Colorimetric Detection of Salivary α-Amylase using Maltose as Inhibitor for Polysaccharide Cleavage

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ABSTRACT This paper describes an approach for colorimetric detection of salivary α-amylase, one of the potential biomarkers of autonomic nervous system (ANS) activity, for enabling assessment of fatigue. The ability of α-amylase to cleave α-bonds of polysaccharides is utilized for developing a colorimetric assay. In the proposed approach, 2-chloro-4-nitrophenyl-α-D-maltotrioside as substrate releases a colored by-product upon cleavage by salivary α-amylase. Introduction of maltose as a non-competitive inhibitor yields desirable linear responses in the physiologically relevant concentration range (20-500 μg/mL) with a limit of detection (LOD) of 8 μg/mL (in aqueous solution). The concentrations of substrate and non-competitive inhibitor are subsequently optimized for colorimetric detection of salivary α-amylase. A facile paper-based "strip" assay is proposed for analysis of human saliva samples with marginal interference from saliva components. The proposed assay is rapid, specific and easy-to-implement for colorimetric detection of salivary α-amylase between 20-500 μg/mL. Complementary RGB (red, green, blue components) analysis offers quantitative detection with a LOD of 11 μg/mL. The two assay formats are benchmarked against the Phadebas test, a state of art method for spectrophotometric detection of α-amylase. The reported paper-based methodology possesses a high potential for estimation of altered ANS responses towards stressors that possibly could find applications in assessment of fatigue and for monitoring onset of fatigue.

Keywords: α-Amylase; paper-based strip; colorimetric detection; maltotrioside; non-competitive inhibitor.

Research groups around the globe have devoted significant efforts for assessment of fatigue by screening biological fluids such as plasma, saliva and urine for biomarkers that could indicate onset of fatigue. Saliva is considered to be one of the ideal biological fluids for assessment of fatigue due to its constant accessibility and minimal invasiveness of the sample collection processes. Moreover, saliva comprises relevant biomarkers of fatigue such as free fractions of cortisol and testosterone, immunoglobulins and α-amylase [1-4]. α-amylase is known to follow diurnal patterns of variations [5] with lowest concentration in the morning and gradual increase during the day. However, fluctuations in salivary α-amylase levels are also directly caused by ANS response to parasympathetic and sympathetic stimulation. Parasympathetic stimulation of salivary glands through cognitive processes usually leads to secretion of watery saliva, which is poor in α-amylase. On the contrary, sympathetic stimulation through physical activities is followed by secretion of saliva containing higher levels of α-amylase compared to parasympathetic stimulation. In addition, the levels of α-amylase are also known to be associated with depression [6], stress [7-8], panic disorders [9], ADHD [10] etc. With fatigue development being a consequence of altered ANS responses to physiological processes [11-12], constant monitoring of α-amylase is required to enable assessment of fatigue and could possibly be used to predict onset of fatigue.

The physiological range of α-amylase in saliva typically varies from 50 to 500 μg/mL, presented usually in units/mL [13]. However, expression of enzymatic units depends on type of substrate that is used for detection under certain temperature, thus, the numbers may vary significantly. Given, that conditions for maintaining constant activity of the enzyme are abided all the time, utilization of mass units is more applicable in this study.

It has been reported that the evaluation of salivary α-amylase concentration is influenced by the adopted sample collection and analytical methodologies except for enzymatic assays that provide reliable quantification of α-amylase [13]. Current developments in the elaboration of enzymatic assays for the α-amylase detection include electrochemical (potentiometry [14-15], amperometry [16-17], cyclic voltammetry [18]), piezoelectric methods [19-20] and spectroscopic methods [21-30]. More information on analytical parameters of some of these systems are provided in Supporting Information, Table S1.

Enzymatic assays typically require trained personnel and instrumentation for accurate analysis of salivary α-amylase. One of the commercially available assays for detection of α-amylase activity is the Phadebas test that comprises water-insoluble starch-based granules incorporated with a blue dye, which slowly releases into solution upon cleavage of α-bonds by α-amylase [31, 32]. Although this method is reliable and sensitive, heterogeneous structure of starch comprising different

portions of amylopectin and amylose could influence the responses obtained within different batches. Besides, this method requires high volumes of saliva and a bench top spectrophotometer to record the obtained responses. Thus, it is essential to develop simple and rapid assay platforms that enable continuous or frequent monitoring of α-amylase concentration levels in saliva, especially for correlating changes in salivary α-amylase concentration to any cognitive or physical activity.

The ability of α-amylase to cleave α-bonds of polysaccharides has been widely explored for colorimetric assaying. Oligosaccharides were coupled to a small dye, which turned from white to yellow upon cleavage by α-amylase. Maltopentaosides were initially utilized as substrates for spectrophotometric and electrochemical detection of α-amylase [15, 23, 33]. However, cleavage of α-bond between saccharide and dye required additional enzymes that complicated the assay and prolonged the assay time. Subsequently, maltotrioside was proposed as a substrate for detection of α-amylase enzymatic activity [28-29], without requiring additional enzymes. Although the assay yielded linear responses for low concentrations of α-amylase, the obtained dynamic range was beyond the physiological range of salivary α-amylase. Increasing the substrate concentration could offer an avenue to increase the dynamic range. However, the reaction may be very rapid, compromising detection of α-amylase at higher concentrations. Introduction of competitive inhibitor (a substance that is structurally similar to a substrate, but not recognized by enzyme as a substrate, e.g. maltose [34]), that slows down reaction velocity would enable more accurate measurements of α-amylase at higher concentrations, and would potentially expand the dynamic range of the assay. In this study, we aim to study the kinetics of α-amylase in order to reveal the catalytic mechanism of the enzyme on substrate and to investigate the effect of an inhibitor on reducing reaction rate and on extending the dynamic range for α-amylase detection. The concentrations of substrate and inhibitor are eventually optimized for visual estimation of α-amylase concentrations in saliva using a colorimetric paper-based assay.

