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A Sensitive and Simple Assay of Saliva on Stamps

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Summary. Identification of saliva on stamps or envelope flaps remains yet a not widely studied problem. In most forensic laboratories it is seldom carried out, but this fact does not reduce the importance of the assay.

Most authors consider amylase a sufficiently specific marker of the presence of saliva; really, the only other human body fluid that contains high amounts of this enzyme is the pancreatic juice (and therefore feces).

Here we present a simple and sensitive assay for the determination of α-amylase that uses a commercially available and well-known substrate. It is hydrolyzed by amylase with the production of soluble blue fragments, that can be measured by photometry, obtaining objective results.

The presented assay identifies 1×10^{-6} diluted saliva or that present on 0.5 mg of a stamp; 16-year-old samples can also be identified.

Intra-assay and day-to-day CV resulted in 10.8% and 13.7%, respectively. Owing to the high sensitivity of the test, handling samples or reagents can introduce contamination with saliva traces, giving false-positive results. Addition of EDTA 0.1 mol/l to the incubation mixture, lowering the sensitivity to 1×10^{-3} diluted saliva, overcomes this problem.

Key words: Saliva, identification – Stamps, saliva identification

Zusammenfassung. Die Identifizierung von Speichel auf Briefmarken oder Briefumschlägen ist ein nicht extensiv untersuchtes Problem. In der Praxis wird sie nämlich in den meisten Laboratorien selten durchgeführt. Doch diese Tatsache schmälert nicht die Wichtigkeit dieser Untersuchung.

Die meisten Autoren betrachten die Amylase als ein ausreichend spezifisches Merkmal für das Vorkommen von Speichel; die einzige andere menschliche Körperflüssigkeit, die diese Menge von Enzym in hoher Konzentration enthält, ist der Bauchspeicheldrüsensaft (Pankreassaft) – (und deshalb auch Kot).

Hier präsentieren wir eine einfache und empfindliche Untersuchung zur Bestimmung der α-Amylase, zu der man ein im Handel erhältliches Substrat benutzt. Dieses wird durch Amylase hydrolisiert, so daß blaue lösliche Fragmente entstehen, die man photometrisch messen kann. Hierdurch lassen sich objektive Ergebnisse erzielen.

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Die vorgestellte Untersuchung erlaubt es 1×10^{-6} verdünnte Speichelproben oder die auf 0,5 mg einer Briefmarke vorhandene Menge zu identifizieren; 16 Jahre alte Proben könnten auch identifiziert werden.

Untersuchungen zur Variation innerhalb des gleichen Ansatzes sowie zur Variation von einem Ansatz zum anderen (Tag zu Tag) ergaben Variations-Coeffizienten von 10,8% bzw. 13,7%. Dank der hohen Empfindlichkeit des Tests können manuell berührte Proben oder Reagenzien eine Kontamination durch Speichelspuren aufweisen, wodurch falsch-positive Resultate entstehen.

Der Zusatz von EDTA 0,1 mol/l zur Inkubationsmischung beseitigt dieses Problem, indem hierdurch die Empfindlichkeit zu einer Speichelverdünnung von 1×10^{-3} verringert wird.

Schlüsselwörter: Speichel, Identifizierung – Briefmarken, Speichelnachweis

Detection of saliva traces and group-specific antigens on stamps or envelope flaps have considerable importance in medicolegal investigations, but till now it remains a not widely investigated problem. It is well-known that very sensitive immunologic methods [1] are available for the identification of specific group antigens in stains. Contrarywise, human saliva or its specific components cannot be detected by immunologic techniques so that its identification is still a problem, mainly in old samples or in exhibits that must be almost entirely preserved.

Lacking specific antisera, some authors proposed tests for detecting saliva components, such as nitrite, thiocyanate, and alkaline phosphatase, fully described by Nelson and Kirk [2] and α-amylase; really, it is present in considerable amounts almost only in saliva and in pancreatic juice [3].

Alpha-amylase activity can be revealed by the classical technique of Nickolls [4]; nevertheless, it is extremely tedious, time-consuming, and not sufficiently sensitive.

More recently, Willot [5] proposed a method based on the hydrolysis of insoluble starch-dye complexes with the production of water-soluble blue fragments.

Other authors [6] described a different method for the detection of saliva stains based on the hydrolysis of soluble amylopectin-procion red complexes pre-precipitated onto filter paper. It detects amylase without destruction of the exhibit, but seems to be little sensitive (1/10 diluted saliva) and reliable, given the difficulty of reproducible preparation of activated paper sheets and of obtaining an homogeneous contact between sample and activated paper. Moreover, the subjective evaluation of results based on the visual identification of decoloration areas hampers the objectivity of this test.

Here we present a re-evaluation of Willot's method to identify saliva on stamps, specifically modified to improve sensitivity and reduce inteferences.

