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Development of an Enzyme-Linked Immunosorbent Assay for Determination of Lysozyme in Human Parotid and Submandibular-Sublingual Salivas

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The specificity of lysozyme determinations in human parotid and submandibular-sublingual salivas of two subjects was assessed by comparison of lysozyme concentrations in native acidified salivas with purified enzyme obtained by immunoadsorbent fractionation of the salivas. Lysozyme concentrations were measured by the turbidimetric catalytic method and by a newly developed enzyme-linked immunosorbent assay (ELISA). The validity of the assays was established by comparing assay results with enzyme concentration values determined from optical density-extinction coefficient calculations of the purified lysozyme peak. Values for purified enzyme were found to be similar, irrespective of the assay used to determine lysozyme concentrations, and were in agreement with extinction coefficient calculations. Based on the ELISA technique, recoveries of lysozyme from both parotid and submandibular-sublingual salivas were greater than 75 and 90%, respectively. Similar recoveries were noted for parotid saliva when determinations were based on the turbidimetric assay. However, the ELISA and turbidimetric assays differed with respect to lysozyme levels in submandibular-sublingual saliva because of the apparent presence of an enhancement factor which gave rise to higher lysozyme values in the catalytic assay and therefore resulted in low recoveries of purified enzyme. This catalytic enhancement factor was present in the nonadsorbed fraction of both subjects, as higher lysozyme activities were noted when nonadsorbed fractions were added to affinity-purified lysozymes. Lysozyme levels were also determined in the parotid and submandibular-sublingual salivas of caries-resistant and -susceptible adults. In general, levels of lysozyme in parotid saliva were lower in comparison to submandibular-sublingual saliva; however, significant differences in enzyme concentration were not evident between the caries-resistant and caries-susceptible subjects. Standard errors were within ±10% by the ELISA method.

In recent years, determination of lysozyme concentrations in oral and other biological fluids has been carried out for comparison of normal versus pathological states (1, 12, 17, 18, 20, 23, 26). However, the validity of using the generated experimental data for diagnostic purposes has sometimes been complicated by reports by different investigators of the presence of widely different lysozyme levels for the same biological source. As has been pointed out by many investigators, differences in reported lysozyme concentrations may arise because of the assay systems used to quantitate the presence of the enzyme in the biological fluids (6, 7, 15, 19, 21, 24). There are several problems, including the use of a hen egg-white lysozyme standard instead of purified human lysozyme, which gives significantly higher values of unknown enzyme concentrations and is further complicated by the variability noted among the different assay systems (8). Two commonly used methods, the spectrophotometric and lysoplate assays, employ *Micrococcus luteus* as a substrate, although the latter technique seems to measure the lysozyme concentration on the basis of diffusion rather than catalytic activity (9). In addition, both of these systems have the advantage and at the same time have been suggested to suffer from the fact that total enzyme concentration may not be measured in the biological fluid unless a pretreatment step is applied because of the capacity of lysozyme to complex with other biological molecules (8, 27). To overcome this problem, immunological methods have been developed, and they have been reported to measure total enzyme concentration (10, 11, 21). However, these methods may also suffer from a requirement for diffusion in agarose which, like the lysoplate method, may give rise to erroneous results.

A major difficulty, therefore, may be uncertainty whether the lysozyme assay being used provides accurate concentrations of lysozyme in the biological fluid or tissue being examined. By demonstrating that lysozyme can be selectively removed from biological fluids in a single step (14), it is possible to compare the concentration of purified lysozyme with the concentration of enzyme in the biological fluid. Moreover, an assessment of the accuracy of the assay can then be made, since the purified enzyme concentration can be calculated independently of the assay through a determination of the human lysozyme extinction coefficient. In this communication, we describe the development of a sandwich enzyme-linked immunosorbent assay (ELISA) to accurately quantitate lysozyme in human parotid and submandibular-sublingual salivas.

