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TECHNICAL NOTE

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An Elisa Method for the Identification of Salivary Amylase*

ABSTRACT: An ELISA method for the detection of salivary amylase in dried stains using a monoclonal anti-human salivary amylase antibody was developed. Studies demonstrated the assay to be sensitive down to 0.0002 Sigma units and showed a linear response between absorbance and salivary amylase activity between 0.002 and 0.2 units. The assay showed no cross reactivity with either commercially purchased human pancreatic or bacterial amylase. Sample studies utilizing swabs from several human body fluids showed that 100% of all saliva containing swabs (sixteen of sixteen) and 13% of non-saliva human body fluid swabs (eight of sixty-three) showed a net absorbance with the method. Of these eight non-saliva swabs yielding a net absorbance, none exceeded a salivary amylase activity of 0.003 units. In contrast, only three of the sixteen saliva-containing swabs (swabs produced from saliva diluted 1:5, 1:6, and 1:10, respectively) showed an activity below 0.2 units. Of these swabs, the 1:100 dilution showed the lowest activity (0.048). This value is still more than ten times that of the non-saliva containing swab with the highest activity.

KEYWORDS: forensic science, saliva, salivary amylase, immunosorbent assay

There are two forms of amylase found in human body fluids: pancreatic and salivary. Both are structurally different amylases that are encoded by separated loci on chromosome 1 and are designated AMY_1 or salivary locus and AMY_2 or pancreatic locus. Although the distribution of AMY_1 and AMY_2 can vary in body fluids, it has been reported that salivary amylase is found in saliva, perspiration, and breast milk and pancreatic amylase is found in semen, feces, and vaginal secretion (1–3). Both gene products have also been reported in serum, urine, and in male and female reproductive tissue (3–5).

The presumptive identification of saliva has traditionally been performed by the identification of amylase using methods such as the Phadebas® assay (6) or radial diffusion in a starch/agarose gel (7). Amylases are commonly found in microorganisms, plants, and many animals. These traditional methods for amylase testing cannot differentiate between amylases, so the sources of amylase activity on items of physical evidence cannot be determined with these tests. In addition, the presence of bacterial amylase in and on the human body, for example in the vaginal cavity (as well as amylase from vaginal secretion that is pancreatic in origin) can lead to measurable amylase activity on swabs taken from sexual assault victims. There have been a number of attempts to develop more specific methods such as using selective anti-sera (8), chemical inhibitors (9), and inhibiting monoclonal antibodies (10).

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An indirect ELISA technique (11) employing a monoclonal salivary amylase antiserum has been developed that can differentiate human salivary amylase from other types of amylases. In addition to offering greater specificity for the identification of saliva, the ELISA method is capable of analyzing several samples simultaneously offering the advantage of increased throughput.

Methods and Materials

Collection and Preparation of Samples

Included in the study were eight saliva swabs, eight semen swabs, fifteen urine swabs (eight from females and seven from males), nine vaginal secretion swabs, five blood swabs, seven fecal swabs, fourteen perspiration swabs, and five swabs containing post-mortem stomach contents. In addition, individual swabs were made from whole saliva diluted 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:10, and 1:100 with phosphate buffered saline (PBS). Samples were made on individually wrapped cotton sterile swabs that were soaked or rolled in the particular body fluid. After preparation, the swabs were air dried for one hour before storage at 2–5°C in manila coin envelopes until use. The samples were stored under non-desiccated conditions. All sample swabs were free of other foreign body fluids. Four swabs were prepared as above containing approximately 260 Sigma units of type XII-A bacterial amylase from *Bacillus licheniformis* (Sigma, St. Louis, A3403).

One-half of each swab was cut and soaked in 150 μL of PBS for 30 min prior to centrifugation at 14,000 × g for 5 min. One hundred μL of each extract was used for assay. Lyophilized samples of human pancreatic amylase (Sigma, A9972) and human salivary amylase (Sigma, A1031) were reconstituted with sterile water. Dilutions used to make standard curves were made with PBS. None of the four stains containing bacterial amylase produced a positive net absorbance.

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ELISA Procedure

Assays were performed in Dynatech Immulon 2 microELISA plates (ISC Bio Express, Kaysville, UT, D-1750-3) and is a modification of an existing P30 ELISA method (12). Sample wells were first coated with 100 μL of one of three solutions. The first two solutions, PBS and mouse IgG1 kappa chain antibody (MOPC 21 Sigma M7894; 6.6 mg reconstituted in 1 mL sterile water), were used to detect and compensate for non-specific binding. The third coating solution was mouse monoclonal anti-human salivary amylase antibody (Roche, Indianapolis, 1543 598), prepared by first dissolving 2 mg of the monoclonal antibody in 2 ml of normal saline and then diluting this stock solution 1:200 in PBS. The coated plates were then incubated overnight at 4°C.