Materials and Methods

The used substrate was a water-insoluble, cross-linked starch polymer carrying a blue dye, commercially available as „Phadebas amylase test with BSA", furnished by Pharmacia

Diagnostics AB (Uppsala, Sweden). Other used chemicals were provided by Carlo Erba (Milan, Italy).

The analytic procedure is described here synthetically:

- 40 mg substrate was suspended in 200 µl 0.9% saline solution or in the same medium containing 0.1 mol/l EDTA;
- a few milligrams (down to 0.5 mg) exhibit (stamp or envelope flap) was incubated with the substrate for 9–20 h in a water bath at 37°C;
- 1 ml 0.1 mol/l NaOH was then added and the suspension contrifuged at 3,000 rpm for 5 min to produce a clear supernatant;
- the OD of supernatants was measured at 620 nm wavelength;
- the OD of assayed samples was compared with that of negative controls introduced in the same run.

If possible, five to ten fragments from different areas of the same exhibit, five reagent blanks, and ten negative controls were assayed simultaneously.

We considered that exhibit positive the mean OD of the fragments of which was significantly higher than the negative control mean OD. Statistical evaluation was carried out using Student's *t*-test.

All the tests for validating this method were carried out on stamps wetted with saliva and dried at room temperature for one week, save the assay of old samples that were obtained from latters home-stored in uncontrolled conditions.

Results

Kinetics or Starch Hydrolysis

Samples weighing 6 mg, cut from the same stamp, were incubated with substrate for increasing times. The tests were carried out in saline and in the presence of 0.1 mol/l EDTA. The OD of supernatants was plotted vs. the relative incubation times. The results are shown in Fig. 1.

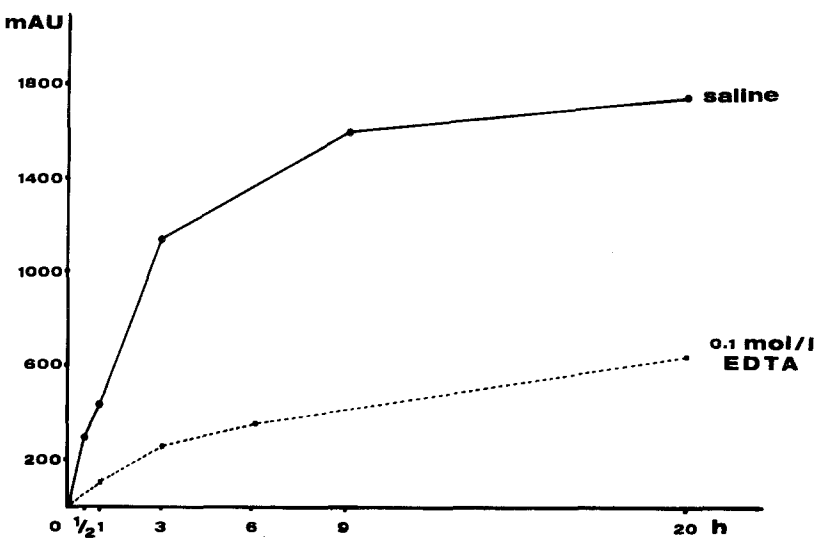

Fig. 1. Kinetics of starch hydrolysis by saliva present on 6 mg of stamp. The reaction was carried out in saline and in 0.1 mol/l EDTA *(dotted line)*.

Table 1. Sensitivity test on diluted saliva

Dilution factor	Saline OD (mean ± SD) mAU	0.1 mol/l EDTA OD (mean ± SD) mAU	
1×10^{-1}	1,497.5 ± 211.4	1,189.7 ± 109.7	(n = 5)
1×10^{-2}	1,427.0 ± 158.3	1,056.0 ± 106.8	(n = 5)
1×10^{-3}	1,353.6 ± 111.7	838.3 ± 74.9	(n = 5)
1×10^{-4}	899.3 ± 28.8	56.6 ± 5.1	(n = 5)
1×10^{-5}	439.5 ± 71.4	19.1 ± 3.8	(n = 5)
1×10^{-6}	92.0 ± 12.1	14.2 ± 1.5	(n = 5)
neg. contr.	21.2 ± 5.0	16.5 ± 1.7	(n = 10)

Table 2. Assay of old samples

Age	Amount (mg)	OD (mAU; mean ± SD)	
		Saline	0.1 mol/l EDTA
1 day	4	1,200 ± 114.0	1,230 ± 135.3
1 yr	6	1,142 ± 116.4	1,230 ± 124.2
2 yr	7	1,098 ± 100.5	1,125 ± 130.7
4 yr	7	1,086 ± 121.3	1,148 ± 117.8
8 yr	6	1,015 ± 111.6	1,211 ± 135.7
9 yr	7	1,086 ± 100.9	508 ± 61.4
13 yr	8	1,022 ± 114.4	–
16 yr	6	444 ± 48.8	20 ± 3.0
Neg. contr.	6	23 ± 5.1	17 ± 2.1

Sensitivity

Human saliva was progressively diluted up to 1×10^{-6} with saline; 50 µl of every dilution was assayed according to the described procedure. The same test was also carried out in the presence of 0.1 mol/l EDTA. The results are shown in Table 1.