MATERIALS AND METHODS

Biochemicals. Human lysozyme was purified from leukemic urine as described previously (14). The concentration of leukemic lysozyme was determined from the extinction coefficient of $E^{1\%}_{1\ cm} = 26.9$ at 280 nm (13). The immunoglob-

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ulin G (IgG) populations of sheep and rabbit antisera to human leukemic lysozyme were prepared as reported (14). Sheep IgG, directed solely towards human lysozyme, was obtained through immunoadsorption affinity chromatography (B. L. Grossbard, J. J. Pollock, and V. J. Iacono, published data). The disodium salt of *p*-nitrophenyl phosphate and type VII alkaline phosphatase were purchased from Sigma Chemical Co., St. Louis, Mo. Goat anti-rabbit IgG was from Miles Laboratories, Elkhart, Ind. Chemicals used in the preparation of buffers were of the highest purity available.

Salivary lysozyme. Parotid salivas were obtained by indirect cannulation with modified Carlson-Crittenden collecting devices (3) and use of 2% citric acid or sour lemon drops (Regal Crown) as stimulants of salivary flow. Stimulated submandibular-sublingual saliva was collected by indirect cannulation of the ducts of these glands with modified Truelove collection devices (25). When parotid and submandibular-sublingual salivas were to be used in affinity chromatography studies to isolate purified lysozymes, 50 ml from each gland per sitting was collected in screw-cap plastic centrifuge tubes that were chilled on ice. The salivary samples were then acidified to pH 4.5, centrifuged to remove precipitated material, and stored frozen at −20°C until sufficient volumes were collected. Before fractionation by immunoadsorption affinity chromatography, a second centrifugation was carried out to remove additional precipitated material that had formed during storage. Salivary samples (ca. 5 ml) from caries-susceptible and caries-resistant adult subjects were generously provided by Irwin Mandel, Columbia University Dental School, New York. Freshly collected salivas were similarly acidified and stored frozen until assayed for lysozyme by either the ELISA or spectrophotometric techniques.

Lysozyme purification. Human parotid lysozyme was purified from parotid saliva by immunoadsorption affinity chromatography as recently reported (14). The enzyme was similarly isolated from submandibular-sublingual saliva. Immunoadsorbents were prepared by coupling an IgG fraction of goat anti-human leukemic lysozyme to epoxy-activiated Sepharose 6B (14). Briefly, affinity columns were successively eluted with 0.05 M phosphate buffer (pH 6.7) containing 0.15 M NaCl, with 0.05 M phosphate buffer (pH 6.7) containing 1 M NaCl and distilled water, and finally with 0.2 M acetate hydrochloride buffer (pH 1.8). Lysozyme was desorbed from the columns by acid treatment. Both the isolated parotid (14) and submandibular (unpublished data) lysozymes have been determined to be homogeneous.

Immunoassay. A four-layer sandwich technique (4) was used to quantitate the human leukemic lysozyme standard and the lysozyme in submandibular-sublingual and parotid salivas. The assay was performed by using a Gilford PR 50 EIA automated analyzer from Gilford Instruments, Oberlin, Ohio. Cuvettes (EIA Cuvette Paks, Gilford Instruments) were coated with 400 μl of a 1:100 dilution of 320-μg/ml sheep anti-human leukemic lysozyme-specific IgG for 2 h at 37°C. Dilution of the IgG was done in 0.1 M sodium carbonate, pH 9.6, containing 0.02% sodium azide. Lysozyme was then added for 1.5 h at 37°C either as a purified human leukemic enzyme standard or as a naturally occurring component of parotid or submandibular-sublingual saliva. Salivas and standards were initially assayed by the turbidimetric assay (2) to determine enzyme concentrations. Dilutions were then made for the ELISA in 0.02 M phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20 and 0.02% sodium azide such that enzyme concentration values of unknown samples fell with a standard curve ranging from 2.5 to 15 ng per ml. Subsequent to the addition of lysozyme, 400 μl of a 1:200 dilution in phosphate-Tween-azide buffer of 5-mg/ml rabbit anti-human lysozyme IgG was added to each cuvette for a further 1.5 h. Between each addition, cuvettes were washed three times with 800 μl of 0.9% sodium chloride containing 0.05% Tween 20. After the 1.5-h period with rabbit antibody, 400 μl of phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20 and 0.02% sodium azide was added to each cuvette. Cuvettes were sealed with waterproof tape to prevent evaporation and were stored overnight at 4°C. On the following day, cuvettes were washed and incubated with 400 μl of a 1:800 dilution of goat anti-rabbit IgG (Miles Laboratories) coupled to alkaline phosphatase (type VII; Sigma Chemical Co.) as described by Engvall and Perlmann (5). The disodium salt of *p*-nitrophenyl phosphate (Sigma), dissolved in 0.05 M sodium carbonate (pH 9.8) containing 1 mM magnesium chloride, was then added as a substrate. Generation of *p*-nitrophenol was read at 405 nm in the Gilford PR 50 EIA after a suitable time, usually 2 h. Appropriate controls were run without lysozyme by using one or more of the sheep, rabbit, and goat anti-rabbit antibody preparations or with lysozyme in the absence of either the rabbit or goat anti-rabbit antibodies. All controls proved negative in color development.

