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Direct Potentiometric Determination of Ptyalin in Saliva

Nikola Sakač[1], Lidija Regušić[1], Milan Sak-Bosnar[1], Marija Jozanović[1], Nevenka Breslauer[2]*

[1]Department of Chemistry, Josip Juraj Strossmayer University of Osijek, F. Kuhača 20, HR-31000 Osijek, Croatia
[2] Polytechnic of Međimurje in Čakovec, Bana Josipa Jelačića 22a, HR-40000 Čakovec. Croatia
[*]E-mail: nsakac@kemija.unios.hr

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The application of platinum redox electrode for potentiometric determination of salivary α-amylase (ptyalin) is described. Ptyalin, an enzyme present in human saliva, catalyses the hydrolysis of starch. The nonhydrolysed starch, which amount is inversely proportional to the ptyalin concentration, forms a complex with triiodide reducing accordingly initial triiodide concentration. The change of triiodide ion concentration causes the change of the triiodide/iodide redox-couple ratio, which results with the changes of the electrode potential of a redox-sensor. The changes are directly correlated with ptyalin concentration. The experimental data were compared with an appropriate theoretical model in which the sensor and analyte properties were optimized with Solver using the least-squares criterion to fit a theoretical curve to the experimental data set. The influence of physical activity on the ptyalin level in saliva was also investigated. It was found that ptyalin concentration in the rowers saliva strongly increases after their exposing to stronger physical activity (training), even by 2,6 to 7 times in comparison with ptyaline level before training. The influence of alcohol consumption to the ptyalin level in saliva has opposite effect, i.e. after alcohol consumption the ptyalin activity/concentration dramatically desreases by 9,5 - 24,9 times compared to its activity before alcohol consumption.

Keywords: ptyalin, saliva, direct potentiometry, stress

1. INTRODUCTION

Ptyalin is one of the principal salivary proteins. It is a salivary isoform of α-amylase present in the digestive systems of humans and many other mammals. This calcium-containing metalloenzyme degrades starch by hydrolyzing its linear α-1, 4-glycosidic linkages, producing successively smaller polysaccharides known as dextrins and ultimately maltose [1]. Ptyalin is synthesized and secreted by acinar cells of the salivary glands in to the oral cavity, where it serves a role of enzymatic digestion of carbohydrates, but it is also important for mucosal immunity in the oral cavity, as it inhibits the

adherence and growth of bacteria [2]. Elevated levels of the enzyme are associated with oral and dental diseases [3], hence ptyalin determination has been recognized as an important diagnostic tool for many years. Since ptyalin secretion [4] is regulated by the sympathetic nervous system which stimulates acinar cells of the salivary glands via beta-adrenergic receptors, ptyalin activity varies under different conditions [5].

There have been numerous reports on impact of physical exercise on ptyalin activity. All have found a significantly higher concentration of ptyalin during intense exercise in comparison to a control period [6,7,8]. In order to examine the correlation between physical and ptyalin activity, a saliva samples from a group of a healthy young adult male that took a pedal training of 45 min duration were collected. The physical exercise was expected to cause an increase in ptyalin activity of their saliva [6].

Several studies [9,10] have reported significant decrease in ptyalin activity after alcohol consumption. In order to estimate the effect of acute alcohol consumption on ptyalin activity, the ptyalin activity was measured in saliva samples of healthy nonalcoholic volunteers, taken after alcohol consumption.

The influence of cigarette smoke inhalation on ptyalin activity was investigated by several authors who documented lower ptyalin activity in habitual smokers [11, 12, 13]. This can be explained by direct noxious effects of tobacco smoke on salivary proteins including ptyalin. Specifically, aldehydes, which are a major component of tobacco smoke, react with and modify sulphydryl groups of salivary enzymes, leading to functional impairment [14,15]. Ptyalin activities within the group of habitual smokers were measured.

