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A Flow Injection Assay System for Online Quantification and Calibration of Saliva α-Amylase Activity

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Abstract

Salivary α-amylase (sAA) as a non-invasive marker for sympathetic nervous system (SNS) activity has drawn more attention in recent years. The determination of sAA activity is very useful in the mental health assessment. A completely automated analytical system was developed for sAA activity using a flow injection-spectrophotometric analysis system (FIA)-basing on an enzyme degradation reaction of starch and the color reaction of starch with iodine. Without any pretreatment, the system can monitor the samples sequentially with high precision and accuracy. When the equipment is functioning, the starch and iodine solution were brought to the confluence point from each line and then dispersed toward the detector. Enzymatic reaction started in the starch solution line at the moment of the sample being injected through the injection valve. An online method was established for calibrating standard α-amylase activity.The α-amylase standard and starch solution at a known concentration was mixed and then brought into the line, met with iodine and reacted at the detector. The absorbance of the reaction solution was reduced continuously until the starch degraded completely. The accurate value of α-amylase activity can be calculated by the time periods of the absorbance reducing. A comprehensive study was initiated to optimize FIA step, such as the concentration of starch and iodine, the speed of the peristaltic pump. The optimal analytical performance was achieved including a wide dynamic range of 1607~19284U/ mL, detection limits of 200U/mL and precision (as RSD%) lower than 5% for both intra-day and inter-day assays. This method was applied for the determination of α-amylase activity in human saliva sampled from the subjects at different ages . The result show that the saliva α-amylase activity of volunteers at age of 20 (7557±3799, n=49) is lower (P<0.05) than that of 5 (9656±5782, n=61). der Pauw method which increases (0.85-3.91 S/cm) with increasing sintering temperature. This study opens the potential for the synthesized clay-based ceramic/carbon composite to be developed as carbon-filled filter or be used in electronic applications.

Keywords: α-amylase, Enzymatic reaction curve, Flow injection analysis.

Contribution of study

This study provided a convenient, rapid and precise FIA method for quantification of saliva α-amylase activity which was an important biomarker.

1. Introduction

Saliva α-Amylase (sAA) is a enzymes present in the digestive systems of humans and many other mammals. This calcium-containing metalloenzyme degrades starch by hydrolysing its linearα-1,4-glycosidic linages, producing successively smaller polysaccharides known as dextrins and ultimately maltose (Janeček and Svensson et al.). As sAA inhibits the adherence and growth of bacteria, it is also important for mucosal immunity in the oral cavity (Scannapieco and Torres et al., 1993). Meanwhile, numerous studies have established that sAA serves as a non-invasive and an easily obtained surrogate marker of autonomic nervous system(ANS) activity in adults and children (Nater and Rohleder et al., 2005; Granger and Kivlighan et al., 2007; Nater and Rohleder, 2009).

There are various methods available for determination of sAA activity, such as sepctrophotometry (Foo and Bais, 1998), fluoromety (Sakač and Sak-Bosnar, 2012), chromatography (Battershell and Henry,

1990), immunological methods (Svens and Käpyaho et al., 1989), electrochemistry (Sakac and Regusic et al., 2014) and etc. Most of the methods need diluted saliva samples, and somehow time consuming. Flow injection analysis was a convenient, repeatable and rapidly method, which have been used in the sAA activity analysis by our and other groups (Chen and Kang et al., 2012; Ohtomo and Igarashi et al., 2013). Unfortunately, up to now the standard method for the measurement of sAA activity has not been established.

In this study, a completely automated analytical system was developed for sAA activity using a FIA- spectrophotometric system basing on an enzyme degradation reaction of starch and the color reaction of starch with iodine. Without any pretreatment, the system can monitor the samples sequentially with high precision and accuracy. An online method was also established for calibrating standard α-amylase activity.

2. Experimental

Instruments and reagents. TDL-80-20 centrifuge (Shanghai Anting Scientific Instrument Factory) was used for getting salivary sample from salivettes (Sarstedt,Nümbrecht,Germany). IFIS-D smart flow injection injector (Xianruimai Analytical Instruments co., Ltd.), HH-2 electric-heated thermostatic water bath (Jintan Ronghua Instrument Manufacturing Co., Ltd.) and 723 vis spectrophotometer (Shanghai Jinghua Technology Instruments Co., Ltd.) was all used in the FIA- spectrophotometric system system.

Potassium iodide, iodine (analytical grade) was purchased from Shanghai Shenbo Chemical Industry Co., Ltd. Soluble starch (analytical grade) α-amylase standard (biochemical, 40-80U/mg) was purchased from Sinopharm Chemical Reagent Co., Ltd. The water used throughout the experiments was double distilled.

Solution. Starch solution was prepared by dissolving 1.6 g of dry starch in 1 L distilled water and heated to become transparent. The iodine solution was composed of 40g potassium iodide and 20g iodine in 1 L distilled water, which was sonicated until complete dissolution. α-amylase standard solution was first formulated as a 10 mg/ml and then diluted to 0.25, 0.5, 0.75, 12 mg/ml.