Turbidimetric assay. Lysozyme activity of salivary samples was assayed turbidimetrically by measuring the decrease in absorbance at 700 nm of a suspension of *M. luteus*

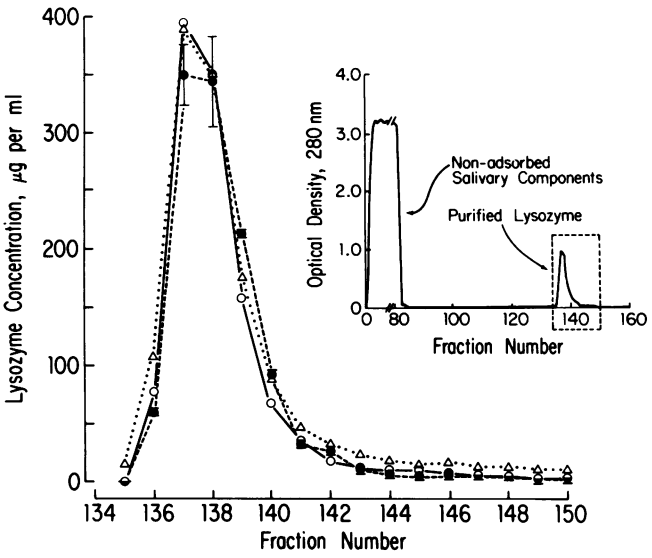

FIG. 1. Comparison of ELISA and turbidimetric assays with extinction coefficient determination of affinity-purified human parotid lysozyme. Human parotid saliva (795 ml) of subject A was applied to an immunoadsorbent column (1.6 by 4 cm) specific for human lysozyme (see text for details). The inset shows the optical density profile for the fractionation of human parotid saliva. The boxed area of the inset is enlarged in the graph and represents purified lysozyme eluted by low pH treatment. Fractions of 10 and 1 ml were collected for the nonadsorbed and purified lysozyme peaks, respectively. The extinction coefficient of human leukemic lysozyme was used to calculate the concentration of parotid lysozyme in micrograms per milliliter for each optical density value (△); the concentration of lysozyme was determined from turbidimetric assays (○). Values represent the average of duplicate determinations and those determined from ELISA (●). Values represent the mean of four determinations ± the standard error.

with purified human leukemic lysozyme as a standard (2, 14). The standard curve ranged from 1 to 15 μg of lysozyme per ml.

RESULTS

Affinity-purified lysozymes. An examination of the low pH eluate regions of both acidified parotid (subject A, Fig. 1) and submandibular-sublingual (subject B, Fig. 2) salivas demonstrates that the lysozyme concentration in each fraction can be determined from either extinction coefficient calculations, turbidimetric assays, or the ELISA technique. Although there were concentration variations for individual fractions, particularly for purified submandibular lysozyme, the curves for each assay approximated each other. The turbidimetric assay was two to three orders of magnitude less sensitive than the ELISA, and replicates were within 10% of each other. Standard errors for the ELISA, however, were observed to show greater variation since means were determined by making different dilutions of individual fractions to yield a series of values at different points on the standard curve. Large dilutions were required to bring the unknown lysozyme concentrations of the saliva fractions within the nanogram range of the ELISA standard curve.

