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Flow injection spectrophotometry determination of salivary α-amylase for stress evaluation

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Abstract— **Salivary α-amylase is a hot research area of stress; many studies have confirmed that the substance can be used as the marker of stress-related autonomic nervous system. This paper presents a fast and simple method for determination of salivary α-amylase by flow injection spectrophotometry. The cycle of a sample using this method is only 58s; this method can be automated operation with a high precision, which can avoid big differences of determination caused by improper manual control on the operation time. The established method was successfully applied to detection of salivary samples from an exercise stress test. The stress process was better monitored by this method with good reproducibility.**

Keywords- Flow injection; Salivary α-amylase; Stress

I. INTRODUCTION

Salivary α-amylase (SAA) is one of the main protein components of saliva. Studies have generally concerned about the function of enzyme digestion of carbohydrates, as well as the role of inhibition of bacterial growth [1]. However, many observations in stress filed makes SAA quickly become a hot research in recent years. SAA is directly secreted by salivary gland cell controlled by the sympathetic nervous system (SNS). When the SNS is stimulated, the secretion of SAA could be rapid increased from 1 to a few minutes. Since 1996, Chatterton [2] research group reported in an experimental writing exercise found that SAA and norepinephrine were in response to exercise, there was a significant positive correlation, which suggests the start of the SAA can be used as an activity marker of sympathetic nervous system. In recent years, several experiments have confirmed that SAA can be regarded as an activation sign of sympathetic nervous system, responding to psychological stress and physical stress [3-4].

Currently available methods for determination of amylase have been compiled as many as 200 species [5]. These methods can be categorized into two types: natural starch composition of the substrate and clear molecular structure of the substrate. Although the iodine-starch colorimetry is difficult to standardize due to the substrate, and it is not considered as an ideal method caused by not a zero order reaction and other shortcomings, however, this method has been used widely in the developing countries because of simple, rapid, sensitive and inexpensive. Another kind of methods of clear molecular structure of the substrate, maltopetaose and maltotetraose as the representative, are more or less defective (interference by endogenous sugar and poor stability of the substrate), which has also not been accepted by IFCC.

Flow injection analysis (FIA) has been established as a key tool to obtain data with real time and effectively. Several techniques combined with FIA have been proposed for the assay of a-amylase [6–9]. However, there is no report of testing the activity of SAA by the flow injection combined with the iodine-starch method. Therefore, this study still chooses present widely used simple iodine-starch colorimetry to further research, with the existing flow injection equipment to achieve automation, which can avoid big differences of determination caused by improper manual control on the operation time. Moreover, this study can achieve rapid detection with good reproducibility and quantitatively evaluate vital reactions to stress.

II. MATERIALS AND METHODS

CMFIA-1 multi-functional computerized flow injection analytical instrument (Shandong dian xun qi chang Co., Ltd.), constant temperature water bath (self assembly), TGL-16C centrifuge (Shanghai Anting Scientific Instrument Factory).

A -amylase standard (purchased from Beijing Soledad Technology Co., Ltd., the enzyme activity 3700 U/g); 10 mg/mL of stock solution of amylase was prepared by dissolving appropriate amount of enzyme in water, and working solutions of 0, 5, 12.5, 20 and 50 μg/mL were prepared daily by an appropriate dilution of the stock solutions with water. Soluble starch (AR, Sinopharm Chemical Reagent Co., Ltd.), the starch solution was prepared by dissolving 0.8 g of dry starch in 1000 ml of 6.89 phosphate buffer (pH=6.89) and gently boiling for 1 min. Iodine solution: potassium iodide, iodine (AR, Shanghai Shenbo Chemical Industry Co., Ltd.), 50 mM of the iodine solution was prepared by accurately weighing 6.5g iodine and 17.5g potassium iodide into 1000ml of water. Doubly distilled water was used throughout.

Flow injection spectrophotometry device and the manifold were shown in Fig. 1. The set of experimental parameters and data processing was completed by the operation software of CMFIA-1 multi-functional computerized flow injection analytical instrument. Starch

solution and sample (water blank, diluted saliva samples or α -amylase solution) through the injection valve were brought to the reaction coil by the carrier water. The mixed solution in reaction coil reacted for 20s at 37 ℃ water bath and continued to be brought to the second confluence point to react with the iodine solution. Finally, the reaction mixture was brought to the detector, measured the absorbance at 660 nm. Absorbance value of A_0 is obtained when the sample is replaced by water, while the absorbance value of A is obtained when the sample flows through the pipeline and reacts with starch solution. ΔA (ΔA= A_0 -A) could be used to construct the calibration curve.

Saliva samples were acquired by spitting directly into the tube after mouth rinsing and gargling with plain water. About 100 μL of saliva could meet the testing requirements. Samples were centrifuged at 2000 r/min for 10 min to obtain clear saliva, 10 μL of saliva supernatant was precise amounted and diluted to 10 mL, which is testing solution. All studies with samples from human were approved by the Institutional Review Board of the Southeast University.

