Introduction

Stress was firstly defined in 1935, as a reaction of humans to external stimuli. In the modern way of life and every day actions, the term stress is very common and has a significant influence on human reactions, health, work ability and mood. There is also a direct connection between stress and depression in humans. Apart from adults, recent studies show that preschool children are also under stress, and some helping support strategies could reduce it. Thus, it appears that quantification of stress is a challenging issue, not only in humans, but also in animals. Stress response is inducing the sympathetic nervous system and hypothalamic-pituitary-adrenal system, where hormone regulation is included (norepinephrine and cortisol). Invasive techniques measure hormone levels in the blood as an indicator of stress, but these levels are very low and hard to measure, and response time is too long and delayed by 20–30 minutes in response to loading stress.

The use of salivary biomarkers has gained increased popularity over the past decade in psychological and biomedical research. While the measurement of free cortisol in saliva has proven to be in correlation with stress, salivary amylase activity has appeared as a new highlight in stress measurement.

When secretion of the salivary amylase is stimulated by direct innervations, its response is very quick, generally within one to a few minutes, a markedly quicker response than that induced by hormonal regulation. Salivary glands amplify the low levels of norepinephrine and give a faster and more sensitive response to the psychological stress than cortisol. To date, there are no international standard measurement methods for human salivary amylase activity. Salivary amylase has a high activity, ranging from several thousands to hundred thousand units per litre.

According to literature, in human and animal science, amylase activity rises remarkably under autonomic system activation, such as response to a) physical stressors like exercise, exposure to a high-pressure chamber, running, bicycle exercise, sky diving or cold exposure; and b) psychological stress or relaxation interventions. While it seems clear that alpha-amylase levels rise following physical stress, the response to psychological stressors appears to be more inconsistent. This might be due to the psychological nature of the stressors employed or other methodological details. Furthermore, concerning medical aspects, salivary amylase activity has also been employed as a marker for schizophrenia, since several researches indicate that the autonomic nervous system dysfunctions in patients with schizophrenia, in patients with chronic relapsing pancreatitis, the activity of the salivary amylase was significantly lowered and recently, there have been attempts to suggest salivary amylase...
lase as a new biomarker for cardiovascular diseases, like heart failure.20
Saliva collection methods have many variations and include absorbent materials, like cotton21; commercial serviettes; passive drooling and spitting, and other methods22.
Published methods for salivary amylase activity include variations of biosensors, usually glucose biosensors, like disposable screen-printed amylase biosensor based on ferrocene as an electron transfer mediator23, a disposable point-of-care, colorimetric biosensor strip24, biosensor microfluidic SIA systems25, and spectrophotometric determination of salivary amylase activity with p-nitrophenyl maltoside as substrate26. In addition, many other analytical methods for alpha amylase determination could be employed27,28,29.

The aim of this investigation was to test a new methodology for direct potentiometric determination of salivary amylase, based on our previous work30, and quantify human stress as a function of salivary amylase activity at different stress conditions: physical activity – exercise, and psychical (mental) activity – student exam.

**Experimental part**

**Reagents**

α-Amylase (EC 3.2.1.1) from *Aspergillus oryzae* (its activity was 36 U mg⁻¹) was purchased from Fluka (Switzerland) and used as the standard amylase reagent without purification. Soluble starch, calcium chloride, sodium chloride and iodine were purchased from Kemika (Croatia). Potassium iodide was purchased from Sigma-Aldrich (SAD), glacial acetic acid from Panreac (Spain), sodium acetate trihydrate (CH₃COONa·3H₂O) from J. T. Baker (Holland). All chemicals were p.a. grade.

**Solutions**

**Starch solution**

An amount of 5 g of dried soluble starch was mixed in 0.1 mol L⁻¹ acetate buffer solution (pH 6.0) in 100 mL flask. After heating and stirring, the starch had completely dispersed. The solution was cooled to room temperature to perform the measurements. Starch solutions were prepared daily to avoid microbial degradation and retrogradation.

**Conditioning solution**

The conditioning solution was prepared in 500 mL volumetric flask by dissolving CaCl₂ (6 mmol L⁻¹) and NaCl (20 mmol L⁻¹) in a 0.1 mol L⁻¹ acetate buffer solution (pH 6.0).

**Saliva sample solutions**

Saliva sample solutions (1 %) were prepared by adding 0.5 mL of each saliva sample to a 50 mL volumetric flask and then diluting it to the mark with a conditioning solution. The resulting solution was stirred without heating until the saliva had completely dissolved. The conditioning solution was used to maintain the constant volume and stability. The addition of CaCl₂ and NaCl to the saliva solution serves to stabilise enzyme activity.