Recovery of purified lysozymes. For human parotid lysozyme of subject A (fractions 135 to 150, Table 1), recoveries were 75, 76, and 86% for spectrophotometric, ELISA, and extinction coefficient determinations, respectively. Based on the spectrophotometric and ELISA techniques (data not shown), similar recoveries were noted for the parotid lysozyme of subject B. Therefore, almost all of the recoverable enzyme, as determined from the optical density-extinction coefficient calculation, could be accounted for by either the turbidimetric or ELISA assays. Moreover, the concentration of lysozyme in the parotid saliva was observed to be the same, irrespective of which assay was used for the determination (Table 1).

For human submandibular-sublingual lysozyme of subject B, recoveries based on the ELISA column input ranged from 84 to 91% (fractions 65 to 100, Table 2). All of the enzyme essentially could be accounted for by either the turbidimetric or ELISA method. However, although the turbidimetric and ELISA values for lysozyme were almost identical in the purified enzyme region of the column, the values for lysozyme in the column input were surprisingly different. This

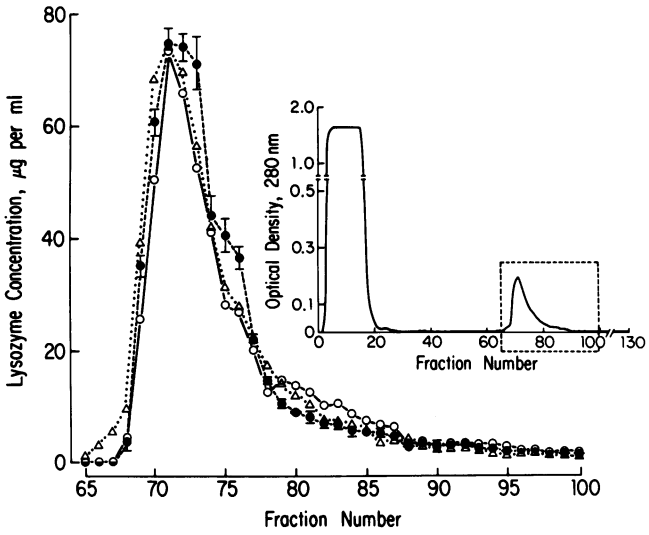

FIG. 2. Comparison of ELISA and turbidimetric assays with extinction coefficient determination of affinity-purified human submandibular-sublingual lysozyme. Human submandibular-sublingual saliva of subject B (131 ml) was applied to an immunoadsorbent column (1 by 23 cm) specific for human lysozyme (see text for details). The inset shows the optical density profile for the fractionation of human submandibular-sublingual lysozyme. The boxed area of the inset is enlarged in the graph and represents purified lysozyme eluted by low pH treatment. Fractions of 10 and 1 ml were collected for the nonadsorbed and purified lysozyme peaks, respectively. The extinction coefficient of human leukemic lysozyme was used to calculate the concentration of submandibular-sublingual lysozyme in micrograms per milliliter for each optical density value (△); the concentration of lysozyme was determined from turbidimetric assays (○). Values represent the average of duplicate determinations and those determined from ELISA (●). Values represent the mean of four determinations ± the standard error.

TABLE 1. Recovery of purified lysozyme from human parotid saliva

Sample	Total lysozyme (μg)	% Recovery
ELISA column input	1,542[a]	100[a]
Turbidimetric assay column input	1,582[b]	103
Fractions 135 to 150		
Optical density at 280 nm	1,330[c]	86
ELISA	1,165	76
Turbidimetric assay	1,156	75

[a] A total volume of 795 ml of acidified human parotid saliva from a single donor was applied to the immunoadsorbent column (Fig. 1). By the ELISA technique, the lysozyme concentration in the parotid saliva was determined to be 1.94 μg/ml, yielding the total shown. Recoveries of purified lysozyme are based on the ELISA.

