt treatment^a

Assay method and sample	Amt of lysozyme ($\mu\text{g/ml}$ of saliva) in samples that were:		
	Untreated	Salt treated ^b	Acid treated ^c
Lysoplate			
Whole	16.3	27.3	20.0
Centrifuged	8.9	28.8	23.3
Turbidimetric			
Whole	6.4	9.3	10.3
Centrifuged	3.4	10.1	9.3

^a Human colostrum lysozyme was used as the standard.^b Whole saliva was diluted 1:1 with 1.0 M NaCl.^c Whole saliva was dialyzed against 0.1 M Tris acetate (pH 4.5) for 1 h. The whole saliva sample was removed before centrifugation and neutralized with solid Tris. The centrifuged sample was obtained by neutralization of the supernatant fraction after centrifugation.

were about 0.10 higher than the Pearson scores and were significant ($P = 0.0112$ to 0.0001) for all comparisons.

In the saliva samples subjected to treatment with 0.1 M Tris acetate (pH 4.5) before assay, the values for lysozyme activity were comparable to those obtained when the same whole saliva samples were mixed 1:1 with 1.0 M NaCl before assay (Table 3).

DISCUSSION

It is clear based on our results that the assessment of lysozyme activity in human whole saliva is influenced by a number of factors. Several of these factors were investigated in this study. Of particular importance is that our observations represent the first direct comparison of the effects of these factors on the same set of human whole saliva samples.

Assay methods and standards. The values for lysozyme concentration for all saliva samples were higher with the lysoplate assay than with the turbidimetric assay. These results were obtained by using an identical standard human lysozyme preparation in both assay systems. Greater values with the lysoplate assay as compared with the turbidimetric assay were also obtained by Klass and co-workers (5) for lysozyme concentration in blood serum but with hen egg white lysozyme as the standard for both assay systems. Under our assay conditions, the relative activities of human colostrum lysozyme and hen egg white lysozyme were similar for both assays. A strong linear relationship occurred, with concentrations of 8 to 84 $\mu\text{g/ml}$ for human colostrum lysozyme and 12.5 to 100 $\mu\text{g/ml}$ for hen egg white lysozyme. Consequently, either standard should be appropriate to obtain relative lysozyme activity in test samples in either the lysoplate or the turbidimetric assay system in the absence of any other factors which might differentially affect lysozyme activity.

Because the use of the same lysozyme standard (either human or hen egg white) yielded markedly different values for the concentration of lysozyme in saliva in the two assay systems, it is apparent that the observed activity of lysozyme in human saliva was influenced by the assay conditions in one or both of the systems. This quality of effect has been observed with the assay of lysozyme in blood (3, 5, 9, 15). In the absence of rigorous structural analysis of human salivary lysozyme, it is possible that there are molecular differences not detected by polyclonal antibodies which result in different activity in the two assay systems under our experimental conditions. Although this is unlikely, it must be considered until more information regarding the molecular properties of the human salivary protein is available. A more likely possibility is that there are substances in human saliva which differentially affect the observed activity of the enzyme in the two assay systems. Our laboratory recently saw such effects with amines and polyamines (J. W. Jenzano and R. L. Lundblad, abstr. 298, Annu. Meet. Int. Assoc. Dental Res., 1986).

Sample preparation. Values for lysozyme activity in saliva were greatly reduced in our study if the whole saliva was centrifuged before assay and the supernatant fraction was used as the sample, such that lysozyme activity was only 20 to 25% that of whole untreated saliva. Furthermore, correlation of these values with those of the other variations of sample preparation was low. Apparently, a considerable amount of active lysozyme is removed by centrifugation, and the factors responsible for the apparent binding and removal of lysozyme appear to be separate variables, such that the resulting information has questionable value.

The treatment of whole saliva with 0.5 M NaCl increased measurable lysozyme activity in both of the assay systems. The difference between the values obtained before and after salt treatment could reflect the amount of the enzyme in the oral environment that is inactive or unavailable because of binding to other salivary constituents, such as mucins or bacterial cells. A similar argument was previously advanced by Virella and Goudswaard (13) and by Virella and co-workers (14). The values obtained by salt treatment in the present study (the uncentrifuged salt-treated saliva values) exhibited high correlation with those for untreated saliva and were not significantly altered if the samples were subsequently centrifuged and the supernatant fractions were used as samples in either assay system. The results with salt treatment were comparable to those obtained with the more complex procedure of pH adjustment by dialysis against pH 4.5 Tris acetate (13). Of particular note is that for salt-treated samples, a comparison of lysoplate and turbidimetric results showed that the magnitude of the increase was much greater with the lysoplate assay. Although some of the difference may reflect a modest effect of increased ionic strength on the activity of lysozyme in the two assays, it is apparent that other factors influenced the observed lysozyme activity. The difference between the centrifuged and centrifuged salt-treated saliva values in Table 1 most likely reflects the activation of bound lysozyme in whole saliva and probably provides a reasonable estimate of the total lysozyme activity in saliva which can be assayed by the lysis of "*M. lysodeikticus*" in either assay system.

Conclusions. (i) The lysoplate assay provides higher values for lysozyme concentration in human saliva than does the turbidimetric assay with either human colostrum lysozyme or hen egg white lysozyme as the standard in each assay.

(ii) The use of human colostrum lysozyme instead of hen egg white lysozyme in both the turbidimetric and the lysoplate assay system gives lower values for lysozyme concentration in human saliva.

(iii) The value for lysozyme concentration in the supernatant fraction obtained by the centrifugation of untreated whole human saliva both is low with respect to the amount of lysozyme in whole saliva and appears to be unreliable with respect to values obtained with the treatment of whole saliva.

(iv) Treatment of whole saliva with 0.5 M NaCl increases the amount of lysozyme available for assay in both the turbidimetric and the lysoplate system.

(v) Evaluation of the results of studies relating to changes in lysozyme concentration in whole saliva must involve the consideration of assay methods, standards used, and sample preparation.

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