III. RESULTS AND DISCUSSION

The iodine-starch colorimetry experiments have been reported in more studies [10-11], the experimental conditions are ripe, and so we conducted the pre-test according to the reagent concentration reported in the literature at the beginning of the experiment. The starch solution was found in the 0.8 g/L concentration of the most appropriate value of around 1.0 absorbance. Blank values cannot be too high or too low; otherwise it will over-range or lead to a small linear range. So 0.8 g/L starch solution was the final choice as the working concentration. Iodine solution at 50 mM reported in the literature proven feasible by the experiment. Additionally, because amylase enzymatic reaction is affected by temperature, it is needed to control the reaction temperature. 37 ℃ reported in the literature is still chosen as the incubation temperature, and self assembly constant temperature water bath was used to control the reaction temperature.

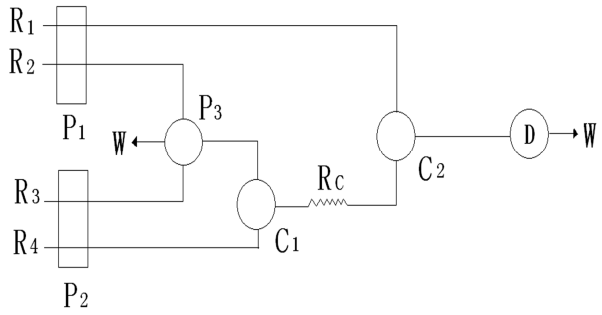

Figure 1. Schematic diagram of the flow system for determination of salivary α-amylase
P_1, P_2 (Peristaltic pump); P_3 (multi-channel injection valve); D (detector); C_1, C_2 (three-way valve); Rc (reaction coil); W (waste); R_1 (iodine solution); R_2 (starch solution); R_3 (sample); R_4 (water).

As enzymatic reaction requires an incubation time, it is necessary to make a choice about reaction time in the flow injection system. Because the starch solution flowing through the pipeline is limited, the incubation time of enzymatic reaction cannot refer to conventional reaction time. Enzyme standard solution was incubated with starch solution at 5, 10, 20, 30, 40 and 60 s to observe the absorbance values of remaining starch. Fig. 2 showed that no significant reaction was found when the incubation time was too short; while few residual starch existed if reaction time becoming long , which would influence the linear range. Therefore, 20 s was selected as the best choice of the retention time after full consideration.

In addition, the choice of flow rate and stopping flow time of the peristaltic pump is also critical; the experiments were optimized to obtain a set of experimental parameters in Table 1.

A series of experiments were employed for achieving the parameters of analytical performance. The results showed that when the concentration of α-amylase was in the range of 0 -50 μg/mL; a good linear relationship was found. The linear regression equations for the calibration plot for the α-amylase: ΔA = 0.02C-0.026 (r = 0.9990).

The reproducibility of the presented method was assessed by analyzing six replicates of water spiked with analyte at three different concentrations (5, 10, 40 μg/mL), the average coefficients of variation of within-day and between-day assays were respectively 1.2% and 2.8%.

Recoveries were determined as percentages of the measured concentrations (after subtraction of endogenous concentration) against the spiked concentrations for salivary samples spiked at 5, 10, and 40μg/mL (n = 3 for each). The relative recoveries of 97.8% to 105.9% were obtained respectively for all of the spiked saliva.

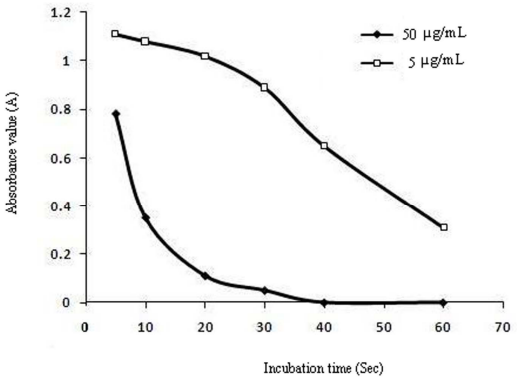

Figure 2. Enzymatic reaction time graph

Table 1. Optimal conditions for flow injection

Experimental parameters	Optimization results
Flow rate / mL· min^{-1}	6.7
Loading time /s	6
stopping time /s	20
Reaction coil length /m	2.5

The concentration of the SAA is obtained by enzyme standard curve in this method. Because the end result of experiments needs to be marked in the form of enzyme activity, the enzyme concentration needs to be converted to enzyme activity units. Since the activity of commercial enzyme would be changed due to environment and the storage conditions. As a result, an enzyme activity unit was defined in this article, and then used for standardization of the activity of α-amylase in saliva samples. The test conditions of enzyme activity: 2 mL of starch solution (1.6 g/L) and 10 μL of enzyme solution (0.1 mg/mL) were incubated at 37 ℃ for 10 min, and then 1 mL of iodine solution (50 mM) was added in, and diluted with water to 25 mL. The absorbance value was tested at 660 nm.

