The Establishment of Flow Injection Analysis Method for Saliva α-Amylase and Its Application in the Psychological Stress

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***Abstract*-This study established a novel non-invasive and efficient analysis method which can be applied in research of individual's psychological stress. Because in the different state of psychological stress, the oral secretion of salivary α-amylase will be changed under the control of the hypothalamus-pituitary-adrenal medulla secretion system. Many studies have confirmed that salivary α-amylase measurements may be employed as a non-invasive measure of autonomic (sympathetic) nervous system activation and are related to a variety of behavioral, social, health, and cognitive phenomena in human subjects. In this experiment, a flow injection system for sample analysis and data collection was established to determine the activation of salivary α-amylase. Because flow injection system has the merits of rapid analysis, high precision and accuracy, easily operating and popularizing, it could meet the requirements of quickly and accurately determining of salivary α-amylase. This study collected salivary samples of individual at different times of a day, and drew an individual's salivary α-amylase secretion curve according to the measured results (one hour for the sampling interval). In addition, after sampling analysis of volunteer's samples before and after the examination, it was verified that individual's salivary α-amylase secretion would be changed under psychological stress, which suggested that salivary α-amylase could be used as a biochemical marker of individual's psychological stress changes. The founded method in this paper is easy to be universalized because of its non-invasive and quick features.**

Key words- Flow Injection; Salivary α-Amylase; Stress

I. INTRODUCTION

The term of change with each passing day can be a good response to today people's living conditions. Compared to the previous two decades, it is not difficult to find that we are in an era of change. As modern human, the accelerating pace of life and increasing pressure from all sides increase the individual's psychological stress. Psychological stress tends to induce a variety of mental illnesses, the busy city life makes many people suffer from mental illness such as depression, mania, fantasy, and some even can not afford their own psychological stress to select a suicide [1]. Therefore, convenient monitoring of individual's psychological stress is necessary.

The research communities have long confirmed that the level of cortisol in saliva is an effective indicator to reflect the body's stress levels [2, 3]; however, salivary α-amylase (SAA) is to be concerned as a sensitive biomarker just in recent years [4-6]. SAA can be regarded as an activation sign of sympathetic nervous system, responding to psychological stress and physical stress [7]. The concentration of cortisol in saliva is too low (the content is about 1–8 ng/ml), and many determination methods require several time-consuming manipulation steps and sophisticated instruments; in contrast, analysis of SAA is convenient (higher concentration) due to the fact that there is no need for sophisticated instruments and tedious separation procedure.

Flow injection analysis (FIA) is an analytical technique appeared in the past 20 years, which has greatly promoted the development of automated analysis and instrumentation by combining with other analytical techniques [8, 9]. Therefore, flow injection analysis has become a new type of micro-, high-speed and automated analytical techniques. Although several flow injection analysis methods have been applied in the detection of SAA, there are few reports of testing the activity of SAA by the flow injection combined with the iodine-starch method [10-13]. There is no ideal method for α-amylase determination to meet the needs proposed by the International Association for Clinical Chemistry (IFCC) [14]. Although the iodine-starch colorimetry is difficult to standardize due to the substrate, and it is not considered as an ideal method because of being not a zero order reaction and other shortcomings, this method has been used widely in the developing countries because of being simple, rapid, sensitive and inexpensive. Therefore, in this paper, a FIA method was developed based on widely used simple iodine-starch colorimetry, which can avoid big differences of determination caused by improper manual controls on the operation time. This study collected saliva of individual at different times of a day, and drew an individual's SAA secretion curve according to the measured results (one hour for the sampling interval). In addition, after sampling analysis of volunteer samples before and after the examination, it is verified that individual's SAA

secretion will be changed under psychological stress, which suggests that SAA can be used as a biochemical marker indicating individual's psychological stress changes. It seems that a simple and an accurate analytical method proposed in this paper can be applied in research of individual's psychological stress, which is easy to be universalized because of its non-invasive and quick features.

II. MATERIALS AND METHODS

All reagents used in this work were of analytical grade, α-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 mmol/l of the iodine solution was prepared by accurately weighing 6.5 g iodine and 17.5 g potassium iodide into 1000 ml of water. Doubly distilled water was used throughout.

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

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 20 seconds 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. The 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.