Ptyalin activity has been employed as a marker for schizophrenia, since several researches indicate that autonomic nervous system (ANS) dysfunctions in patients with schizophrenia [16].

There are several different assay methods available for determination of α-amylase activity. Different industries tend to rely on different methods, including spectrophotometry [17], fluorometry [18], amperometry [19], electrophoresis [20], isoelectric focusing [21], chromatography [22] and immunological methods [23], also automated [24]. All of them regard mainly on four common types of assays: (1) assays based on measuring the amount of reducing sugars by the dinitrosalicylic acid (DNS) assay [25] or the Nelson–Somogyi [26] method; (2) assays based on the decreased staining value of blue starch–iodine complexes, which was developed by Fuwa [27] and based on color development that results from iodine binding to starch polymers; (3) a colorimetric method in which starch is covalently labeled with various dyes [28] and assays based on the decrease in viscosity of starch solutions during enzymatic reaction [29].

Unfortunatly, up to know the international standard measurement method has not been established on activity of human ptyaline. In this work, a direct potentiometric method for the determination of ptyalin activity in saliva was proposed. The analytical methodology is based on the direct potentiometric determination of free triiodide ion released from starch-triiodide complex after its biocatalytic degradation by ptialyn. The quantity of released triiodide ions is directly correlated with ptyalin activity.

2. MATERIALS AND METHODS (EXPERIMENTAL)

2.1. Reagents and solutions

Soluble starch was purchased from Kemika (Croatia). Iodine (I_2), purchased from Kemika (Croatia), and potassium iodide (KI), purchased from Sigma–Aldrich (SAD), were used to prepare the potassium triiodide solution. Glacial acetic acid (CH_3COOH) was purchased from Panreac (Spain), sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) was purchased from J. T. Baker (Holland), calcium chloride ($CaCl_2$) was purchased from Kemika (Croatia) and sodium chloride (NaCl) was purchased from Sigma–Aldrich (SAD). α-Amylase (EC 3.2.1.1) from *Aspergillus oryzae* (its activity was 36 U/mg) was purchased from Fluka (Switzerland) and was used as the standard amylase reagent without purification.

2.1.1. Starch solution preparation

The initial 5 g/L starch solution was prepared by dissolving 5 g of dried soluble starch in 0.1 M acetate buffer solution (pH 6.0) in a closed 100 mL volumetric flask. After heating and stirring the solution for approximately ten minutes, starch was completely dissolved. After cooling to room temperature the volumetric flask was filled with deionised water to the mark. A new starch solution was prepared daily to avoid microbial degradation and retrogradation.

2.1.2. Preparation of α-amylase solution

A standard α-amylase solution was prepared and standardised as described elsewhere [30].

2.1.3. Preparation of saliva sample solution

Saliva 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 conditioning solution. The resulting solution was stirred without heating until the saliva was completely dissolved.

2.1.4. Preparation of conditioning solution (CS)

A conditioning solution was prepared by dissolving solid salts to provide final concentrations of 6 mM $CaCl_2$ and 20 mM NaCl in 500 mL of a 0.1 M acetate buffer solution (pH 6.0). The addition of $CaCl_2$ and NaCl to the saliva solution serves to stabilise enzyme activity.

2.1.5. Preparation of acetic acid triiodide solution

Potassium triiodide solution was prepared by dissolving solid iodine (c = 100 μM) in potassium iodide solution (c = 0.05 M). Glacial acetic acid was added to this solution and diluted with deionized

water until the final concentration reached 1M. This acetic acid–triiodide solution (ATIS) was used for the inhibition of ptyalin activity and the reaction of triiodide with the nonhydrolyzed starch.

2.2. Equipment

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) 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). The system was equipped with an IJ64 platinum redox electrode (Ionode, Australia) and an Ag/AgCl reference electrode (Metrohm, Switzerland).