Definition of enzyme activity unit: 1 unit (U) is the amount of α-amylase that catalyses the reaction of 10 mg of starch substrate within 30 minutes in 1mL of solution incubated at 37 ℃.

The formula is:

$$\text{SAA Unit} = \frac{A_0 - A}{A_0} \times \frac{3.2}{10} \times \frac{30}{10} \times \frac{1}{0.01} = \frac{A_0 - A}{A_0} \times 96$$

A_0: absorbance value of water blank; A: absorbance value of sample

IV. EXERCISE STRESS TEST

It was designed running exercise stress to verify whether the process of the stress response could be monitored by this new established method. Three saliva samples of Volunteers (n=3) were collected (10 minutes before exercise, the end of the exercise, 10 minutes after the end). Centrifuged saliva was diluted and detected by the established method, and the result was shown in Fig. 3 (Finally, the results of concentration calculated from the linear equation were then converted to enzyme activity units.).

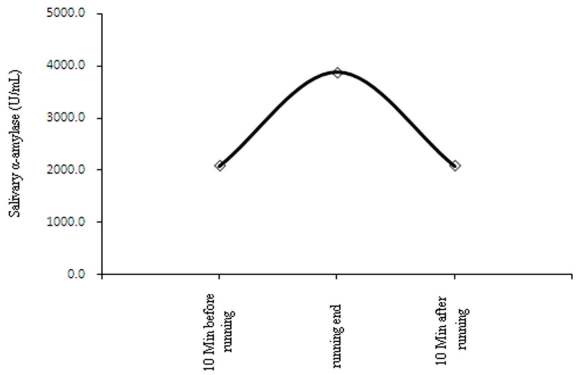

Figure 3. Result of a preliminary experiment on running exercise stress test.

It can be seen from Figure 3 that the activity of salivary α-amylase was in a rapid increase in the stress process, while it rapidly restored to its original level after the end of stress.This report is consistent with the literature [12], which shows that the establishment of the fast, easy and automated flow injection spectrophotometry method could be applied to determination of salivary α-amylase. This experimental study can provide research tools and support for stress researches.

ACKNOWLEDGMENT

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REFERENCES

[1] B.J. Baum, “Principles of saliva secretion,” Ann. NY Acad. Sci. vol. 694, 1993, pp. 17-23.

[2] R.T.J. Chatterton, K.M. Vogelsong, Y.C. Lu, et al. “Salivary alpha-amylase as a measure of endogenous adrenergic activity,” Clin. Physiol. vol .16, 1996, pp. 433–448.

[3] J.A. DECARO, “Methodological Considerations in the Use of Salivary a-Amylase as a Stress Marker in Field Research,” Am. J. Hum. Biol. vol . 20, 2008, pp.617–619.

[4] U.M. Natera, R.L. Marcaa, L. Florina, et al. “Stress-induced changes in human salivary alpha-amylase activity—associations with adrenergic activity,” Psychoneuroendocrino. vol .31, 2006, pp.49–58.

[5] S.M. Geng, Z.R. Bai, J.C.Yuan, “Discussion on difficulty to standardize of Amylase determination method,” China Practical Medicine. vol .4, 2009, pp.206–207.

[6] P.W. Hansen, “Determination of fungal α-amylase by flow injection analysis,” Anal. Chim. Acta vol .158, 1984, pp.375.

[7] R. Schindler, B. Lendl, R. Kellner, “Simultaneous determination of a-amylase and amyloglucosidase activities using flow injection analysis with fourier transform infrared spectroscopic detection and partial least-squares data treatment,”Anal. Chim. Acta vol .366, 1998, pp.35.

[8] J.F. van Staden, L.V. Mulaudzi, “Flow injection spectrophotometric assay of a-amylase activity,” Anal. Chim. Acta vol .421, 2000, pp.19–25.

[9] M. Yamaguchi, M. Kanemaru, T. Kanemori, et al. “Flow-injection-type biosensor system for salivary amylase activity,” Biosensors and Bioelectronics vol .18, 2003, pp.835–840.

[10] N. Sun, Y.S. Zhang, W.H. Zhang, et al. “An improvement and application of starch iodine colorimetry for amylase,” Heilongjiang Medical Journal. vol .10 , 1993, pp.42–43.

[11] J.F. Yuan, “Rapid colorimetric method for determination of amylase,” Medical Journal of Communications. vol .12 , 1998, pp.255.

[12] N. Rohleder, U.M. Nater, J.M. Wolf, et al. “Psychosocial stress-induced activation of salivary alpha amylase: an indicator of sympathetic activity?” Ann. NYAcad. Sci. vol .1032, 2004, pp.258–263.