The next day sample wells were aspirated (using the Tecan Minilyser with a Columbus washer, San Jose, CA) and washed twice with 100 μL Tween-20 (0.2% in deionized water) (Sigma, P9416). Each well was then blocked with 100 μL Blocker™ Casein (Pierce, Rockford, IL, 37528) and incubated at room temperature for 1 h. After incubation, the casein was aspirated and sample wells were washed twice with 100 μL Tween-20.

In succession, 100 μL aliquots of each sample and standard to be assayed, 100 μL of sheep anti-human alpha-amylase polyclonal antibody (26.6 mg/mL) (Biodesign, Saco ME, K90041C) diluted 1:1000 in phosphate buffered saline, and 100 μL of donkey anti-sheep IgG antibody conjugated with alkaline phosphatase (4.7 mg/mL) (Sigma, A5187) diluted 1:30,000 in PBS were added to each well. After each addition, the plate was incubated at room temperature for 1 h. The contents from each well were then aspirated and washed three times with 100 μL of Tween-20.

One hundred (100) μL of p-nitrophenyl phosphate (p-NPP) substrate (Sigma, N2765) prepared by dissolving 20 mg of p-NPP in 20 mL of alkaline substrate buffer (12) (1.0M diethanolamine, 0.02% sodium azide, and 0.5 mM 6-hydrate magnesium chloride, pH 9.8) (Sigma, D8885, S2002, M0250 respectively) were added to each well and incubated at 37°C for 45 min. The absorbance for each well was read at 405 nm with a reference wavelength at 655 nm using a microtiter plate reader (Tecan Minilyser with a Spectra reader). Absorbance values for salivary amylase standards were used to create standard curves (absorbance v. log units of amylase) to determine units of salivary activity in body fluid swabs.

Background Absorbance and Plate Threshold Determination

For each plate, background absorbance was determined by taking the mean value of uncoated blank wells substituting PBS for the monoclonal antibody, sample, and polyclonal antibodies. Blank wells were washed with Tween-20 and treated with p-NPP substrate. Non-specific binding of the added sheep and donkey antibodies was determined by measuring the absorbances of the MOPC 21 and monoclonal antibody coated wells in the absence of amylase activity.

The plate threshold was obtained by adding three times the standard deviation of the monoclonal-coated blank wells to their mean value. Net absorbance values for each sample and standard were determined after the plate threshold was subtracted from the raw absorbance. Sources of interference specific to individual samples and standards containing amylase can be controlled for non-specific binding, endogenous alkaline phosphatase activity, and so forth with sample wells coated with MOPC 21, rather than the monoclonal antibody, and subtracting net absorbance from these wells from the above net absorbance figures.

Results

Linearity and Specificity of Method

Using the commercially obtained human salivary amylase standard, the assay was tested to determine the range of enzyme activity showing a linear relationship with absorbance. Net absorbance values were determined for salivary amylase activity ranging from 0.0002 to 11 units. As shown in Table 1 and Fig. 1, the assay demonstrates the greatest degree of linearity between 0.002 and 0.2 units. For two of the three example assays, the correlation coefficient (R^2, Excel) exceeds 0.99 while the third yielded a value of 0.91. Absorbance values do increase at 2, 5, and 11 units but at a much lower rate. These results are consistent with a saturation of monoclonal antibody binding sites at higher enzyme activities. The calibration curve for the more dilute salivary amylase standards below 0.002 units also did not continue to be linear, although net absorbance at 0.0002 units shows that the sensitivity of the assay extends down to this level.

An operational range of 0.002 to 0.2 units of salivary amylase standards was therefore utilized for construction of the calibration curves used to determine enzyme activity levels in samples tested in the study. During validation experiments, a monoclonal antibody dilution of 1:200 was found to produce the most linear response in this range of enzyme activities.

Specificity Studies

Similar dilutions using the commercially prepared human pancreatic amylase were prepared and tested for cross reactivity with the assay. At no activity up to 10 units (7 swabs tested) did the pancreatic amylase yield a net absorbance.

Results of the assay on various types of body fluid swabs (including swabs containing human pancreatic and bacterial amylase) are given in Table 2. All eight neat saliva-containing swabs gave a

TABLE 1—*Data for each of 3 runs used to determine curves in Fig. 1.*

Units Salivary Amylase/100 μL	Net Absorbance Assay 1	Net Absorbance Assay 2	Net Absorbance Assay 3
11	1.410	2.118	2.203
5.5	1.530	2.177	1.813
2	1.362	2.087	1.939
0.2	1.279	1.997	1.654
0.02	0.755	0.598	0.980
0.002	0.153	0.181	0.235
0.0002	0.089	0.154	0.044
R^2	0.998	0.911	0.999

R^2 values for each run are calculated from 0.2–0.002 units.