Previously reported protocols utilizing maltotrioside required sophisticated instrumentation and are therefore not ideal for onsite applications. A substrate consisting of two glucose and one galactopyranose residue was recently proposed, which disabled transglycosylation and hydrolysis that are inherent processes for other substrates [24, 35]. This substrate was successfully employed for testing paper strips to evaluate development of fatigue within individuals [25-26]. However, the detection required additional instrumentation for reading the signal intensity, again limiting the usage for onsite applications. Herein, we propose a simple paper-based assay that enables rapid and semi-quantitative visual detection of α-amylase activity within the physiological range (20-500 μg/mL). Subsequent RGB analysis offers a quantitative detection methodology with a competitive LOD of 11 μg/mL. The proposed approach does not require sophisticated instrumentation nor additional reagents for onsite assaying. Therefore, we foresee applications for onsite visual screening of α-amylase in response to external factors as a consequence of changes in ANS activity which could enable estimation of fatigue onset and progression.

Materials and Methods

All the materials and reagents were purchased from Sigma-Aldrich (Singapore) and used without further purification. Deionized (DI) water was used in all experiments. Qualitative filter paper (Toyo Roshi Kaisha, Japan) was used to prepare circular test paper membranes with a diameter of 10 mm. Spectrophotometric analysis for Phadebas test was carried out using a plate reader, Infinite M200 Pro (Tecan, Switzerland), and Lambda 35 (Perkin Elmer, USA).

Preparation of 2-(N-morpholino)ethanesulfonic acid (MES) buffer. MES was dissolved in DI water to obtain a concentration of 50 mmol. pH was adjusted to 6.85 by 5 M NaOH. MES buffer was used for dissolving 2-chloro-4-nitrophenyl-α-D-maltotrioside (G3-CNP) to obtain a concentration of 20 mg/mL. MES buffer that contained 6.7 mmol $CaCl_2$ and 400 mmol NaCl was used for dissolving α-amylase to obtain required concentrations. MES buffer is prepared at room temperature. Buffer pKa is recognized to change minimally with the temperature. MES buffer is very stable indicated by no visual changes in coloration, aggregation or precipitation during 5 months of storage.

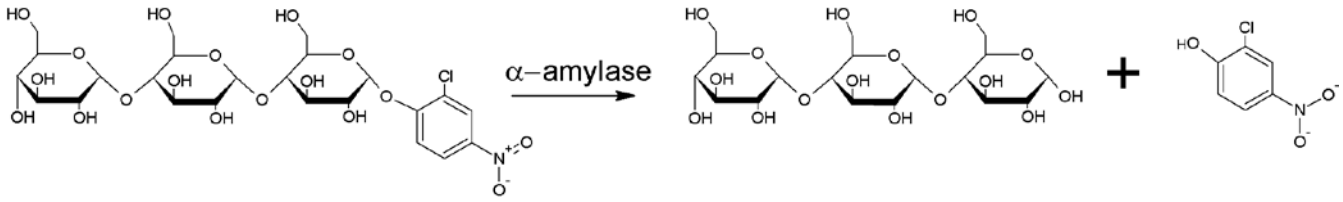
Preparation of solutions of α-amylase. α-Amylase from human saliva, type IX-A, dissolved in MES buffer to obtain a concentration of 5 mg/ml was aliquoted and stored at -20 °C. Before use, the aliquot was thawed and fresh solutions with required concentrations were prepared in MES buffer.

Study of kinetics of enzymatic reaction. The solutions for kinetics study were prepared by mixing increasing concentration of G3-CNP (0.02 – 5 mg/mL) with constant amount of α-amylase (200 μg/mL) in order to establish the amount of substrate for which reaction rate is independent of substrate concentration. The absorbance of released dye was recorded during 20 min. In order to evaluate the dynamic range of the assay, amount of substrate was kept constant (5 mg/mL) while amount of α-amylase was gradually increased (from 5 μg/mL to 500 μg/mL). Maltose (1 mM, 2 mM and 5 mM) was added to the solution along with varying concentrations of α-amylase and the absorbance of released product was recorded during 20 min.

Preparation of test paper membranes. Test membranes were prepared by dropping required amount of G3-CNP and maltose in MES buffer. After drying under air stream, paper membranes were utilized for testing the α-amylase activity.

RGB analysis. For digital image analysis, tested paper membranes were placed on a white background and the images were taken with a digital camera ILCE 7R (Sony) fixed at a constant distance and angle from the samples. Subsequently, the images were transferred to a computer. Central part of the test paper membranes (75x75 pixels) was analyzed by ImageJ software for quantification of obtained colorimetric responses. The illumination for RGB analysis was kept constant (500-550 lum).