[b] By the *M. luteus* turbidimetric assay, the lysozyme concentration in the parotid saliva was determined to be 1.99 μg/ml, yielding the total shown.

[c] Calculated by using the extinction coefficient of purified human leukemic lysozyme. See the text.

TABLE 2. Recovery of purified lysozyme from human submandibular-sublingual

Sample	Total lysozyme (μg)	% Recovery
ELISA column input	641[a]	100[a]
Turbidimetric assay column input	1,028[b]	160
Fractions 65 to 100		
Optical density at 280 nm	554[c]	86
ELISA	587	91
Turbidimetric assay	541	84

[a] A total volume of 131 ml of acidified human submandibular-sublingual saliva from a single donor was applied to the immunoadsorbent column (Fig. 2). By the ELISA technique, the lysozyme concentration in the submandibular saliva was determined to be 4.90 μg/ml, yielding the total shown. Recoveries of purified lysozyme are based on the ELISA.

[b] By the *M. luteus* turbidimetric assay, the lysozyme concentration in the submandibular-sublingual saliva was 7.85 μg/ml, yielding the total shown.

[c] Calculated by using the extinction coefficient of purified human leukemic lysozyme. See the text.

was true also for the submandibular saliva of subject A (data not shown). Turbidimetric assays yielded an average concentration of lysozyme of 7.85 μg/ml, whereas the mean of the ELISA values was 4.90 μg/ml (Table 2). When purified submandibular lysozyme from subject B, isolated by the immunoadsorbent technique (Fig. 2), was mixed with the nonadsorbed fraction (Fig. 2), which exhibited no enzyme activity by itself, a similar enhancement in the *M. luteus* assay was noted, although no change in the ELISA assay was observed (data not shown). The same was true for subject A. Moreover, when purified enzyme and nonadsorbed fractions of both subjects were crossed, similar enhancement values were obtained (data not shown). No differences in either assay were seen when phosphate-buffered saline replaced the nonadsorbed fraction in the combination with purified lysozyme.

Caries-susceptible and caries-resistant salivas. After the dilutions required for each saliva in preliminary ELISA runs were ascertained, one dilution was selected for each acidified saliva to minimize the errors due to averaging values from the standard curve using two or more dilutions. Calculated standard errors fell within ±10% and were usually less than ±5% (Table 3). There was considerable variation in the salivary lysozyme levels, and no significant differences in enzyme concentration were noted between the caries-resistant and caries-susceptible adults. Although acidified salivas were used throughout this study, no differences in ELISA-determined lysozyme values were noted between native salivas and those which were acidified before measurement (data not shown). Turbidimetric determinations for lysozyme in these salivas generally correlated with the values obtained by the ELISA method. However, in some cases, turbidimetric values were lower, whereas in others, enzyme concentrations were determined to be higher than values obtained by the ELISA. No attempts were made to ascertain whether catalytic enhancement factors were present in these salivas.

DISCUSSION

By employing immunoadsorption affinity chromatography to selectively purify parotid and submandibular-sublingual lysozymes (Fig. 1 and 2), we have demonstrated that the ELISA and turbidimetric assays measure only lysozyme in these acidified salivas. Although other assays for lysozyme, including the turbidimetric assay reported here, would in all probablity also be specific for lysozyme determinations in these oral fluids (7–11, 15, 16, 18, 19, 21, 24, 27), the ELISA does offer several advantages. Compared to currently used immunochemical and catalytic assays, the ELISA is orders of magnitude more sensitive. In the case of the ELISA technique, identical lysozyme values were obtained irrespective of whether the salivas were collected and used immediately or were acidified before assay. This is not true for turbidimetric measurements of parotid lysozyme which give lower values for freshly collected salivas (data not shown) in agreement with previous investigators (27) but surprisingly lower enzyme values for acidified submandibular-sublingual salivas (data not shown). Therefore, catalytic assays performed on biological fluids may yield erroneous data because of lysozyme binding to other fluid components.