**Standard α-amylase solution**

A standard α-amylase solution was prepared and standardised as described by Sakac et al. An amount of 5 mg of α-amylase (Fluka, Switzerland) (0.36 U mL⁻¹) was dissolved in 500 mL of the conditioning solution.

**Acetic acid-triiodide solution**

The acetic acid–triiodide solution (ATIS) was prepared by dissolving solid iodine (c = 100 µmol L⁻¹) in potassium iodide solution (c = 0.05 mol L⁻¹) and adding glacial acetic acid to the final concentration 1 mol L⁻¹. ATIS was used for the inhibition of ptyalin activity and the reaction of triiodide with the nonhydrolyzed starch.

**Apparatus**

A Metrohm 780 pH meter, a 728 Stirrer, a Metrohm 765 Dosimat (all from Metrohm, Switzerland), homemade software and a platinum redox electrode IJ64 (Ionode, Australia) were all used for the response measurements. A silver/silver (I) chloride reference electrode (Metrohm, Switzerland) with 3 mol L⁻¹ KCl inner solution was used as a reference. A thermostat (PolyScience, USA) was used for the amylase incubation.

Direct potentiometric measurements were performed on an eDAQ 186 Quad Amp pH/mV amplifier connected to an eDAQ ecorder 821 8-channel data acquisition system operated by the eDAQ Echem 1.5 software (all from eDAQ, Australia).

**Groups and sampling**

The experiment included two groups of people. The first group were candidates involved in physical activity – 30 minutes active exercise for young healthy adults, males and females, age range from 22–30 years. The saliva samples were taken before (relaxed) and immediately after training (stressed). The second group of candidates were focused on mental stress and included students in the time of writing an exam. Here a group of young healthy adults, age range from 20–24 years, were sampled.
just before writing an exam (stress) and on a day at the same time when they had no college obligations (relaxed). We considered both the male and female population.

Saliva was sampled by cotton adsorbent attached on the capillary tube, placed under the tongue, and collected in a tube\(^6\). Sampling took up to five minutes, and the result is transparent and filtrated saliva. The collecting tubes were stored in the refrigerator at \(-20\) °C until analysis. The time lag between the occurrences of mentally stimulated saliva amylose was one to three minutes; the recovery of normal salivary amylose levels after stress reduction is very rapid: the duration of amylose elevation is only about 10 minutes\(^2\), which makes this sampling method suitable.

**Measurement of the salivary amylose activity**

Test tubes containing the same volume of starch solution were heated in a water bath at controlled temperature of 45 °C. The buffered saliva solution was incrementally added to the starch solutions in test tubes at volumes ranging from 0 to 2.5 mL. The volume of the reaction mixture was held constant by a periodic addition of conditioning solution. After 10 minutes of heating at 45 °C, the reaction was terminated by adding 5 mL of ATIS. The incubation time was 10 minutes. Test tube solutions were cooled to room temperature using a water bath. Redox sensor was used for direct potentiometric measurement of the redox potential of the solutions. As a comparator, a standard solution of \(\alpha\)-amylose was used, following the same procedure.

**Data elaboration and optimization strategy using Solver**

The experimental data were elaborated using MS Excel and a Solver add in. The calculations were based on the model previously presented\(^9\). **Solver** is a spreadsheet optimization modelling system incorporated into Microsoft Excel that can be used for solving different linear and nonlinear problems.

**Solver** was activated by choosing **Add ins...** in the **Tools** menu. It was used to compare an array of data predicted by the model with an initial set of parameter values over a range of dependent variable values with a set of experimental data. Then, the sum of squared residuals (SSR) between the two arrays was calculated by varying the parameter values to minimize the error (SSR) between the two data sets.

The **Solver** optimization consists of the following steps:

a) Generation of a worksheet containing the data, fit with an independent variable \(E\) (redox potential in mV) and dependent variable \(mAmy\) (mass of ptyalin in µg).

b) Insertion of a column containing \(E_{calc}\), which was calculated by means of Eq. 7 to describe the response of the platinum amylose sensor and include the appropriate number of parameters to be varied (Changing Cells). These parameters include the sensor slope (\(S\)), the constant potential term (\(Const\)), and the proportionality factor (\(k\)). Instead of a very low \(k\) value on the order of 1·10\(^{-4}\), the log \(k\) value should be used. Providing different sets of initial conditions ensured that **Solver** found a global minimum.

c) Insertion of a column for calculating the squares of the residuals, \(E−E_{calc}\), for each data point.

d) Calculation of the sum of the squares of the residuals (Target Cell).

e) The use of **Solver** to minimize the sum of the squares of the residuals (Target Cell) by changing the selected parameters of Eq. 2 (Changing Cell). No constraints were applied to the variables.