Preparation of standard paper membranes. Standard paper membranes were prepared by dropping 10 μL of a mixture of 20 mg/mL G3-CNP and 2 mM maltose and dried in air stream. 5 μL of α-amylase (20, 50, 75, 100,



Scheme 1. Schematic mechanism of cleavage of G3-CNP by α-amylase

200, 300, 400 and 500 μg/mL) were then dropped on the prepared membranes. After 15 min, 5 μL of 1 M NaOH were added to stop the reaction. Obtained color intensities of the membranes were used as standards (standard membranes) to obtain semi-quantitative estimate of α-amylase concentrations in saliva samples. Membranes were prepared freshly before each experiment that required estimation of α-amylase in saliva samples. Several membranes were also stored in dark for up to 70 days for stability analysis.

Collection of saliva samples and exposure to membranes. Saliva samples were collected from volunteers in different physiological conditions (saliva sample collection from volunteers and their activities prior collection of samples are described in Supporting Information) in order to cover a wide range of salivary α-amylase concentration levels. The samples were collected upon their verbal consent. Volunteers were asked to avoid consumption of food at least for 1 h (unless stated different) and to rinse their mouths with water prior to saliva collection. The collected saliva samples were then used (within 30 min) as is for evaluation of α-amylase response without any further pretreatment. Five μL of saliva was added to membranes deposited with 20 mg/mL G3-CNP and 2 mM maltose. In order to estimate α-amylase level semi-quantitatively, after 15 min of enzymatic reaction, the resulting color was compared to the standard membranes. Saliva from a volunteer was stored for different periods of time in room temperature in order to explore the effect of storage on α-amylase activity.

Evaluation of α-amylase concentrations in saliva samples using Phadebas test kit. 200 μL of freshly collected saliva samples were transferred into falcon tubes and diluted with 4 mL of DI water. Tubes were pre-incubated at 37 °C water bath for 5 min. One tablet of the Phadebas test kit was added into each tube, vortexed for 10 s and incubated in a well stirred water bath at 37 °C for 15 min. The reaction was stopped by adding of 1 mL of 0.5 M NaOH. Tubes were centrifuged at 1500 g for 5 min. Obtained blue supernatant was transferred into a cuvette. Spectroscopic measurements were carried out at 620 nm. All procedures were repeated with a blank solution that had all constituents except saliva. The actual amount of α-amylase was retrieved from the calibration table provided in the Phadebas test kit.

Benchmarking of the developed assay against Phadebas test Kit. Five μL of saliva was added to membranes deposited with 20 mg/mL G3-CNP and 2 mM maltose. Semi-quantitative estimation of α-amylase level was performed according to procedure described in section "Collection of saliva samples and exposure to membranes". The concentration of salivary α-amylase is then estimated based on the obtained color intensities of the test membranes with reference to color intensities of standard membranes. The estimated salivary α-amylase concentrations are subsequently validated using responses from the Phadebas tests. Quantitative estimation of α-amylase concentrations obtained from the RGB analysis also was benchmarked against the Phadebas test.

Results and Discussion

The main function of α-amylase is to cleave α-bonds of polysaccharides, yielding short chain by-products such as maltose and maltotriose [36]. G3-CNP is utilized as substrate in this study. It is known to be effectively cleaved by α-amylase at the dye-glycoside bond [28]. A schematic mechanism of the cleavage reaction is illustrated in Scheme 1.

Enzyme kinetics. In order to understand the substrate cleavage by α-amylase, the kinetics of enzymatic reaction is examined. The Michaelis-Menten kinetics provides the description of the velocity of reaction between enzyme and substrate. Given that the amount of enzyme is fixed, the increase of substrate concentration yields linear augmentation of the reaction rate until saturation of enzyme with substrate molecules, resulting in a plateau after which no further change in velocity is observed. At this point all active sites are occupied by a substrate and the enzyme works at the maximum possible rate. Therefore, for experiments that aim to study the reaction rate as a function of increasing enzyme concentration, the substrate concentration should be significantly higher than the enzyme concentration. Under these conditions is the reaction independent of substrate concentration, resulting in release of product (CNP) in accordance with the enzyme concentration.

With the view of investigating the reaction velocity with increasing substrate concentration, the absorbance values of the released product (CNP) were monitored at 405 nm for a fixed concentration of α-amylase (200 μg/mL) cleaving G3-CNP in the concentration range of 0.01 – 5 mg/mL (Figure 1A). The absorbance values display a complex (non-linear) behavior with increasing substrate concentration, and they reach a plateau at around 4 mg/mL. The absorbance curve does not resemble a classic hyperbolic that is inherent for enzymes that obey Michaelis-Menten kinetics. On the contrary, the absorbance curve displays intermediate velocity profiles, which could be attributed to cooperative binding of by-products (oligosaccharides) formed during enzymatic reaction. These by-products are prone to compete with the substrate molecules during binding to the enzyme. Although oligosaccharides with less than three residues are not recognized as a substrate [37], they could sterically block the active site of α-amylase, thus inhibiting the access for substrate molecules. When products bind to the active site and thereby inhibit the binding of a substrate molecule, the