2.3. Procedures

2.3.1. Measurement of the ptyalin concentration/activity of the saliva samples by direct potentiometry

A series of test tubes with the same volume of starch solution were submerged in a temperature-controlled water bath. 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 min of heating at 45 °C, the reaction was terminated by adding 5 mL of ATIS. After allowing the mixture to cool to room temperature, the redox potential was measured using the platinum redox electrode system described above. The calibration procedure was carried out analogously by using a series of standard solutions of α-amylase (2.1.2.).

2.3.2. Saliva sampling

We fabricated a saliva-collecting device in order to collect the saliva by capillary action automatically. Device includes a glass bottle, a plastic tube and a cotton roll. To the tip of the tube, medical cotton was wrapped in a paraffin film. This cotton was expected to act as a filter of contaminants in saliva, making therefore saliva pretreatment unnecessary, whilst also preventing the surfactant in the tube flowing backward into the mouth. The saliva-collecting device was placed under the tongue, and the saliva was led into a glass bottle by a length of polyethylene tubing. Saliva samples were refrigerated within 2 h of collection and stored in the refrigerator at -20 °C until analysis.

The candidates enrolled in this study were young healthy adults, males, age range from 20-25 years. We considered only male population, since there is a difference in amylase activity between genders. Usually, men show higher salivary α-amylase levels than women do at baseline and the time

lag between the occurrence of a mental stimulation salivary ptyaline is one to three minutes, and the recovery of normal salivary α-amylase levels after stress reduction is very rapid: the duration of ptyaline elevation is only about 10 min [31, 32] which makes this sampling method suitable.

Since the influence of physical activity and alcohol consumption were studied, participants were divided into groups based on type of interest. One sample per member of the group was collected. Only within the group where the effect of physical activity was investigated saliva was collected in each subject before and after paddle race of 45 min duration.

2.3.3. Optimization using Solver

Solver is a spreadsheet optimization modeling system incorporated into Microsoft Excel (licenced version *MS Office 2003* was used) that can be used for solving of 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 m_{pty} (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 amylase 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 $1x10^{-4}$), 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 calculation 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. 7 (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).

3. RESULTS AND DISCUSSION

3.1. Response mechanisms of platinum redox sensor toward ptyalin

Starch forms a violet-blue complex with the triiodide ion which color depends on the nature of the starch [1].

The formation of starch-triiodide complexes can be described by the following equation:

$\left(C_{I_3^-}\right)_0 + \text{Starch} \rightleftharpoons \left[\left(C_{I_3^-}\right) \times \text{Starch}\right] + \left(C_{I_3^-}\right)_f$ (1), where

$\left(C_{I_3^-}\right)_0$ = initial triiodide concentration, and

$\left(C_{I_3^-}\right)_f$ = free, (unbound) triodide concentration.

Potassium triiodide was obtained by dissolution of iodine in potassium iodide solution:

$$I_2 + I^- \rightleftharpoons I_3^- \quad (2).$$

A large excess of iodide should be present in order to maintain constant triiodide concentration due to the low value of the formation constant (K_f = 710) of the reaction (2).

$$E = E^0 + \frac{RT}{2F} \ln \frac{\left(C_{I_3^-}\right)}{\left(C_{I^-}\right)^3} = E^0 + S \log \frac{\left(C_{I_3^-}\right)}{\left(C_{I^-}\right)^3} \quad (3), \text{ where}$$

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

After transformation of equation (3), the following expression was obtained:

$$E = Const + S \log\left(C_{I_3^-}\right) \quad (4), \text{ where}$$

$$Const = E^0 - S \log\left(C_{I^-}\right)^3 = E^0 - 3S \log\left(C_{I^-}\right) \quad (5).$$

Ptyalin catalyzes the hydrolysis of starch from a starch-triiodide complex and releases the triiodide ion. The increase of the triiodide ion concentration increases the triiodide/iodide redox couple ratio resulting with the increase of the electrode potential of the redox sensor according to the Nernst equation (Eq. 3 and 4). Thus, the increase of the electrode potential relates to the ptyalin concentration/activity.