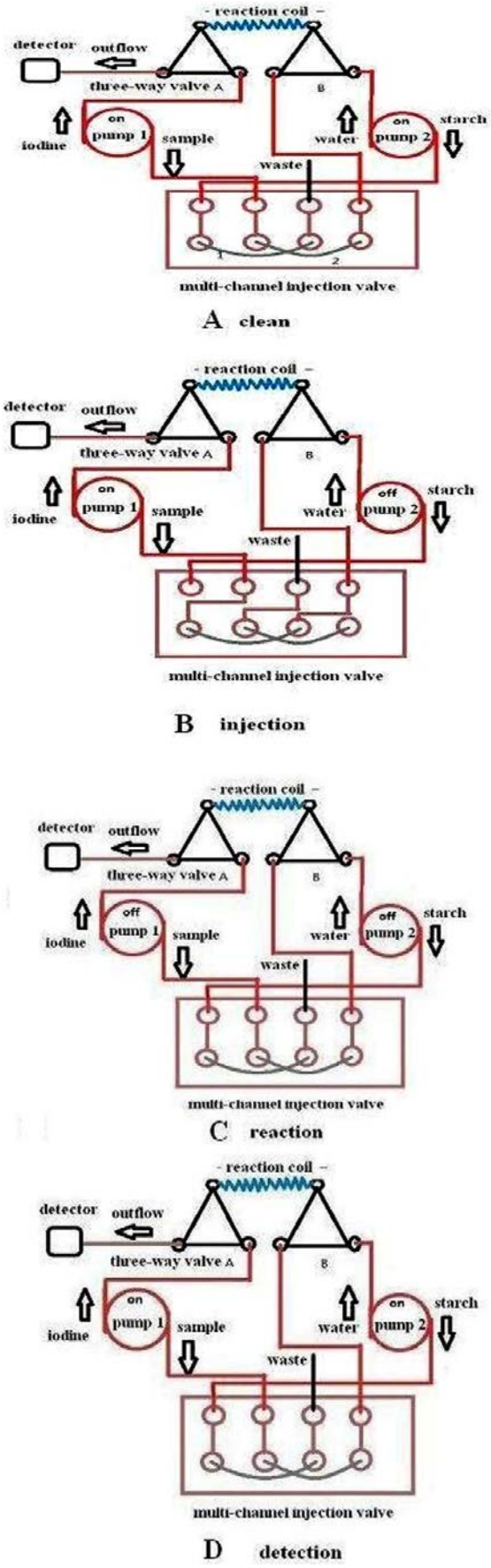

Fig. 1 Schematic diagram of the flow system for determination of salivary α-amylase

III. RESULTS AND DISCUSSION

Fig. 1 showed the schematic diagram of the flow system for determination of SAA, the set of testing procedure including four steps are clean, injection, reaction and detection. (1) Clean. The warm-up operation of the machine before the formal test, the role of clean is to eliminate the residual bubbles in the pipeline in order to ensure higher experimental accuracy. Furthermore, Pipeline 1 can be filled with starch solution during the cleaning operation, and the clean time is generally set to 60 seconds. (2) Injection. The Pump 1 is running (on) and the Pump 2 is not running (off) at this time, the pipeline status as shown in Fig. 1.B. The starch solution can be injected into the reaction tube by the flowing of saliva samples when the multi-channel injection valve turned to the injection location. (3) Reaction. The starch solution is inputted into the waste by the rotation of the multi-channel injection valve, and the quantitative input of starch and saliva samples will be reacting in the reaction tube. The two pump stall to give sufficient reaction time for starch and saliva samples. (4) Detection. The two pumps are rotating (on) after enzymatic reactions. The remaining starch solution after the enzymatic reaction is reacting with iodine at the three-way Valve A, and then the blue reaction solution is transmitted to the detector for the determination of absorbance value.

The flow injection visible absorbance spectrum of iodine - starch resultant is shown in Fig. 2. From the Fig. 2, it can be seen that a test period of SAA is about 70 seconds, and the retention time of the iodine-starch resultant is about 50 seconds. The entire visible absorption spectrum shows peak symmetry, and no other miscellaneous peak interferes.