The macro **SolvStat** provided the regression statistics for **Solver** by calculation of the standard deviations of the parameters, correlation coefficients and standard errors of the \(y\) estimate SE(\(y\)).

**Results and discussion**

**Modelling the response mechanism**

Salivary amylose catalyses the hydrolysis of starch from a starch-triiodide complex and releases the triiodide ion. The increase in the triiodide ion concentration increases the triiodide/iodide redox couple ratio, resulting with an increase in the electrode potential of the redox sensor according to the Nernst equation\(^5\):

\[
E = E^0 + \frac{RT}{2F} \ln \frac{C_{I_3}}{C_I} = E^0 + S \log \frac{C_{I_3}}{C_I}^3 \quad (1)
\]

\(E^0\) = constant potential term, \(S\) = slope of the sensor (mV/decade of activity), \((C_{I_3})\) = triiodide concentration, \((C_I)\) = iodide concentration (considered to be constant).

Thus, the increase in the electrode potential relates to the ptyalin concentration/activity. The referred model has been adopted for salivary amylose activity determination.

Equation 2 represents the Nernst equation after salivary amylose addition to the system:

\[
E = Const + S \log \frac{C_{I_3}}{C_{I_3}} \quad (2)
\]

where \((C_{I_3})\)\(_f\) represents free triiodide concentration (mol L\(^{-1}\)), which is a summary of initial triiodide ions in solution \((C_{I_3})_0\) and a triiodide ion released by the salivary amylose activity \((C_{I_3})_f\).
This can be described:

\[
(C_{I_3})_{f} = (C_{I_3})_{0} + (C_{I_3})_{l}
\]  

(3)

Salivary amylase hydrolyses the starch and releases triiodide, which increase in concentration is proportional to the amylase quantity added:

\[
(C_{I_3})_{f} = k \cdot m_{Amy}
\]

(4)

where

\[k = \text{proportionality factor}\]

\[m_{Amy} = \text{amylase quantity (μg)}\]

By inserting Eq. (4) into (3), the following expression was obtained:

\[
(C_{I_3})_{f} = (C_{I_3})_{0} + k \cdot m_{Amy}
\]

(5)

The salivary amylase quantity can be calculated from Eq. (5):

\[m_{Amy} = \frac{(C_{I_3})_{f} - (C_{I_3})_{0}}{k}
\]

(6)

The adopted model was used for experimental data evaluation and salivary amylase activity calculation. A section of the spreadsheet that displays the potentiometric measurements and the model parameters for the response characteristics of a platinum redox electrode sensor to exposure to the range of volumes of 1 % saliva solution is shown in Table 1. In Table 1, the Solver modelling using the proposed model and experimental data set is presented as a MS Excel spreadsheet. The calculated variables were slope (S), proportionality factor (k) and initial triiodide ion in solution \((C_{I_3})_{0}\). The Solver uses the least-squares criterion to fit a theoretical curve into the experimental data set, and the concentration of free triiodide, a triiodide ion \((C_{I_3})_{f}\), and released triiodide, a triiodide ion \((C_{I_3})_{l}\), could be calculated (Eq. (3)). The concentration of \((C_{I_3})_{f}\) at the beginning of the measurement was 0, since there was no amylase action. When the salivary amylase solution was added, the amylase took action, resulting in an increase in \((C_{I_3})_{f}\) and \((C_{I_3})_{l}\), respectively. Then, by using Eq. (6), the salivary amylase quantity and concentration could be calculated.

Following the model and Eq. 6, information on the amount of salivary amylase could be expressed. This is shown in Figure 1, where the logarithm of the amount of salivary \((\ln(m_{Amy}))\) amylase is dependent on the redox signal potential change. The linear equation for the corresponding dependence was \(y = 0.1062x - 0.2492\), with the correlation coefficient \((R^2)\) value 0.9828.

Table 1 – Selected potentiometric measurements and model parameters for the response characteristics of a platinum redox electrode sensor to exposure to a range of volumes of 1 % saliva solution. (ssr – sum of squared residuals; sr – squared residuals for the experimental data, S – sensor slope; \(E_0\) – standard redox potential; \(E\) – experimental redox potential; \((C_{I_3})_{f}\) – free triiodide concentration (Eq. 5), \(C_{Amy}\) – salivary amylase concentration, \((C_{I_3})_{l}\) – liberated triiodide ion.