The electrode potential before ptyalin addition (at fixed starch concentration) was defined as:

$$E = Const + S \log\left(C_{I_3^-}\right)_0 \quad (6)$$

where

E = electrode potential (mV),

S = slope of the sensor (mV/decade of activity), and

$\left(C_{I_3^-}\right)_0$ = initial triiodide concentration (mol/L).

After addition of ptyalin at a concentration C_{pty}, the following expression was obtained:

$$E = Const + S \log\left(C_{I_3^-}\right)_f \quad (7), \text{ where}$$

$$\left(C_{I_3^-}\right)_f = \left(C_{I_3^-}\right)_0 + \left(C_{I_3^-}\right)_l \quad (8).$$

After insertion of Eq. (8) in (7), the following expression was obtained:

$$E = Const + S \log\left[\left(C_{I_3^-}\right)_0 + \left(C_{I_3^-}\right)_l\right] \quad (9), \text{ where}$$

$\left(C_{I_3^-}\right)_l$ = increase of triiodide concentration (mol/L).

Ptyalin hydrolyses the starch and releases triiodide which increase of concentration is proportional to the ptyalin quantity added:

$$\left(C_{I_3^-}\right)_l = k \times m_{pty} \quad (10), \text{ where}$$

k = proportionality factor
m_{pty} = ptyalin quantity (μg).

By inserting of Eq. (10) in (8) the following expression was obtained:

$$\left(C_{I_3^-}\right)_f = \left(C_{I_3^-}\right)_0 + k \times m_{pty} \quad (11).$$

The ptyalin quantity can be calculated from Eq. (11):

$$m_{pty} = \frac{\left(C_{I_3^-}\right)_f - \left(C_{I_3^-}\right)_0}{k} \quad (12).$$

3.2. Determination of ptyalin concentration/activity in saliva samples

The responses of the platinum redox sensor to different concentrations of α-amylase are presented in Fig. 1.

