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Development of sympathetic nervous system activity monitor using salivary amylase activity

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Abstract

In order to understand human sensitivity and emotion, it is considered useful to evaluate the functions of the autonomic nervous system (the sympathetic nervous system and parasympathetic nervous system). In addition, it is understood that the salivary amylase activity reflects the sympathetic nervous system activity based on the results of recent studies. In order to easily monitor the sympathetic nervous system activity in daily life, a dry chemistry type salivary amylase activity analyzer was then developed. By developing a method for quantitatively sampling a small amount (on the microliter volume scale) of saliva, the technology for measuring the salivary amylase activity using colorimetry has been established. Functions to eliminate the effects of the ambient temperature and the pH of the saliva on the measured value are included in this instrument. The measuring range is 10-140kU/L, and the measuring accuracy of R^2=0.99, which exceeds the standard value of R^2 = 0.95 for the portable measuring instrument, have been achieved. This instrument can precisely analyze the salivary amylase activity in about one minute including the time for sampling the saliva.

1 INTRODUCTION

If human emotion and sensitivity can be objectively evaluated, the development of high value-added products, such as a product which is more comfortable and has no sense of incompatibility, and an individual-oriented product, which can change its specification according to the personal ability or favor, becomes possible. A method to evaluate this from a psychological point of view by questionnaires and interviews [1], and a method to evaluate using physiological reactions of the autonomic nervous system, etc. [2], are used as technologies to measure and evaluate the human emotion (**Table 1**).

In addition, it is known that human saliva is secreted from the organ called the salivary gland in the mouth, and that the activity of the salivary glands is dominated mainly by the

Table 1. Characteristics of each measuring method

	Psychological method	Physiological method
Advantage	• Measurement is simple. • Evaluation by a sense, which cannot be measured by a machine, is possible.	• Objective evaluation is obtained. • Reaction in real time can be examined.
Disadvantage	• An objective rating is hardly obtained. • Evaluation of the unconscious stimulation is impossible.	• Large scale measurement is required. • Restraint of the subject is compelled.

sympathetic nervous system. Consequently, it is considered that the secretion of amylase, which is an enzyme abundantly present in saliva, is also similarly dominated by the sympathetic nervous system. Therefore, it is considered that the salivary amylase activity, which indicates the working rate of amylase in saliva, can be used as an index to evaluate the neural activity of the SAM system (Sympathetic nervous-adrenomedullary system) [3-4]. Because saliva can be collected by a noninvasive method and is easy of frequently measure, it is considered that the measurement of the sympathetic nervous system activity using saliva is extremely useful in evaluating the emotion and the sensitivity at a practical level in daily life.

The authors have already worked on the development of a portable-type salivary amylase activity analyzer in order to establish the measuring technology of the sympathetic nervous activity using saliva [5]. The analysis technique of the salivary amylase activity by the dry chemistry method is adopted for this instrument, and miniaturization compared with the conventional liquid-type analysis method has been achieved using a reagent and a clinical automatic analyzer. However, in order to achieve the expected features, such as on-demand character, immediacy, and convenience, at a practical level, it is required to add the functions of quantitatively collecting a small amount (on the microliter volume scale) of samples and controlling the reaction time.

In order to solve these above-mentioned problems, a saliva collection tool and a quantitative transcript mechanism were newly designed in this study, and a portable salivary amylase activity analyzer equipped with these mechanisms was developed. In order to eliminate the effect of the ambient temperature and the pH of the saliva on the measured value of the salivary amylase activity, functions to experimentally obtain their temperature dependence and pH dependence and to automatically calibrate the activity value based on these characteristic values were installed. Moreover, the calibration curve was prepared in order to know the measuring range and the accuracy of the measuring instrument, and the utility as a sympathetic nervous system activity monitor was examined.