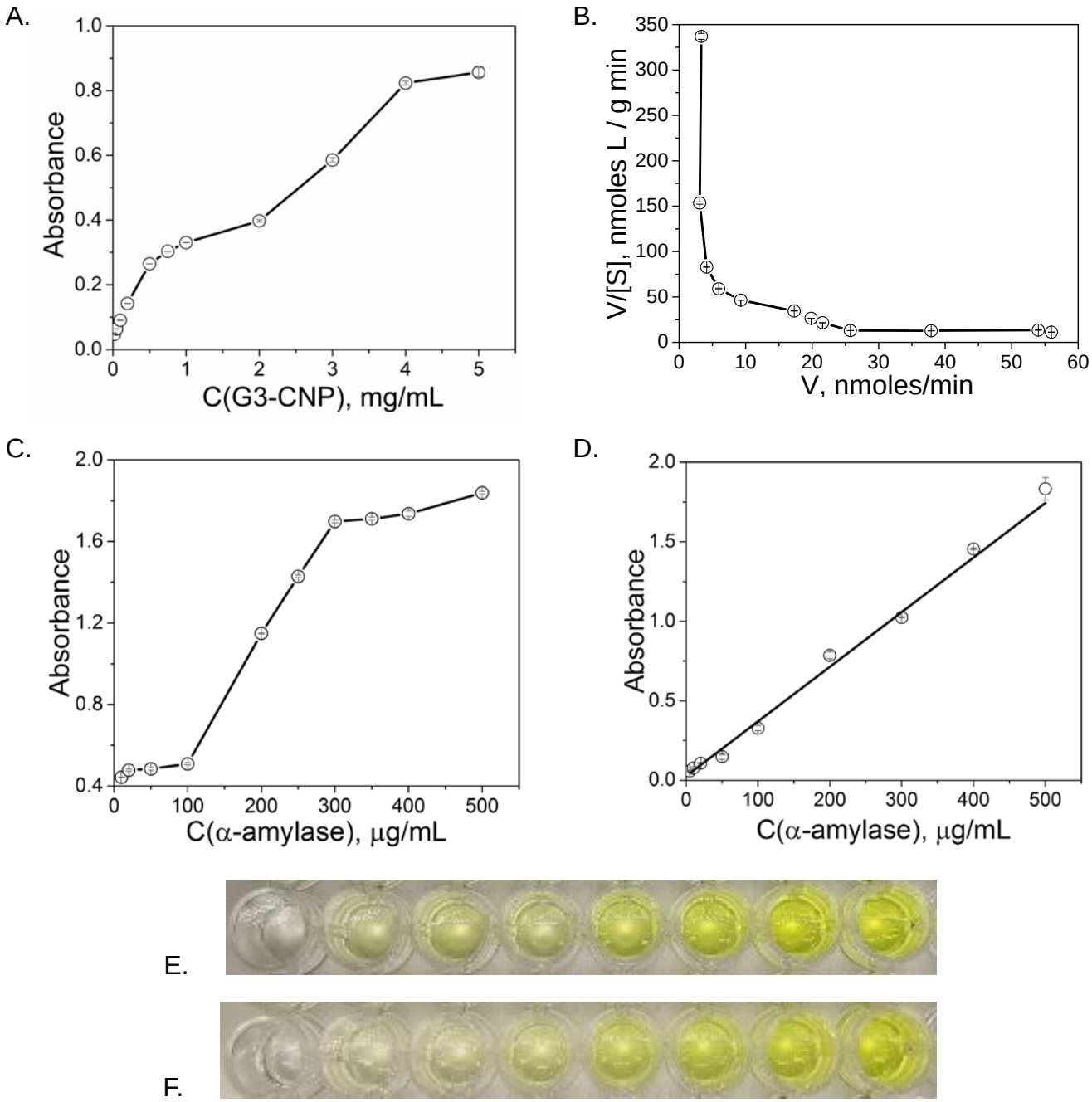


Figure 1. (A) Absorbance curve of released dye (CNP) illustrating the enzymatic digestion of increasing concentration of substrate (G3-CNP) by α-amylase (200 μg/mL). (B) Modified Scatchard plot to address the nature of the cooperative binding between α-amylase (200 μg/mL) and G3-CNP. Absorbance curves of released dye as a function of amount of α-amylase (C(α-amylase)=5-500 μg/mL) (C) without maltose as non-competitive inhibitor, and (D) with 2 mM maltose as non-competitive inhibitor, concentration of substrate C(G3-CNP)=5 mg/mL. (E) Colorimetric response of enzymatic reaction between 5 mg/ml G3-CNP and α-amylase (20, 50, 75, 100, 200, 300, 400 and 500 μg/mL, from left to right) without maltose and (F) with 2 mM maltose inhibitor. The curves (A) to (D) and images (E) and (F) are recorded after a reaction time of 3 min. The samples for curves (A) to (D) were prepared for n=3.