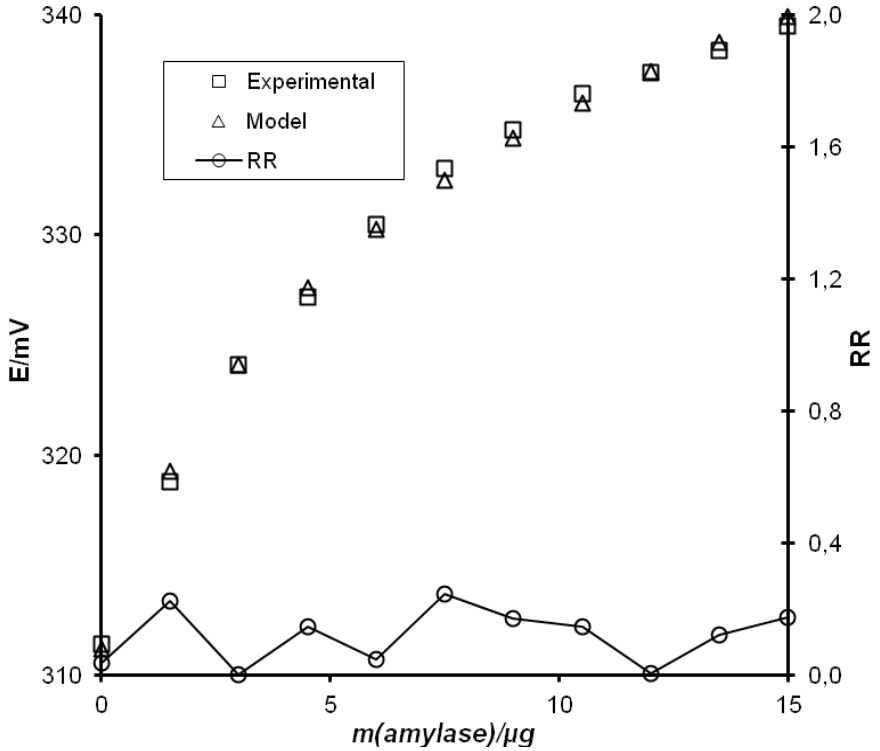

Figure 1. Comparison of experimental (□) and modeled (Δ) response characteristics of a platinum redox sensor toward α-amylase (triiodide concentration 100 μM, iodide concentration 0.05 M, starch concentration 5 mg/mL). The values of the squared residuals RR (○) are presented on the secondary Y-axis.

The experimental data (Fig. 1) were compared with an appropriate theoretical model (Eq. 7) in which the sensor and analyte properties (sensor slope *S*, constant potential term *Const*, proportionality factor *k*) were optimized with Solver using the least-squares criterion to fit a theoretical curve to the experimental data set. Solver was used for determination of the variables that minimize the sum of the squares of the differences between the theoretical and experimental curve.

The values of those parameters and the corresponding regression statistics obtained by macro SolvStat are given in Table 1.

Table 1. Sensor and analyte parameters obtained by modeling potentiometric data after the optimization procedure using Solver and the corresponding regression statistics obtained by SolvStat.

Slope* / (mV/decade)	Const* / mV	Log(k)*	Correlation coefficient R^2	SE(y)
30.77 ± 3.00	435.4 ± 12.4	-3.399 ± 0.105	0.9934	0.800

* values ± standard deviation, SE(y) = standard error

The theoretical model satisfactorily fit the experimental values for the α-amylase concentrations investigated. The value of the slope obtained by modeling is in satisfactory accordance with the theoretical Nernstian value from Eq. 3.

3.2.1. Influence of physical activity to the ptyalin level in saliva

The influence of physical activity on the ptyalin level in saliva was exemplified through the rowing. Rowing is considered as one of the most demanding sporting discipline.

The investigation was carried out on a group of five healthy young rowers aged between 14 and 18.

The each examinee donated two saliva samples, the one before exposure to 45 minute training and another after this training. The ptyalin concentration in saliva was measured before and after the training. The typical potentiometric responses of a platinum redox sensor in saliva before and after the training are shown in Figure 2.

It can be seen from Figure 2 that redox potential significantly increases due to increase of free triiodide ions after exposing of the rowers to stronger physical activity (training). The triodide ions are released after degradation of starch-triodide complex caused by increasing ptyalin concentration. The results of determinations are given in Table 2.