<table>
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<tr>
<th>Sl.</th>
<th>V(saliva)/mL</th>
<th>Experimental (E)/ mV</th>
<th>(\Delta E = E - E_0)</th>
<th>((C_{I_3})_{f})</th>
<th>((C_{I_3})_{l})</th>
<th>Model (E)/mV</th>
<th>sr</th>
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<td>1.32E-03</td>
<td>326.39</td>
<td>1.93</td>
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</tbody>
</table>

\[C_{Amy} = \mu g mL^{-1}\]
Influence of physical activity

The influence of physical activity on the salivary amylase concentration was exemplified through the intensive exercise. Exercise intensity was medium. The investigation was carried out on a group of ten healthy young people. Saliva was collected before the exposure to the intensive exercise, and after 30 minutes of training it was collected again. The salivary amylase concentration was measured before and after training using the previously presented model. The results of salivary amylase concentration before and after exercise are shown in Table 2.

The salivary amylase activity ranges from 4.51 to 34.04 μg mL\(^{-1}\) for samples taken before training, and 8.89 to 91.53 μg mL\(^{-1}\) for samples taken after training. The minimum increase in salivary amylase activity was shown in sample 8 (151 %) and the highest increase in sample 7 (699 %). Candidates 1, 2, 3, 4, 9, 10, 8 were trained regularly and their salivary amylase activity increase was quite lower than that of untrained candidates. According to the literature, there is no data available on the response difference in “basal” salivary amylase activity and “stress” salivary amylase activity between trained and untrained persons\(^5\). There is a good prospective to continue the research on that issue.

Table 2 – Results of salivary amylase concentration in saliva before and after exercise

<table>
<thead>
<tr>
<th>Series</th>
<th>Before exercise (baseline)</th>
<th>After exercise (stress)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg mL(^{-1})</td>
<td>units</td>
<td>μg mL(^{-1})</td>
</tr>
<tr>
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<td>4.51</td>
<td>0.162</td>
<td>8.89</td>
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<td>12.22</td>
<td>0.440</td>
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<tr>
<td>3</td>
<td>12.99</td>
<td>0.468</td>
<td>29.96</td>
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<tr>
<td>4</td>
<td>27.45</td>
<td>0.988</td>
<td>62.84</td>
</tr>
<tr>
<td>5</td>
<td>22.88</td>
<td>0.824</td>
<td>91.53</td>
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<td>11.70</td>
<td>0.421</td>
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<tr>
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<td>10</td>
<td>25.22</td>
<td>0.908</td>
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</table>

Influence of psychical (mental) activity

The influence of psychical (mental) activity on the salivary amylase concentration was exemplified through the intensive mental effort (student exam). The investigation was carried out on a group of thirteen healthy young people. The salivary amylase activity ranges from 1.73 to 36.56 μg mL\(^{-1}\) for samples taken in relax mode (baseline), and 11.79 to 64.92 μg mL\(^{-1}\) for samples taken before the exam. The minimum increase in salivary amylase activity was shown in candidates 6 and 13 (117 % and 119 %) and the highest increase in candidate 8 (1201 %). Unfortunately, the final exam score was not calculated in the final amylase activity results shown in Table 3.

Table 3 – Results of salivary amylase concentration in saliva before student exam and in relaxation mode (no exam)

<table>
<thead>
<tr>
<th>Series</th>
<th>No exam (baseline)</th>
<th>Before exam (stress)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>units</td>
<td>μg mL(^{-1})</td>
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<tr>
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logical (mental) stressor appears to be more inconsistent, and compared to physical activity, shows higher dispersion in salivary amylase activity increase (Table 3). It is not so simple to define the reason; it is a matter for discussion and further investigation with controlled experimental design.

Conclusion

Salivary amylase activity was determined using direct potentiometric method with redox sensor and starch-iodide complex; and corresponding adapted model in real samples (candidates). Corresponding salivary amylase activity is in relation to human stress and was confirmed by the results for two groups of experiments: a) Physical stress (medium intensity exercise) resulted in relatively high salivary amylase activity increase (up to 699 %) with potential “trained level” dependence; and b) Psychological (mental) stress with dispersed salivary amylase activity increase values (ranging from 117 to 1201 %), which makes it more inconsistent and requires more objective and well-defined experimental groups and environment.

The proposed methodology offers a fast and inexpensive way to determine salivary amylase activity and stress levels in humans. The method has potential in checking body trained levels in sports.

References