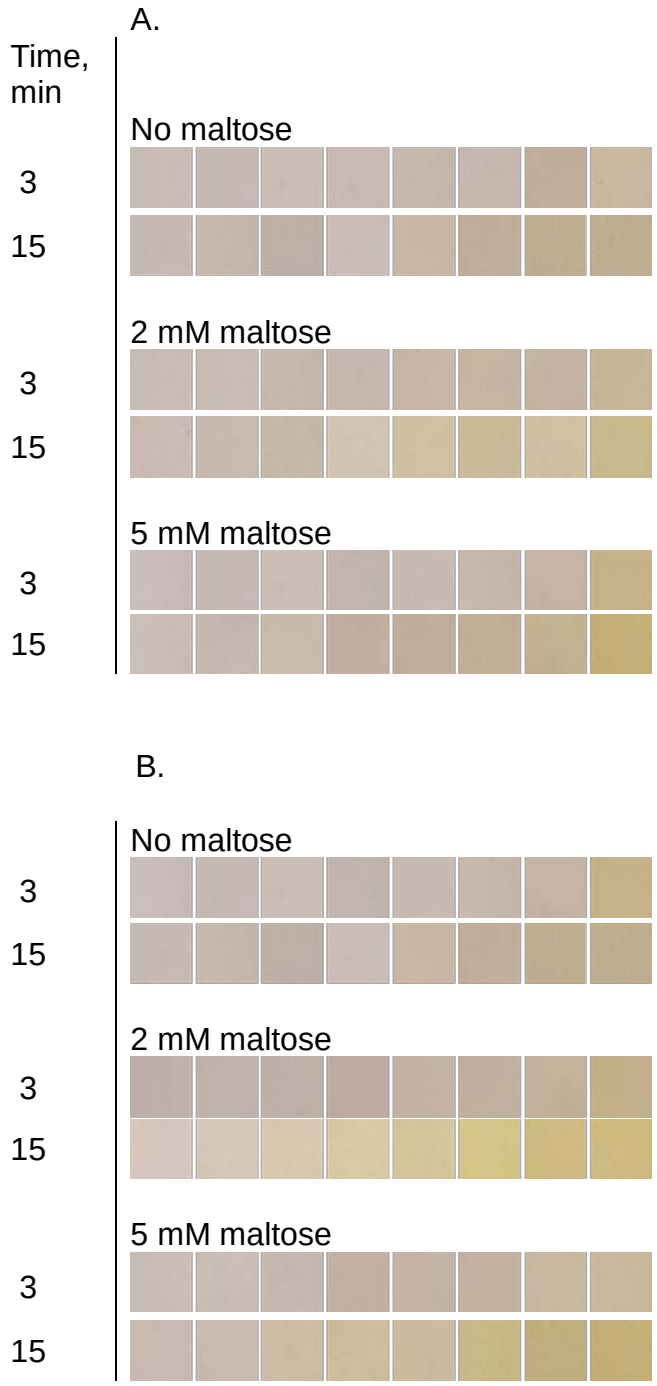


Figure 2. Colorimetric response of paper membranes with concentration of G3-CNP (A) 5 mg/mL; and (B) 20 mg/mL, and for 0, 2 or 5 mM maltose. The reaction time was 3 or 15 min and the α-amylase concentration increases from left to right from 20, 50, 75, 100, 200, 300, 400 to 500 μg/mL, respectively.

interaction becomes negatively cooperative. The shape of V/[S] vs V (modified Scatchard plot) was examined in order to evaluate cooperativity. In the presence of cooperativity, the curve is no longer linear; instead, it is concaved downwards or upwards, in the case of positive or negative cooperativity, respectively [38]. The shape of the curve shown in Figure 1B undoubtedly points toward negative cooperative binding caused by an excess of by-products in the solution. Typically, a longer reaction time could overcome inhibition, as the substrate molecules would have sufficient time to reach and bind to the active sites of the enzyme. This assumption is confirmed by the kinetic curve recorded after 20 min of reaction (Figure S1). Although, the curve remains non-linear, the intermediate step response between 0.5-2 mg/mL appears less pronounced indicating that there is sufficient time for substrate molecules to reach the active sites of the enzyme.

Effect of non-competitive inhibitor. For accurate determination of α-amylase amount in the sample, the dynamic range of developed system should be established. For this purpose, the concentration of α-amylase was varied between 10 and 500 μg/mL with the intent to evaluate response for physiological concentration ranges of α-amylase in saliva. As observed from Figure 1C, the curve is still non-linear, preventing accurate determination of α-amylase over the entire physiological range. Therefore, in order to reduce enzymatic reaction rate and thereby to broaden the dynamic range, maltose is evaluated as a non-competitive inhibitor [34]. Maltose binds nonspecifically to the enzyme (not to the active site), thereby changing its morphology that indirectly influences the binding and cleavage rate of the substrate. When 1, 2 or 5 mM solutions of maltose are added to the reaction mixture, a decrease in absorbance at low α-amylase concentrations is observed, which implies that less products are released from substrate molecules. While 1 and 2 mM maltose appears to have a marginal effect on reaction velocity, addition of 5 mM maltose drastically reduces the product formation (Figure S2a). Although the kinetic curves for reaction between α-amylase and substrate with 1 mM or 2 mM maltose almost coincide, better visual perception of colorimetric responses and higher linearity within the physiological range was observed with the inhibitor concentration of 2 mM. This observation is in agreement with the colorimetric response and RGB analysis (Figure S2b-S2c).

The best linearity for the system is observed for 2 mM maltose after a reaction time of 3 min, with the linear fit equation; $A = 0.03\pm0.02 + (7.43\pm0.08)\cdot10^{-3}\cdot C(\alpha\text{-amylase})$ (Figure 1D). The LOD is evaluated as 8 μg/mL based on 3-fold standard deviation (n=3) of the mean value divided by a slope in the linear fit equation and is lower than natural variation of α-amylase levels in saliva. It is important to stress that the adopted approach enables us to expand the dynamic range of α-amylase assay and to obtain a linear calibration curve (Figure 1D), which was not feasible in the absence of a non-competitive inhibitor (Figure 1A and 1C). Moreover, visual inspection of the corresponding solutions confirms the importance of using the maltose inhibitor to maintain linearity over a broader dynamic range. For example, Figure 1E demonstrates that in the absence of maltose it is difficult to differentiate the color intensities at low concentrations and they are almost indistinguishable at higher concentrations of α-amylase (>100 μg/mL). On the contrary, color changes upon addition of different concentrations of α-amylase within the physiological range could be readily distinguished by naked eye using 2 mM maltose as inhibitor, Figure 1F. Thus, the use of maltose as non-competitive inhibitor undoubtedly provides an avenue to improve the linearity over broad dynamic range and to obtain a competitive LOD of 8 μg/mL for α-amylase.

