A SENSITIVE METHOD FOR SALIVA DETECTION IN FORENSICS USING SALIVARY AMYLASE COUPLED WITH AMPLEX RED OXIDATION

SABINA BUNESCU[1], BOGDAN A. STOICA[1*], DRAGOS PEPTANARIU[2], LILIANA FOIA[3]

Received: 13th of March 2020 / Revised: 23rd of April 2020
Accepted: 27th of April 2020 / Published: 5th of January 2021

Keywords: Forensic Science, salivary amylase, Amplex Red, saliva detection, fluorimetry, body fluids detection, serology
Abstract: A new sensitive method for saliva detection was developed, based on salivary amylase detection but with a final fluorescent product, which increases its sensitivity. After the starch is degraded due to the presence of salivary amylase, glucose is oxidised and generates hydrogen peroxide which is able to transform Amplex Red in resorufin - a highly fluorescent product. The final product is visible both under normal and UV light. The method is fast, accurate, can detect trace amounts of saliva and shows little to no interference with other body fluids. A further increase in sensitivity could be obtained by using horseradish peroxidase in the final step, but this would also lead to an increased background signal and stronger interference with urine.

INTRODUCTION

Among the body fluids analysed in forensic science, saliva is one of the most frequently encountered, being a good source of DNA for subsequent typing (Kuwayama et al., 2016; Carboni et al., 2014; Aps and Martens, 2005). In various casework analysis, saliva traces can be found on cigarette butts, clothing, bite marks as well as different objects found at crime scenes. Saliva identification and subsequent genetic analysis remain crucial evidence in court (Groschl, 2017; Saxena and Kumar, 2017).

There are several choices for saliva detection, each of them using a specific marker. Among the various markers proposed in literature (Nakanishi et al., 2009; Virkler and Lednev, 2009), salivary amylase is the most frequently used. There are some advantages, such as: good sensitivity, relative specificity and cost effective detection. There are also a few drawbacks, including the possibility of obtaining false positive results and the inability to differentiate between species (Saxena and Kumar, 2017).

Although the amylase function was described as early as 1831 by E.F. Leuchs (Zakowski and Bruns, 1985) it wasn't used in casework until 1928 (Mueller, 1928). The enzyme is found in various body fluids (saliva, blood, urine, semen etc.), but the highest concentration is encountered in saliva (10). The biochemical function of this enzyme is to hydrolyse the α-(1,4)-glucoside bonds found in a variety of polysaccharides and this breakdown reaction could be the starting point in saliva identification.

Among the amylase based methods used for saliva identification, many rely on colour changes (the radial diffusion test (Quarino et al., 1993) , Phadebas® test (Wornes et al., 2018) and SALIGaE® test (Park et al., 2015)) which are based on substrate chemical changes after amylase action. The oldest variant uses starch/ iodine for detection of amylase (Myers and Adkins, 2008) and the most common tests today use a dextrin linked to a 4-nitro-phenol moiety (4-nitrophenyl-maltoheptaoside - which releases 4-nitro-phenol, with a yellow colour) (Soyama and Ono, 1983) or a substrate made of insoluble starch coloured blue with a dye marker (Ceska et al., 1969). Some other methods are based on antibody-antigen interactions and have the advantage of human specificity (RSID) (Old et al., 2009).

The present study aims to increase the sensitivity of the amylase based saliva identification method, using enzymatic reactions that are connected to a final highly fluorescent product (Figure 1). The salivary amylase is able to hydrolyse a starch solution with subsequent release of glucose and dextrin formation. The amount of glucose could be increased by adding α-glucosidase. In the next step, glucose-oxidase transforms glucose in D-gluconolactone and hydrogen peroxide. The final step consists of detection of hydrogen peroxide: Amplex Red will be transformed into a highly fluorescent compound – resorufin (oxidation, de-acetylation and double bond rearrangement), the transformation being assisted by horseradish peroxidase. It is worth mentioning that resorufin is also visible under normal light, as a bright pink compound.