Fragments (n = 10) weighing about 0.5 mg, cut from a recently licked stamp, gave constantly positive results (596.5 + 68.7 mAU). The ability of identifying old samples was verified assaying 1- to 16-year-old letters. All the samples were correctly identified when the incubation medium was saline; in the presence of 0.1 mol/l EDTA, only the stamps aged up to 9 years resulted positive. The results are shown in Table 2.

Interferences

Up to 50 different Italian and foreign stamps and envelope flaps were assayed to evidence false-positive or false-negative results, without finding any signifi-

Table 3. Assay of amylase in solution and adsorbed on stamp

	OD (mAU)	
	In saline	On stamp
Level I	937	1,027
Level II	714	753
Level III	585	508

Table 4. Intra-assay precision

	4-mg samples (mAU/mg)	12-mg samples (mAU/mg)
	192	148
	271	152
	257	147
	274	123
	274	140
	276	130
	242	147
	260	150
	221	146
	241	135
Mean ± SD	251.2 ± 27.36	142.0 ± 9.44
CV %	10.8	6.6

cant interferences. Moreover, we assayed stamps previously handled with not recently washed hands; mean OD resulted significantly higher than in negative controls (651 ± 76.8 mAU vs. 22 ± 3.7 mAU; $n = 5$). The addition of 0.1 mol/l EDTA to incubation medium avoided these interferences: mean OD was 24 ± 3.2 mAU in handled samples and 19 ± 3.1 mAU in negative controls.

Accuracy

Three levels of saliva dilutions in saline were assayed simultaneously with equal amounts of saliva, previously adsorbed on stamp and dried under a stream of air; the results were comparable (Table 3).

Precision

Ten samples weighing 12 mg and 4 mg on average, cut from two different stamps, were assayed simultaneously; OD were measured and normalized for 1 mg of sample. Intra-assay variation coefficient was 6.6% for 12-mg samples and 10.8% for 4-mg ones. The results are showed in detail in Table 4.

Table 5. Inter-assay precision

	4-mg samples (mAU/mg)
	297
	218
	221
	278
	269
Mean ± SD	256.9 ± 35.26
CV %	13.7

Day-to-day CV, calculated by assaying on different days five samples weighing about 4 mg prepared as described above, was 13.7%. Table 5 shows analytically the resuls.

Discussion

The presented method, a modification of Willot's one, specifically studied to increase sensitivity, shows interesting features. First of all, the prolonged incubation and the higher concentration of substrate allow the detection of minimal traces of amylase, such as that present in 1×10^{-6} diluted saliva or in 0.5 mg of stamp sticked by licking onto an envelope.

A 9-h incubation time at +37°C of samples weighing 4–6 mg, which allows an almost complete hydrolysis of the substrate, seems to be the most suitable.

Amylase assayed, dissolved in saline, and adsorbed onto stamp develops a comparable activity in hydrolyzing starch-dye complexes. On the basis of these data, it can be hypothesized that saliva amylase keeps a complete activity also adsorbed onto paper, mixed with glue and dried. Intra- and inter-assay precision is slightly worse than in modern clinical chemistry methods, but it must be considered that the distribution of saliva on stamps is rather unhomogeneous, hampering a reproducible collection of samples.

Nevertheless, the sensitivity of this method allows multiple sampling of a single exhibit, permitting a statistical evaluation of results. Owing to the high sensitivity, amylase activity can be found also in up to 16-year-old samples, in spite of a high degree of enzyme inactivation.

Several Italian and foreign stamps, envelopes, and cards did not show any interferences on the results. Other possible interferents, such as serum, urine, feces, and semen have not been investigated because they are reasonably not used for sticking stamps or envelope flaps. Nevertheless, handling of samples or reagents can introduce contamination with saliva, that causes false-positive results, when the sensitivity of the method is stressed.

The addition of 0.1 mol/l EDTA to the incubation medium, lowering the enzyme activity, which depends on Ca^{++} concentration, reduces the sensitivity of the assay to $1 \cdot 10^{-3}$ diluted saliva and allows the elimination of these interferences, yet permitting to identify samples aged up to 9 years.

Moreover, photometric measurement of hydrolysis products allows to gain objectively evaluable results. This method required cheap, stable, standardized, and commercially available reagents allowing reliable assays without a great degree of operator expertise, that seems an important feature for an assay that is seldom carried out in most forensic laboratories.

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