Colorimetric paper-based test. Based on the promising spectrophotometric and colorimetric responses obtained in homogeneous solutions, the assay was then transferred to a paper membrane with an aim to

establish a platform for onsite analysis of saliva samples. Again, we carried out a series of experiments to establish the most favorable assay conditions for naked eye estimation of α-amylase concentrations. This optimization was necessary as the exact concentration of inhibitor might vary when applied to paper membranes because of unknown sorption phenomena, if any. The concentrations of the G3-CNP and maltose were 5 and 20 mg/mL, and, 0, 2 and 5 mM, respectively. Images were captured after 3 min and 15 min of reaction time for further analysis.

As observed from Figure 2, the difference in colorimetric responses recorded after 3 min of reaction time could not be clearly distinguished by naked eye although most linear responses are recorded in aqueous solutions under similar test conditions. This is attributed to the difference in velocities of enzymatic reactions in homogeneous solutions and on paper membranes. Paper membranes may restrict the reaction rate by lowering degrees of freedom and/or by steric hindrance caused by the three dimensional morphology of the paper. However, colorimetric responses recorded after 15 min of reaction time appear to be more applicable for the assay as significant differences in color intensities are observed. Figure 2 illustrates that the systems comprising of 5 mg/mL G3-CNP and 5 mM maltose, as well as 20 mg/mL G3-CNP and 2 mM maltose possess distinct and gradual changes in colors, enabling semi-quantitative detection of α-amylase at physiological ranges. It is also evident from Figure 2 that the use of maltose inhibitor is crucial for obtaining a gradual color change over the entire dynamic range.

It is important to note that the visual estimation of color is highly susceptible to light intensity (see Table S2). Thus, visual estimation should be ideally performed in normal lighting conditions (in the range of 500 lum).

RGB analysis of colorimetric response. In order to objectively select the most appropriate test strip for calibration purposes we performed a detailed RGB and Delta-E analysis. The Delta-E (ΔE) parameter (calculated according to CIE76 algorithm [39]) for the two assay conditions was calculated. A ΔE value of 2 is normally regarded as a cut-off indicator above which the human vision system is capable of differentiating two colors [40]. Therefore, larger differences in ΔE values enable more accurate estimation of α-amylase concentrations. Figure 3A demonstrates a larger difference in ΔE values for the sample containing 20 mg/mL G3-CNP with 2 mM maltose, as compared to the sample with 5 mg/mL G3-CNP and 5 mM maltose. Specifically, the ΔE values for the assay with 5 mg/mL G3-CNP and 5 mM maltose varies between 5.4–6.9 in the concentration range from 20 to 100 μg/mL. Although being sufficient for the human eye to distinguish the difference between colors, the assay with 20 mg/mL G3-CNP and 2 mM maltose provides more distinctive changes in ΔE values (4.6–14.3) within the same concentration range. The difference in the ΔE-values between the two membrane preparations remain the same for concentrations above 200 μg/mL. RGB analysis also was utilized for evaluation of the system with best linearity, which could be used for quantitative estimation α-amylase in saliva samples. The R/G ratio demonstrates insignificant change with increasing α-amylase concentration in contrast to the R/B and B/G ratios. Ratio B/G is chosen for analysis due