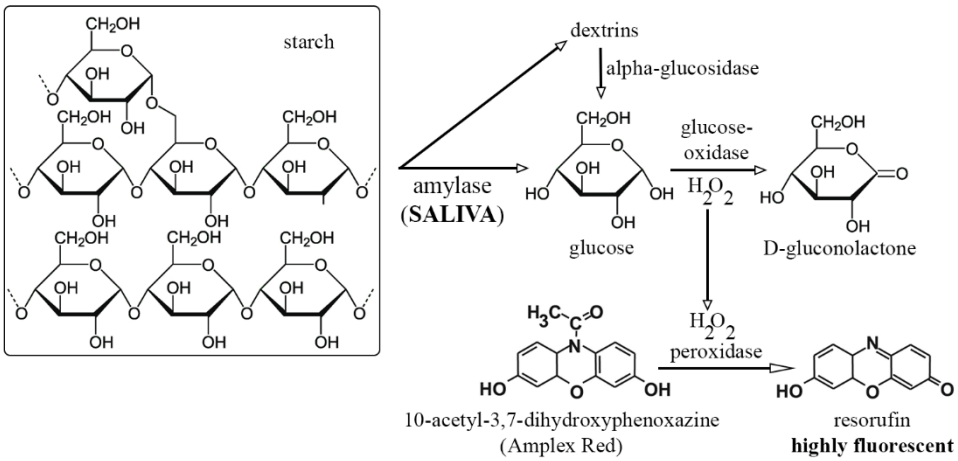

Figure 1. The proposed principle of saliva detection.

MATERIALS AND METHODS

Chemicals. Amylase, glucose oxidase, α-glucosidase, horseradish peroxidase, DMSO and Amplex Red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). After testing three different types of starch, an in-house made soluble form of starch was used (Han and Lim, 2004): 50 g of food grade corn starch were dissolved in 100 ml dimethyl sulfoxide (DMSO) and kept under mild stirring at 37^0 C for 24 hours. Using this procedure, a high percent of the amylose chains is unfolded, resulting in increased solubility and greater susceptibility for amylase. This DMSO modified starch was precipitated with 200 ml cold ethanol 99.8%, vacuum filtered and washed three times with cold ethanol 99.8% and then used as a substrate for the saliva detection experiments. After drying, the modified starch was stored at room temperature and dissolved before conducting the experiments. A glucose-oxidase based kit for glucose detection from Biosystems (Barcelona, Spain) was used for checking the starch quality.

The proposed protocol for saliva detection. In a test tube, the following solutions were mixed: 100 µL saturated solution of the described above soluble starch, 100 µL fresh saliva obtained from a healthy volunteer, 50 µL α-glucosidase (0,2 mg of solid enzyme with 23 units/mg in 1000 µL water) and 25 µL glucose-oxidase (0,8 mg of solid enzyme with 175 units/mg in 1800 µL water). The mixture was vortexed for 20 seconds and incubated at room temperature for 4 minutes. The final step consisted in adding 10 µL of Amplex Red solution (0,8 mg dissolved in 1000 µL dimethyl sulfoxide – DMSO) and 4 µL horseradish peroxidase solution (1,6 mg of solid enzyme with 113 units/mg in 1800 µL water). The final mixture was vortexed for 20 seconds and incubated at room temperature for another 4 minutes.

Spectrophotometric measurements. The resorufin concentration obtained in the presence of saliva was measured using a Piccos Biochemistry Analyser (AMP Diagnostics, Belgium) with a 546 nm filter, taking into account that resorufin light absorbance is near 550 nm (Silva et al., 2016).

Fluorimetric measurements. All the measurements were made using an EnSight Multimode Plate Reader (Perkin Elmer, USA) with a 560 nm excitation wavelength and a 588 nm emission wavelength. Using a 96 well plate, serial dilutions of saliva were analysed in triplicate at different time frames.