ELISA determinations of the salivary concentrations of purified enzymes in the low pH eluates of the immunoadsorbent column demonstrated that the total amounts of the enzymes in the salivas were being measured accurately (Tables 1 and 2). This was true also in the case of the turbidimetric assay for measurement of parotid salivas, since turbidimetric values were virtually identical to ELISA determinations (Fig. 1). However, for the fractionated submandibular-sublingual saliva of two subjects, recoveries of lysozyme by this technique were considerably lower and did not appear to be accurate because of an unidentified catalytic enhancement factor present in the lysozyme-depleted fraction and therefore present in the intact acidified submandibular salivas. Determinations of purified lysozyme in fractionated submandibular-sublingual saliva, in which the enhancement factor was not adsorbed to the immunoadsorbent column, clearly illustrated that both assays yielded identical values (Fig. 2). Because lysozyme assays like the lysoplate assay or rocket immunoelectrophoresis were not performed in this study, it was not possible to assess their accuracy relative to the ELISA.

In a recent report, Morsky and Aine (16) found similar

TABLE 3. Determination of lysozyme concentration in human parotid and submandibular-sublingual salivas by the ELISA technique and the turbidimetric assay

Subject	Lysozyme concn (μg/ml) in:			
	Parotid saliva by:		Submandibular-sublingual saliva by:	
	ELISA	Turbidimetric assay	ELISA	Turbidimetric assay
Caries resistant				
C	0.54 ± 0.01[a]	ND[b]	2.84 ± 0.19	3.28 ± 0.17
D	0.93 ± 0.05	1.39 ± 0.01	17.82 ± 0.97	27.21 ± 1.94
E	2.06 ± 0.09	2.51 ± 0.28	14.15 ± 0.42	11.58 ± 1.05
F	3.08 ± 0.23	2.94 ± 0.02	10.03 ± 0.47	10.20 ± 0.95
G	3.03 ± 0.14	3.24 ± 0.08	9.00 ± 0.39	10.69 ± 0.45
Caries susceptible				
H	1.67 ± 0.09	ND	3.34 ± 0.12	3.95 ± 0.08
I	2.55 ± 0.15	ND	25.14 ± 1.08	21.82 ± 1.87
J	3.10 ± 0.12	3.54 ± 0.03	4.29 ± 0.21	5.52 ± 0.40
K	5.08 ± 0.24	6.03 ± 0.29	4.58 ± 0.18	4.83 ± 0.09
L	10.89 ± 0.32	13.08 ± 0.45	14.88 ± 0.28	7.90 ± 0.17

[a] Values represent the mean of four replicates ± the standard error. Salivas were diluted appropriately to fall within the standard curve of human leukemic lysozyme in the ELISA (see the text).

[b] ND, Not determinable because of salivary agglutination of the *M. luteus* cells.

lysozyme levels in tears by using both an immunoturbidimetric and an optimized *M. luteus* hydrolytic assay. Their results are thus similar to our findings with parotid saliva, and these authors have suggested that such results imply the retention of steric, enzymatic, and immunological activities (16). Although their studies (16) and ours utilized an automated multiple sampling assay, an advantage of our system, as pointed out above, is that the ELISA technique is highly sensitive and can be applied to biological fluids such as gingival crevicular fluid or plaque fluid, in which lysozyme levels would not approach that found in tears.

Analyses of salivas of caries-resistant and caries-susceptible adult patients can easily be achieved by the ELISA method (Table 3) or by other means (22). Excellent replicates were obtained when one particular dilution on the standard curve was employed (Table 3). Noteworthy, with the turbidimetric assay, is that it was not possible to quantitate lysozyme concentrations in all of the parotid salivas because of aggregation of the *M. luteus* cells (Table 3).

The results of this study would suggest that ELISA determinations provide accurate concentrations of lysozyme in human salivas. Further studies are in progress to examine lysozyme levels in the salivas of children and in other body fluids.

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