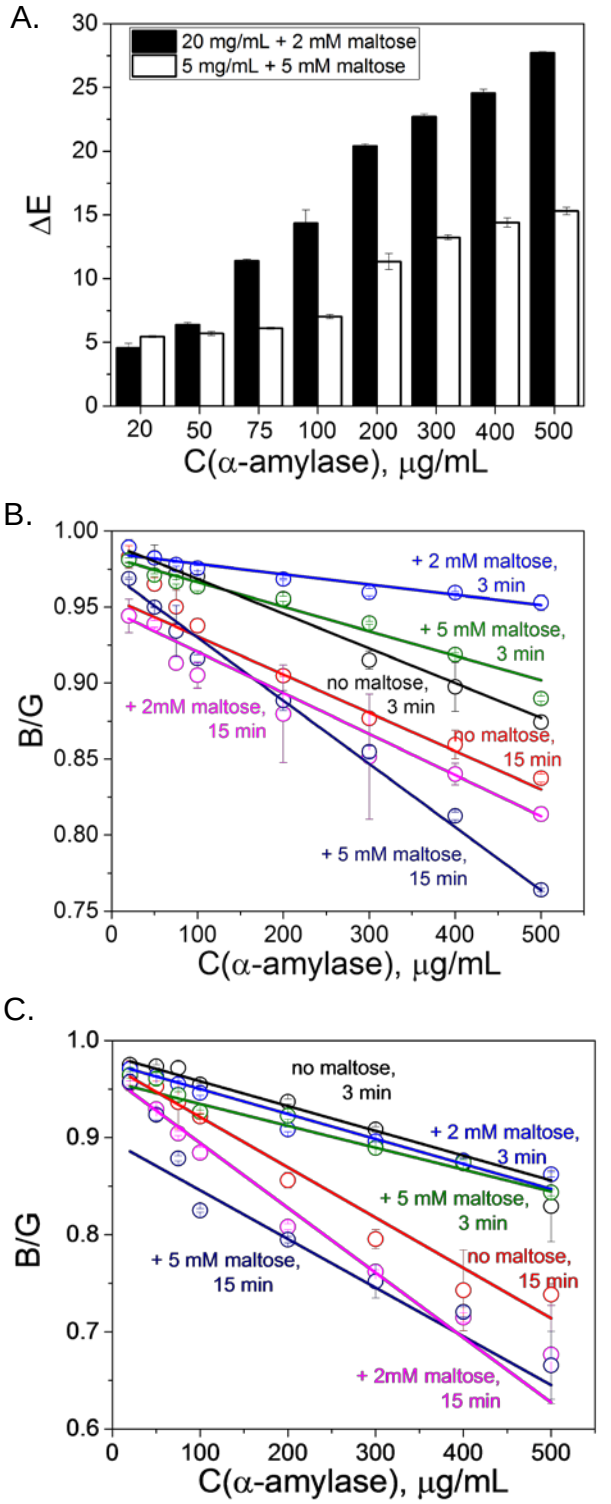


Figure 3. (A) Estimation of expected color difference of paper membranes for visual α-amylase detection assays that include 5 mg/mL G3-CNP with 5 mM maltose (white box) or 20 mg/mL G3-CNP with 2 mM maltose (black box) with reaction time 15 min. Analysis of B/G ratio as a variable of colorimetric response of evaluated assays that consist of (B) 5 mg/mL and (C) 20 mg/mL substrate and inhibitor to different concentrations of α-amylase.

to its distinctive dependence on concentration of α-amylase. The ratio B/G is then calculated for images of membranes with G3-CNP concentrations of 5 and 20 mg/mL, and with 0, 2 and 5 mM maltose, after 3 and 15 min of reaction time. Although the dynamic range of every studied system lies within natural distribution of salivary α-amylase, the optimized assay system should possess a viable combination of three parameters (besides linearity): low standard deviation, low LOD and distinct differences in B/G values obtained from RGB analysis. Figure 3B-C illustrates that the most linear responses are obtained for the strips prepared using 5 mg/mL of G3-CNP with 5 mM maltose, with reaction time 15 min (Figure 3B) and for 20 mg/mL of G3-CNP with 2 mM maltose after 15 min (Figure 3C) within dynamic range 20 – 500 μg/mL of α-amylase. Moreover, the assays based on the above experimental conditions provide the lowest standard deviation for n=3 and yield the lowest LOD, calculated based on 3-fold standard deviation (n=3) of the mean value divided by a slope in the linear fit equation, as 10 and 11 μg/mL, respectively. These LODs are substantially lower as compared to those obtained for experiments with the same amount of substrate but without maltose (60 and 35 μg/mL, respectively, calculated from the black curves in Figures 3B-C). Based on the analysis of B/G ratio, these two systems appear to satisfy all three parameters and therefore suitable for semi-quantitative estimation of α-amylase in buffer.

Taken together, the calibration strip prepared using 2 mM maltose as inhibitor and 20 mg/mL G3-CNP (Figure 2B, Figure 3A and 3C) seems to be the most promising one for visual (semi-quantitative) as well as B/G-based (quantitative) estimation of α-amylase concentration in saliva samples.

Semi-quantitative detection of α-amylase in saliva samples. Saliva samples are known for their matrix complexity, where different constituents could interfere with the detection of target analyte. However, enzymatic reactions are characterized by high specificity of substrate recognition that enables assaying without extensive sample cleanup and pretreatment. Phadebas test was utilized for benchmarking of the results obtained by semi-quantitative estimation of α-amylase concentration in saliva. For validation of the proposed methodology, we selected saliva samples that span the entire physiological range from a study involving a group of volunteers (see Supporting Information). Colorimetric responses obtained for saliva samples from the volunteers are then assessed using the optimized assay condition with 20 mg/mL G3-CNP and 2 mM maltose with a reaction time of 15 min (Figure 2B). The Phadebas test (column 2) was carried out concurrently for validation of obtained colorimetric responses. As illustrated in Table 1 (column 4), the concentration of α-amylase estimated by the paper based test correlates well with responses obtained using the Phadebas test (employed in homogeneous solution), ascertaining that visual estimation of α-amylase concentration is feasible for rapid screening (see also Figure S3). Moreover, it is evident from the Table 1, column 3, the variations in visual responses among 3 membranes are not significant. It is to be noted, however, that semi-quantitative estimation implies lower resolution of the assay. Concentrations of α-amylase can be evaluated only approximately because of variations in subjective judgment of coloration between users, differences in the illumination, etc. The variation in subjective judgement is also a limiting factor for inclusion of intermediate concentrations in the calibration strip (standard paper membranes) to improve the resolution of the assay. Nevertheless, the developed paper-based colorimetric assay provides a rapid, specific and reagents-free assessment of α-amylase concentration in saliva, which facilitates applications in field, without requiring trained personnel and sophisticated instrumentation.

Quantitative detection of α-amylase in saliva samples.