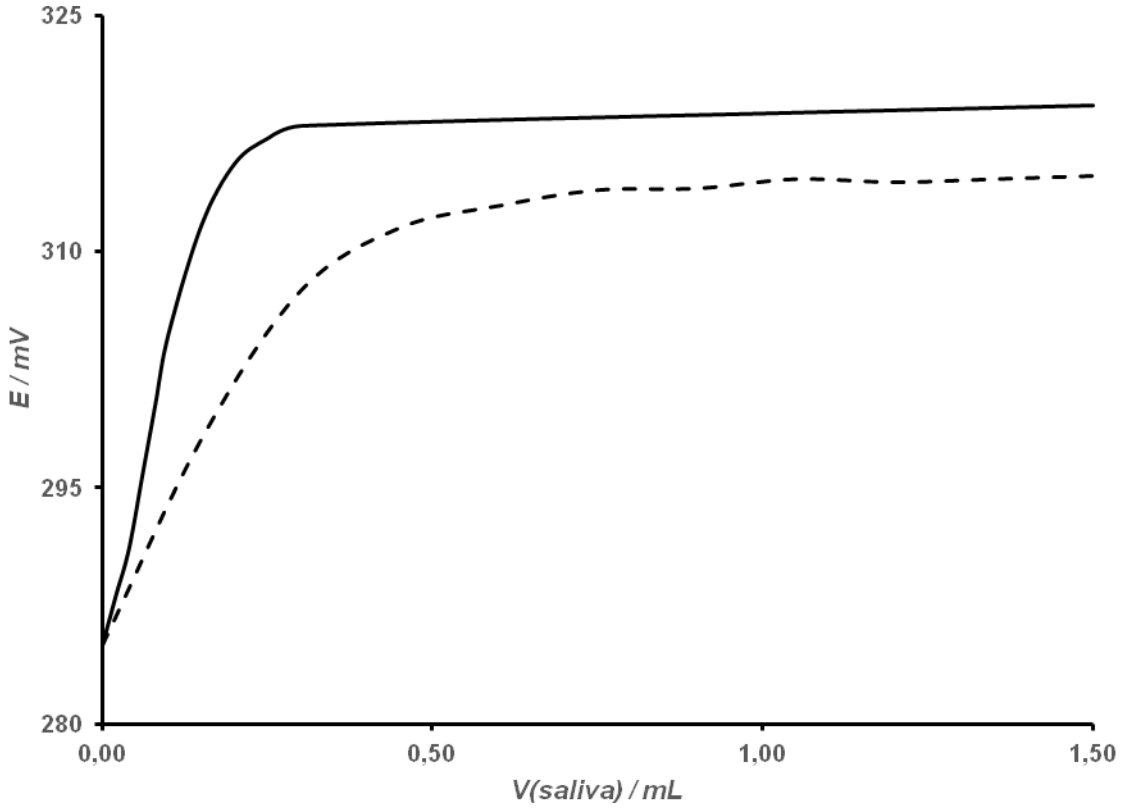

Figure 2. The typical responses of a platinum redox sensor in the rower saliva samples before (dashed line) and after (full line) the training.

It can be concluded that increase of redox potential amounted approx. 30 mV by all examinees, resulting with increase of ptyalin concentration from 264 - 703 %.

Presented results are expected, according to the results on impact of physical exercise on ptyalin activity from the literature [6,7,8].

Table 2. The results of ptyalin concentration in rower saliva before and after training

Series	Ptyalin concentration				Increase (%)
	Before training		After training		
	µg/mL	Units	µg/mL	Units	
1	16,44	0,410	115,55	4,160	703
2	22,53	0,811	45,38	1,634	201
3	15,12	0,544	81,32	2,927	538
4	22,69	0,817	59,24	2,133	261
5	34,75	1,251	91,91	3,309	264

The responses of the platinum redox sensor to different concentrations of ptyalin obtained for the 3 series of measurements in the same rower saliva sample before training are presented in Fig. 3. It can be seen that the obtained results demonstrated good reproducibility.