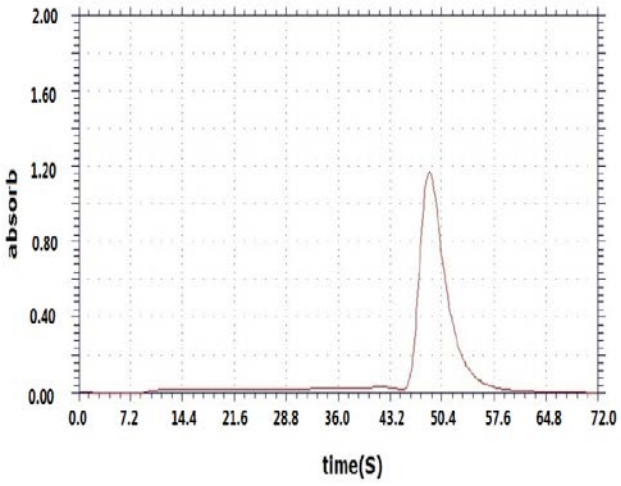

Fig. 2 The flow injection visible absorbance spectrum of iodine-starch resultant

A series of experiments were employed for achieving the parameters of analytical performance. The results showed that a good linear relationship was found when the concentration of α-amylase was in the range of 0-50 μg/ml. The linear regression equations for the calibration plot for the α-amylase: $\Delta A = 0.02C-0.026$ ($r = 0.9990$). 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 40 μg/ml ($n = 3$ for each). The relative recoveries of 97.8% to 105.9% were obtained respectively for all of the spiked saliva.

The concentration of the SAA is obtained by enzyme standard curve in this method. Because the final 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 the 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 mmol/l) 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. APPLICATION IN THE PSYCHOLOGICAL STRESS

In order to verify whether the SAA can be used to monitor the individual's psychological stress, this study designed a simple psychological stress test. The subjects were two students who would take the exam, the SAA contents before and after the exam were detected for analysis of psychological stress changes under the exam stress.

Firstly, we tested the rhythm of SAA secretion to make a preliminary understanding of the difference of SAA in the normal state and stress state. Therefore, this study collected salivary samples of individual at different times of a day, and drew an individual's SAA secretion curve (as shown in Fig. 3) according to the measured results (one hour for the sampling interval). From the SAA secretion curve, it can be seen that the individual secretion of SAA from the two days was similar. Only certain data may be subject to appetite respect and show great differences.

In addition, after sampling analysis of volunteer's samples before and after the exam, it was verified that individual SAA secretion would be changed under psychological stress (as shown in Table 1, the results of concentration calculated from the linear equation were then

converted to enzyme activity units.). The secretion of SAA in two volunteers before the exam was significantly increased and began to decline after the examination, which suggested that SAA could be used as a biochemical marker indicating individual's psychological stress changes. Combined with the score analysis of psychological stress testing (PSTR), the psychological state of two volunteers before the exam is in moderate stress and this state could recover rapidly after the stress. This report shows that the establishment of the fast, easy and automated flow injection spectrophotometry method could be applied to the determination of SAA. This experimental study can provide research tools and support for stress researches.

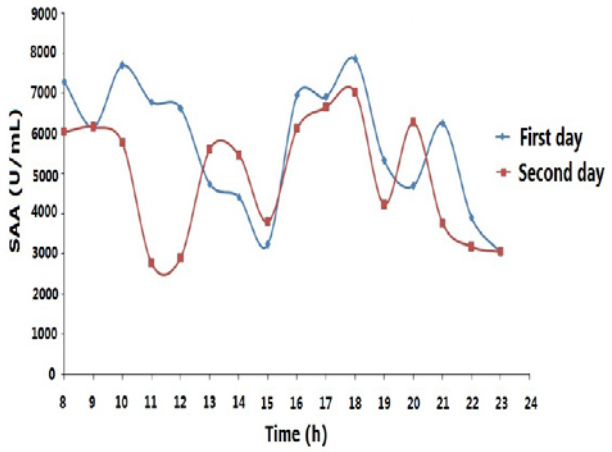

Fig. 3 Individual's saliva α-amylase secretion curve

TABLE I DETERMINATION OF SAA BEFORE AND AFTER THE EXAM

Subjects	Gender	Age	Psychological scores	SAA before exam (U/ml)	SAA after exam (U/ml)
A	Male	24	64	15501	13285
B	Male	23	71	16912	15055

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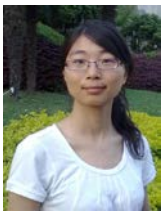

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