In order to quantitatively evaluate the obtained colorimetric responses, RGB analysis of paper membranes with saliva samples was carried out. Ratio B/G was calculated for all the membranes with saliva samples from volunteers and the concentrations of α-amylase were then estimated according to the linear equation ($B/G=0.962\pm0.004 - (109\pm2)\cdot10^{-5}\cdot C(\alpha\text{-amylase})$) obtained for assay with 20 mg/mL and 2 mM maltose (Figure 3C, purple curve). Table 1 (column 5) reveals good correlation between the concentrations of α-amylase obtained by the RGB analysis and by Phadebas test with an accuracy of >95% and an average precision of 5.4% (see also "Correlation with Phadebas" in Supporting Information). The LOD of Phadebas test (2.5 μg/L, as calculated according to the specifications provided in the test kit) is much lower than that of a developed assay (11 μg/mL). Note, however, detection of high concentrations of α-amylase within physiological range of saliva requires an additional dilution step.

We have additionally tested the developed assay for possible interferences from salivary components by performing a series of experiments. The experiments were designed to study the effect of varying salivary components that differ between volunteers and the interferences from salivary components that could potentially hydrolyze the α-bonds of the polysaccharide substrate (please refer to the list of salivary enzymes in Table S3). More details about the interference tests are provided in Supporting Information and the results provided in Tables S4-S6 demonstrate that salivary components do not have a pronounced effect on the α-amylase activity.

It is at this stage important to emphasize that we intentionally do not aim to draw any conclusions on the effect of cognitive or physical activities on salivary α-amylase levels. However, the observations herein provide an opportunity to assess the adequacy of ANS response to different stressors and potentially estimate and predict onset of fatigue using a simple and fieldable methodology.

Storage and stability. Different concentrations of α-amylase (20, 100, 400 μg/mL) were added to membranes stored in dark for 1 (freshly prepared), 42 and 70 days at room temperature. There was no significant differences in visual and RGB responses for membranes stored up to 70 days (see Figure S4). Storage tests beyond 70 days is a subject for further studies. We also studied the influence of storage of saliva on the activity of α-amylase. Storing saliva in room temperature for a prolonged time can affect α-amylase activity due to the presence of bacteria, debris and various substances [41]. Sample from a volunteer was stored in room temperature for different periods of time. It is evident that the activity of α-amylase does not vary significantly

Table 1. α-amylase concentration in human saliva collected from volunteers, n=3 (different membranes), arranged in increasing concentration as determined using the Phabebas test, visual read out and B/G ratio analysis of test strips.

#	Concentration of α-amylase according to Phadebas test, µg/mL*	Image of a colorimetric response from test strip	Concentration of α-amylase obtained by visual read out of the developed strip test, µg/mL**	Concentration of α-amylase obtained by RGB analysis of the test strip, µg/mL
1	70±1		75	73 ±7
2	157±1		150[1]	166±5
3	202±1		200	192±20
4	306±1		300	297±27
5	345±1		350[2]	348±17
6	407±1		400	401±1
7	429±1		450[3]	443 ±1

*** Obtained by Phadebas test kit using benchtop UV-spectrometer**

**** Obtained visually using the calibration strip test in Figure 2B for 20 mg/ml G3-CNP and 2 mM maltose (15 min).**

[1] Obtained colorimetric response was estimated to fall in between 100 µg/mL and 200 µg/mL

[2] Obtained colorimetric response was estimated to fall in between 300 µg/mL and 400 µg/mL

[3] Obtained colorimetric response was estimated to fall in between 400 µg/mL and 500 µg/mL

if the analysis is performed within 1 h (see Table S7 and Figure S5).

It has been reported that the activity of human salivary α-amylase is not greatly affected by changes in temperature within 20-37 °C, but that the activity drops significantly upon incubation for prolonged time at temperatures higher than 40 °C [42, 43]. In order to verify this hypothesis, we performed an experiment for the detection of α-amylase at room temperature 24 °C and at 31 °C. The results are shown in Table S8. No significant variation in visual and RGB responses is observed, indicating that the developed assay could be utilized for measuring α-amylase levels at temperature range 24-31°C. Therefore, the developed assay could be utilized for onsite estimation of α-amylase levels, for instance at users' homes or training sites, at typical room temperature conditions (around 24 °C). However, if the developed assay is utilized in adverse weather conditions, then a calibration may be required, which will be investigated in our future studies.

Conclusion

Having investigated the catalytic behavior of α-amylase, we evaluated the effect of a non-competitive inhibitor that was introduced into the proposed assay in order to extend the linearity for assay in physiological range. The analysis of colorimetric response to variation in concentration of substrate and inhibitor as well as with time was conducted, which enabled optimization of α-amylase assay using membranes. Benchmarking was performed with state of art Phadebas test that requires benchtop spectrophotometer to determine actual amount of α-amylase in saliva. The results of visual (semi-quantitative) and RGB-based (quantitative) estimation of α-amylase level in saliva samples demonstrated good correlation and repeatability with respect to Phadebas test. Developed assay demonstrated LODs and dynamic range that is sufficient for routine analysis of α-amylase in saliva. Thus, the developed paper membrane can be used onsite in field settings or at home without requiring sophisticated instrumentation and trained personnel for analysis. Future work will emphasize on evaluation of α-amylase response to ANS activity caused by external factors for assessment of fatigue.

ASSOCIATED CONTENT

Supporting Information

Current methods for detection of α-amylase in saliva; Information on volunteers and saliva collection; Absorbance curve of enzymatic reaction; Effect of non-competitive inhibitor on reaction velocity; Effect of illumination; Correlation with Phadebas; Interference of saliva components; Storage stability of membranes; Stability of saliva samples; Effect of temperature on α-amylase detection in saliva sample.

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