Subjects. With the concurrence of parents of the children and the subjects, 49 college students at age of 20 and 61 pre-schoolers at age of 5 were recruited. Morning saliva were collected using salivettes , and then centrifuged at 3000 rpm for 10min. Samples were directly stored at -20 ℃ before analysis.This research had been approved by the Ethic Committee of Southeast University.

Flow injection system. The hyphenated flow injection- spectrophotometry schematic diagram was shown in fig.1. The system has two functional parts, quantification (fig.1A) and calibration (fig.1B) of sAA. The wavelength of the detector was 660 nm.The tempreture of the heated water bath was set at 37℃.

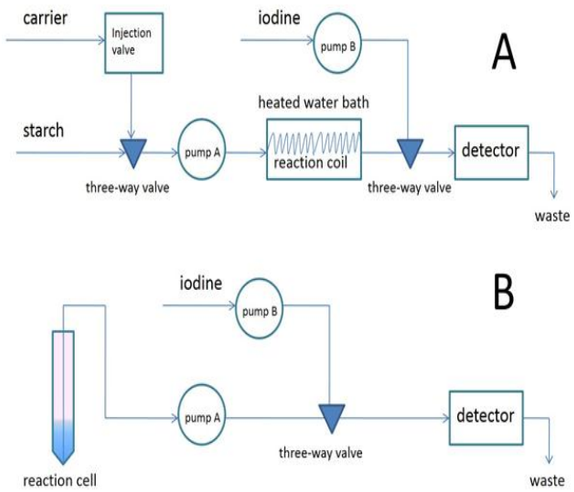

Fig-1. Flow injection analysis system

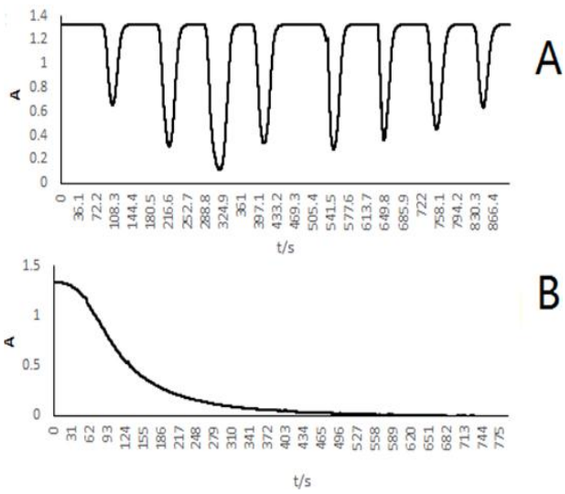

Fig-2. Flow Signals of the FIA

3. Results and Discussion

Procedures. When the quantification part was functioning, the starch and iodine solution were brought to the confluence point from each line and then dispersed toward the detector. Enzymatic reaction started in the starch solution line at the moment of the sample or α-amylase standard solution being injected through the injection valve. The absorbance of the liquid flow was reduced and a downward peak was received on the record softwave (fig.2A). The salivary α-amylase activity was calculated according to the peak area.

Beacause the α-amylase standards always show a activity range instead of accurate value, calibration functional part was designed. In this part, 25mL the starch solution at 1.6 mg/mL and 0.1mL α-amylase standard at 1mg/mL was mixed in the colorimetric tube, and the mixture was brought into the detector after reacted with the iodine solution depend on the FIA. The absorbance of the reaction solution was reduce continuous until the starch was degraded completely (fig.2B). The time of the absorbance reduced can be used for calculating the accurate value of α-amylase activity.

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 the standard solution incubated at 37 ℃. The formula is:

$$\text{sAA Unit} = \frac{30 * m * 1}{t * 10 * V} = \frac{1.2}{t}$$

m: The quality of the consumption of starch; V: the volume of α-amylase standard.

In order to achieve good performance, the two peristaltic pumps speed was considered. When the speed was fast, the time for the enzyme degradation reaction was not long enough. While it would be time consuming and the baseline of FIA was unstable if the speed was slow.15 r/min was set in the softwave for both pumps according to the experiment result and eight samples could be analysed in 15 min in this condition with satisfied absorbed peak.

The concentration of starch was also important because it would be exhausted when the concentration was low, while the sAA with low activity could not form a peak in the detector when the concentration was high. 1.6g/l was chosen because it could meet the needs of the determination for real samples.

Validation of assay: The validity of this method was investigated and optimal analytical performance was achieved including a wide dynamic range of 1607~19284U/ mL. The liner regression equations were y = -0.0005x + 9.1984 (R^2 = 0.9949). The LOD was 200U/mL and precision (as RSD%) lower than 5% for both intra-day and inter-day assays.

Determination of real samples: The real salivary samples which was collected from 110 volunteers were used to examine how this method performed. The result show that the saliva α-amylase activity at age of 20 (7557±3799) is lower ($P<0.05$) than that of 5 (9656±5782). Which was consistent with the previously research (Hill-Soderlund and Holochwost et al., 2015).

4. Conclusions

A completely automated, rapid and accurate method was developed for the quantification and calibration of sAA activity. Experimental results indicated that this method was applicable to real salivary samples.

5. Acknowledgement

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