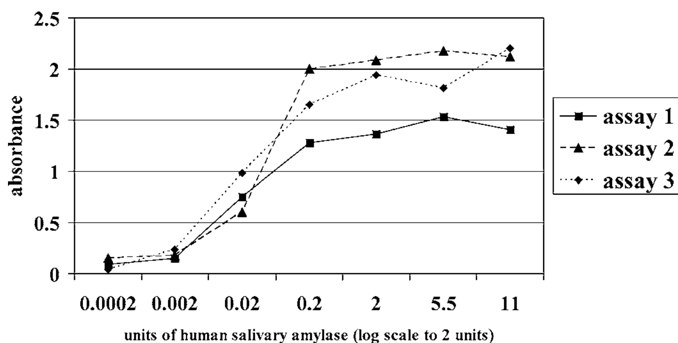

FIG. 1—*Dilution of human salivary amylase standard (3 assays).*

TABLE 2—*ELISA testing of various body fluid swabs (including swabs containing human pancreatic and bacterial amylase) for salivary amylase.*

Sample Type	N	Number of Samples Showing Net Absorbance	Units of Salivary Amylase*
Saliva	8	8	All > 0.2
Semen	8	1	0.003
Urine (male)	7	2	0.003; <0.002
Urine (female)	8	1	<0.002
Vaginal Secretion	9	0	NA
Blood	5	0	NA
Feces	7	2	<0.002; <0.002
Perspiration	14	2	<0.002; <0.002
Stomach Contents	5	0	NA
Human Pancreatic Amylase	7	0	NA
Bacterial Amylase†	4	0	NA

* Results are from multiple assays and include only samples giving a net absorbance. Units of activity for swabs showing a net absorbance above or below the linear range of the standard curve are reported as >0.2 and <0.002, respectively.
†Amylase type XII-A from *Bacillus licheniformis* (Sigma, A3403).
NA = no activity.

TABLE 3—*ELISA assay of dried swabs containing various dilutions of saliva**.

Dilution	Net Absorbance	Units Salivary Amylase
1:1	2.034	>0.2
1:2	1.717	>0.2
1:3	1.774	>0.2
1:4	1.796	>0.2
1:5	1.598	0.18
1:6	1.461	0.12
1:10	1.648	>0.2
1:100	1.191	0.048

*Units of salivary amylase were determined from a standard curve with a R^2 value of 0.999.

net absorbance above the linear range of the standard curve. Most non-saliva containing samples showed no net absorbance. None of the nine vaginal secretion stains, five bloodstains, and five stomach content stains, produced a net absorbance. Only one of eight semen stains, two of seven male urine stains, one of eight female urine stains, two of seven feces swabs, and two of fourteen perspiration swabs produced a net absorbance. Of these eight non-salivary swabs giving a net absorbance, no sample showed units of activity above 0.003 (observed in one semen and one male urine stain).

Swabs containing dilutions of saliva gave positive net absorbances for all dilutions tested with little difference observed from 1:2 to 1:10 (Table 3). Amylase activities were either above or close to 0.2 units. The 1:100 dilution showed lower amylase activity (0.048) but still fell within the upper portion of the linear range of the standard curve.

Discussion and Conclusions

Results of the study show that the assay is highly specific for human salivary amylase and can be used as a confirmatory test for human saliva. Of the eight non-saliva samples giving a measurable net absorbance, five are from body fluids that are reported to contain salivary amylase (two from perspiration and three from urine). The other three swabs are from body fluids reported to contain pancreatic amylase (two from feces and one from semen), although the presence of salivary amylase cannot be discounted since mixtures of amylases are known to exist in body fluids and tissue (3,4). Since salivary amylase has been found in reproductive tissue (4), it seems reasonable to hypothesize that salivary amylase could be found in semen although semen has been reported to contain only pancreatic amylase (13). Furthermore, two of the feces swabs, which were easily distinguished from salivary samples with the ELISA method, showed high levels of amylase activity indistinguishable from salivary samples using the starch/agarose radial diffusion method. In contrast, of all sixteen swabs containing saliva, only one swab yielded an activity less than 0.1 (1:100 diluted saliva swab). This 1:100 diluted sample still produced a salivary amylase activity ten times the highest value obtained for a non-saliva containing swab. This suggests that a confirmatory threshold value for salivary amylase activity can be determined in validation studies. In this study, for example, a threshold of 0.02 (middle point of linear range of standard curve) units would result in positive results for all sixteen saliva-containing swabs and no false positives for any of the non-saliva samples. Proper utilization of the method requires validation to determine a confirmatory threshold value that may not be the same in all cases.

The negative results from the vaginal secretion swabs are illustrative of the potential application of the assay. In many laboratories, amylase testing is conducted on vaginal swabs from sexual assault victims to determine if oral copulation may have occurred. With radial diffusion in a starch/agarose gel or Phadebas® assay, the detection of low levels of amylase on a vaginal swab is not determinative as to possible body fluid origin. At least one study showed that vaginal secretion swabs could have amylase levels that fall within the range of activities (0.013 to 0.183 I.U. for 3 mm^2 stains) shown by saliva stains, albeit toward the lower portion of range (6). The reporting of the presence of amylase on vaginal swabs based on these methods could lead to a false inference that saliva was present. The ELISA method reported here should remove the interpretative ambiguity associated with the more traditional methods in these types of cases.

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