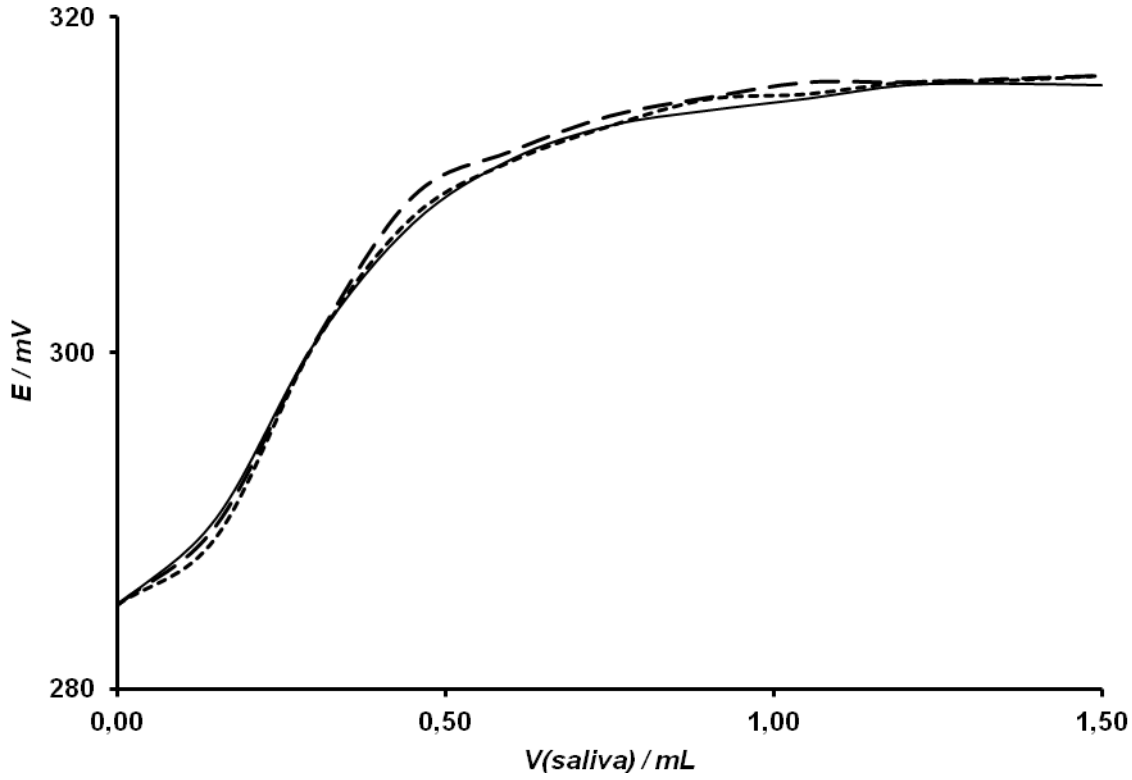

Figure 3. The responses of a platinum redox sensor for the 3 series of measurements in the same rower saliva sample before the training.

3.2.2. Influence of alcohol consumption to the ptyalin level in saliva

A group of 5 examinees was subjected to testing of the influence of alcohol consumption on the ptyalin activity in saliva. The ptyalin level of the all persons was first determined before alcohol consumption. After consumption of three shots of strong drink, the saliva of each examinee was collected and its ptyalin activity determined.

It was notified that saliva collection procedure lasted around three times longer after alcohol consumption than before testing. The typical potentiometric responses of a platinum redox sensor in saliva before and after the alcohol consumption are shown in Figure 4.