Interference with other biological fluids. Different saliva samples were mixed in variable proportions with urine, blood or diluted blood, and serum (mixtures saliva/other biological fluids were 1/3, 1/1 and 3/1 for each fluid). The mixtures or the body fluids alone were then analysed with the proposed method.

RESULTS AND DISCUSSIONS

Influence of starch type used as a substrate for salivary amylase. After testing three different types of starch (Sigma soluble starch, alimentary grade starch and in-house made soluble starch), the best results were obtained with a dissolved and re-precipitated form of corn starch, as described

in materials. Using a glucose-oxidase based kit for glucose detection this type of starch was the only one that was still clearly detectable at 0.02 mg/mL concentration.

Time steps optimisation. Several incubation periods were tested in order to obtain the best colour and fluorescence signal. After changing the time intervals for step 1 from 1 to 10 minutes and from 1 to 20 minutes for step 2, the best incubation times were selected: 4 minutes for step 1 and 4 minutes for step 2. The colour and fluorescence intensity increased after 8 minutes (Figure 2) but this also led to an increase in the negative control. The results were still visible after 24 hours, but with an even stronger increase in intensity for the negative control.

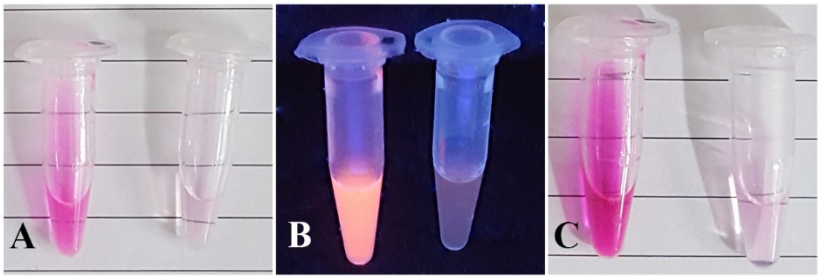

Figure 2. Colour and fluorescence changes with/ without 100 µL saliva – after 4 minutes in visible light (A); after 4 minutes in UV light, 365 nm (B); after 8 minutes in visible light (C).

Limits of detection. The minimum amount of saliva detectable with this method was measured using three different methods: spectrophotometry, fluorimetry and visual macroscopic examination.

Using PBS buffer solution for serial dilutions of saliva ranging from 100 to 0.19 µL/test, the spectrophotometric measurement was able to detect as little as 0,78 µL (Figure 3a). Interestingly, almost the same volume of saliva can also be detected by direct visual examination (Figure 4).

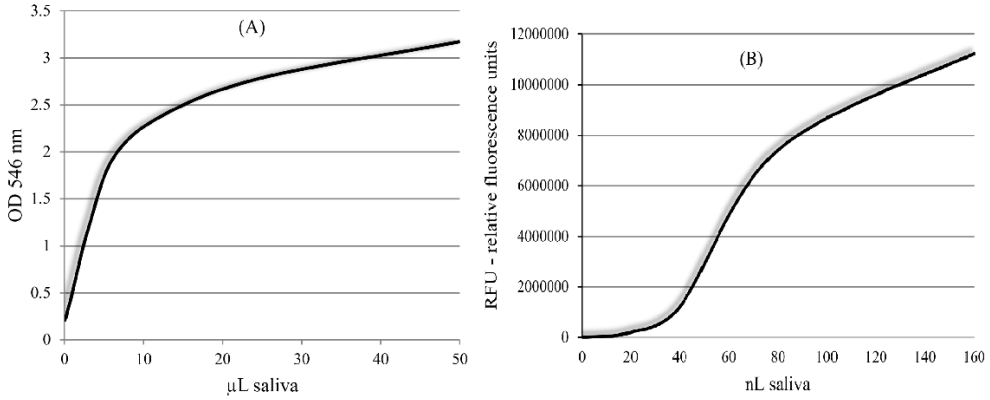

Figure 3. Measurements of light absorbance (OD – optical density) at 546 nm **(A)** and fluorescence intensity - 560 nm excitation and a 588 nm emission - **(B)** for serial dilutions of

saliva. Values are the means of three determinations, and the standard deviation was below 7 % of the mean.

For fluorimetric measurements (Figure 3b) the lowest detection limit reached 20 nL, this result being undetectable using direct examination under UV light. With a saliva volume of 20 nL, the fluorescence signal was still 10 times higher than the negative control.

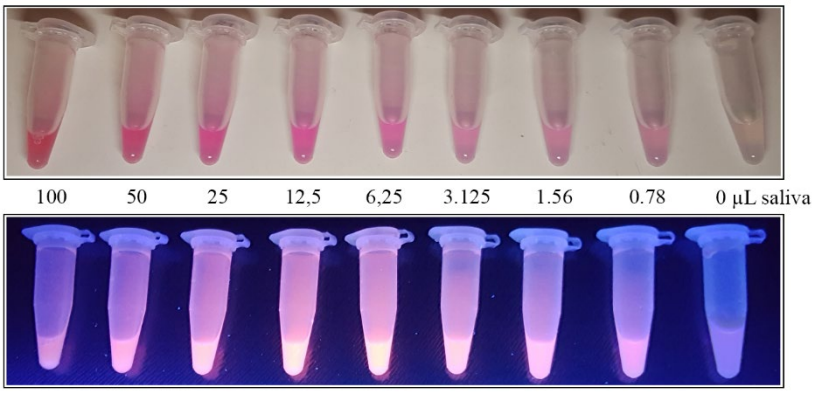

Figure 4. Serial dilution of saliva (ranging from 100 to 0,78 µL) – direct examination under visible and UV light (365 nm).

Interference with other body fluids. As expected, the body fluids that contain certain amount of amylase were able to produce interference with the proposed method of saliva detection. Indeed, after testing various body fluids (alone or mixed with saliva) it was established that the interference was negligible for blood, diluted blood or serum and was stronger for urine. The urine interference could be an important drawback for the method, since the history of forensic cases depicts real situations when these two body fluids must be differentiated. However, this problem could be solved if the final horseradish peroxidase is completely removed from the protocol and the second step incubation time is increased from 4 minutes to 6 minutes. Despite a minor decrease in sensitivity (data not shown), the method without peroxidase was able to make a visible distinction between saliva and urine. Since the removal of peroxidase was the solution for interference, it seems that the problem came from some unwanted substrates found in urine (which are able to produce hydrogen peroxide in the presence of peroxidase) and not necessarily from the urinary amylase. Also, the proposed method was still usable in cases of old saliva (1-28 days) with a minimum decrease of sensitivity, facts which are consistent with literature data (Tsutsumi et al., 1991).

CONCLUSIONS

The method described above could be an interesting alternative for saliva identification in forensic science. The method is sensitive, fast and with little to no interference with other body fluids. Despite being based on a “classical” marker – salivary amylase -, this new method brings at least new standards of sensitivity due to the use of a highly fluorescent final compound visible both in

normal and UV light conditions. This feature could be useful to detect saliva traces on dark surfaces, due to the highly fluorescent properties of the final compound.