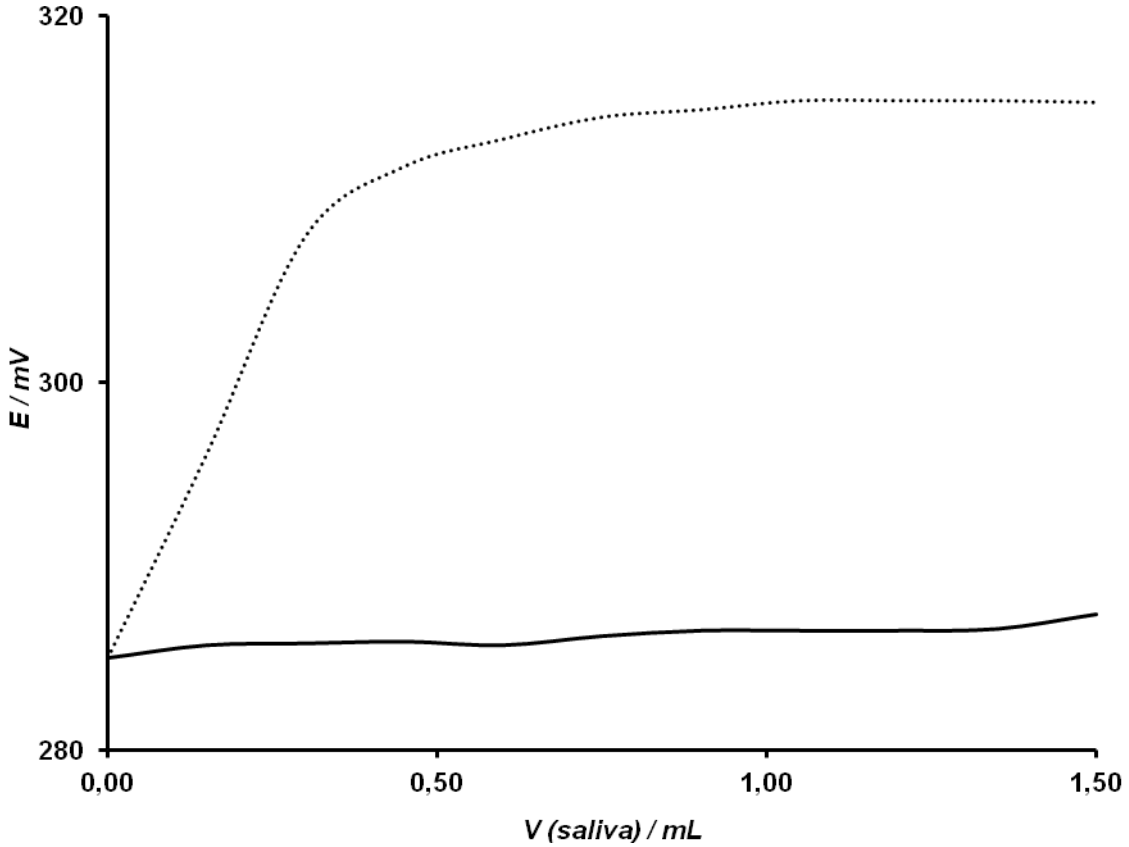

Figure 4. The typical responses of a platinum redox sensor in the saliva samples before (dotted line) and after (full line) alcohol consumption.

As it can be seen from Figure 4, the redox potential in saliva significantly decreases after the alcohol consumption as a result of decrease of free triiodide ions in saliva released after biocatalytic degradation of starch-triodide complex. On the other hand, the decrease of free triiodide ions is a consequence of reduced ptyalin activity. The results of these determinations are given in Table 3.

Table 3. The results of ptyalin concentration in saliva before and after alcohol consumption

Series	Ptyalin concentration				Decrease (%)
	Before consumption		After consumption		
	µg/mL	Units	µg/mL	Units	
1	16,20	0,583	0,650	0,023	96,0
2	20,63	1,634	1,175	0,042	94,3
3	16,31	0,587	1,494	0,054	90,8
4	27,44	0,988	2,896	0,104	89,4
5	29,91	1,077	3,007	0,108	89,9

It can be concluded that decrease of ptyalin concentration by the examinees amounted from 89,4 and 96,0 %, i.e. the ptyalin activity was reduced by 9.5 - 24,9 times.

Presented results are expected, according reported significant decrease in ptyalin activity after alcohol consumption [9, 10]. The mechanism of alcohol influence in ptyalin activity is still uncertain, Maier et all. [33] suggest fat accumulation in the acinar cells of the parotid gland could be the reason, other [34] suggest alcohol induced changes in autonomic innervation of salivary glands would be the explanation. During alcohol consumption sampling, saliva secretion time was extended compared to other sampling time in this paper.

4. CONCLUSIONS

In this work, a direct potentiometric method for the determination of ptyalin activity in saliva was proposed. The ptyalin concentration/activity was calculated from the quantity of triiodide ions released after degradation of added starch-triiodide complex caused by ptyalin present in the saliva samples investigated. A platinum redox electrode was used as a sensor to detect changes in triiodide concentration, which are directly correlated with ptyalin activity.

The use of the electrochemical sensor removes the limitations inherent in titrimetric starch-iodine methods in which the end-point is visually detected. The proposed method requires less work and time than the saccharogenic methods, the procedure is simple, efficient, reliable and rapid. The influence of physical activity as well as of alcohol consumption on the ptyalin level in saliva was investigated.

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