REFFERENCES

Aps, J. K., Martens, L. C. (2005): *Review: The physiology of saliva and transfer of drugs into saliva.* Forensic Sci Int, 150(2-3):119-31.
Carboni, I., Rapi, S., Ricci, U. (2014): *Stability of human alpha-salivary amylase in aged forensic samples*. Leg Med (Tokyo), 16:214-17.
Ceska, M., Birath, K., Brown, B. (1969): *A new and rapid method for the clinical determination of α-amylase activities in human serum and urine. Optimal conditions.* Clin Chim Acta, 26(3):437-44.
Groschl, M. (2017): *Saliva: a reliable sample matrix in bioanalytics*. Bioanalysis, 9(8):655-68.
Han, J. A., Lim, S. T. (2004): *Structural changes of corn starches by heating and stirring in DMSO measured by SEC-MALLS-RI system*. Carbohydr Polym, 55:265-72.
Kuwayama, K., Miyaguchi, H., Yamamuro, T., Tsujikawa, K., et al. (2016): *Effectiveness of saliva and fingerprints as alternative specimens to urine and blood in forensic drug testing.* Drug Test Anal, 8(7):644-51.
Mueller, B. (1928): *Über den Nachweis eingetrockneten Speichels in Tüchern*. Dtsh Z Gerichtl Med, 11:211-24.
Myers, J. R., Adkins, W. K. (2008): *Comparison of modern techniques for saliva screening*. J Forensic Sci, 53(4):862-7.
Nakanishi, H., Kido, A., Ohmori, T., Takada, A., et al. (2009): *A novel method for the identification of saliva by detecting oral streptococci using PCR.* Forensic Sci Int, 183(1-3):20-3.
Old, J. B., Schweers, B. A., Boonlayangoor, P. W., Reich, K. A. (2009): *Developmental validation of RSID-saliva: a lateral flow immunochromatographic strip test for the forensic detection of saliva.* J Forensic Sci, 54(4):866-73.
Park, H. Y., Son, B.N., Seo, Y. I., Lim, S. K. (2015): *Comparison of Four Saliva Detection Methods to Identify Expectorated Blood Spatter*. J Forensic Sci, 60(6):1571-6.
Quarino, L., Hess, J., Shenouda, M., Ristenbatt, R. R., et al. (1993): *Differentiation of alpha-amylase from various sources: an approach using selective inhibitors.* J Forensic Sci Soc, 33(2):87-94.
Saxena, S., Kumar, S. (2017): *Saliva in forensic odontology: A comprehensive update*. J Oral Maxillofac Pathol: JOMFP, 19(2):263-65.
Silva, F. S., Starostina, I. G., Ivanova, V. V., Rizvanov A. A., et al. (2016): *Determination of Metabolic Viability and Cell Mass Using a Tandem Resazurin/Sulforhodamine B Assay*. Curr Protoc Toxicol, 68(4):2.24.1-15.
Soyama, K., Ono, E. (1983): *Pancreatic and salivary amylase determination using a short-chain chromogenic substrate (alpha-4-nitrophenyl-maltoheptaoside) and an amylase inhibitor.* Clin Chim Acta, 131(1-2):149-54.
Tsutsumi, H., Higashide, K., Mizuno, Y., Tamaki, K., Katsumata, Y. (1991): *Identification of saliva stains by determination of the specific activity of amylase.* Forensic Sci Int, 50(1):37-42.
Virkler, K., Lednev, I. K. (2009): *Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene*. Forensic Sci Int, 188(1-3):1-17.
Wornes, D. J., Speers, S. J., Murakami, J.A. (2018): *The evaluation and validation of Phadebas® paper as a presumptive screening tool for saliva on forensic exhibits*. Forensic Sci Int, 288: 81-8.
Zakowski, J. J., Bruns, D. E. (1985): *Biochemistry of human alpha amylase isoenzymes*. Crit Rev Clin Lab Sci, 21(4):283-322.

The institutional affiliation of authors:
[1]Department of Forensic Genetics and Serology, Institute of Legal Medicine, Iasi, Romania
[2]Centre of Advanced Research in Bionanoconjugates and Biopolymers, "Petru Poni" Institute of Macromolecular Chemistry, Iasi, Romania
[3]Department of Biochemistry, "Gr. T. Popa" University of Medicine and Pharmacy, Iasi, Romania

[*] bogdan.stoica@umfiasi.ro

Acknowledgements. This work was supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No 667387 WIDESPREAD 2-2014 SupraChem Lab and from a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS/CCCDI – UEFISCDI, project number PN-III-P3-3.6-H2020-2016-0011, within PNCDI III.