2 DEVICE AND METHOD

2.1 Measuring principle

Amylase is a generic name of the enzyme which hydrolyses starch into maltose, and it is a very prominent enzyme in saliva. There are two methods used to quantitatively analyze this enzyme: a method of measuring the number (concentration) and a method of measuring the working rate of the enzyme. In general, the latter is typically used, and the working rate is defined as the enzyme activity. Though the concentration and the

activity are different unit systems, they are in a proportional relationship under the same conditions (temperature, pH, etc.), and are often used as units which indicate the working rate of the enzyme. The amylase activity of 1 unit is defined as the amount of the enzyme that produces a reducing sugar corresponding to 1 μmol of maltose in 1 minute at 37°C [7].

The prototype portable salivary amylase activity analyzer is a dry chemistry-type measuring system consisting of a test paper impregnated with Gal-G2-CNP (2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosylmaltoside), which is the substrate of salivary α-amylase (AMY). Gal-G2-CNP is a clinical reagent for analyzing the amylase activity in blood. When AMY is added to Gal-G2-CNP, CNP, which is a chromogene, separates due to the hydrolysis effect of AMY, and it turns yellow (Equation 1).

$$\text{Gal-G2-CNP} \xrightarrow{\text{AMY}} \text{Gal-G2} + \text{CNP}(\text{White} \rightarrow \text{Yellow}) \qquad (1)$$

Since the decomposing capacity of AMY is proportional to the number of separated CNPs, the salivary amylase activity can be measured by optically measuring the color density of CNP.

For analyzing the amylase in saliva, a conventional reagent for blood cannot be used as received because the concentration of amylase in saliva is several hundred-fold to one thousand-fold compared with blood. Consequently, pretreatment such as diluting the saliva sample is required for analyzing the salivary amylase activity. In this instrument, a reaction system which does not require diluting was developed by mixing an antagonist with the reagent in order to achieve the measurement at a practical level in daily life. That is, oligosaccharides (maltose monohydrate, pentaose), which have the same effect on the substrate, were added, and the effect which was the same as the dilution was achieved by obstructing the reaction of AMY with Gal-G2-CNP using oligosaccharides.

In this reaction, because AMY does not lose its function as an enzyme, it reacts with new substrates one after another. Therefore, a sufficient amount of Gal-G2-CNP for AMY in order to continue the reaction during the measuring time is supplied, and the enzyme activity is measured by measuring the CNP formation per unit of time. The control of reaction time and the amount of the sample is needed to achieve this step. The saliva collection mechanism, which can collect a given quantity of saliva, and the saliva transcript mechanism, which can control the reaction time by a simple operation, were designed, and the determination of the salivary amylase activity by colorimetry was achieved.

2.2 Portable-type salivary amylase activity analyzer

The measurement system consists of a disposable test strip and the main unit of the measuring instrument (110 x 100 x 40 mm, 350 g) (**Figure 1**). The test strip consists of the collection sheet (120 x 10 x 0.25 mm), in which the saliva collection paper (10 x 10 x 0.23 mm) is installed, and the test paper holder (**Figure 2**). The test paper (4 x 4 x 0.25 mm) is affixed to the back side of the test paper holder. The test paper is impregnated with a pH buffer agent besides the substrate so as not to be easily affected by the pH of the saliva, which is the sample. That is, after Gal-G2-CNP, which is the substrate, is dissolved in Good's buffer in which the buffer capacity was adjusted to pH = 6.5 using MES (2-morpholinoethanesulfonic acid, CAS No.4432-31-9), it is absorbed into the filter paper. It is then dried. The saliva transcript mechanism and the optical measuring unit, which measures the color density of the reagent, are installed in the main section of the measuring instrument.

The collection sheet is inserted into the mouth, and about 20 to 30 μL of the saliva in the sublingual region is directly collected for 10 to 30 sec. (**Figure 3**). Thereafter, the test strip is placed in the main unit of the measuring instrument, and the cover is closed. When the transcript lever is operated, the test paper put on the reverse side of the test paper holder is pressed against the saliva collection paper, and the saliva is transcribed (**Figure 4a**). This time point is detected as the reaction start time, and the alarm sounds when the previously set transcript time has passed (**Figure 4b**). When the collection sheet is then removed, the color density of

Figure 1. Main unit of measuring instrument and test strip

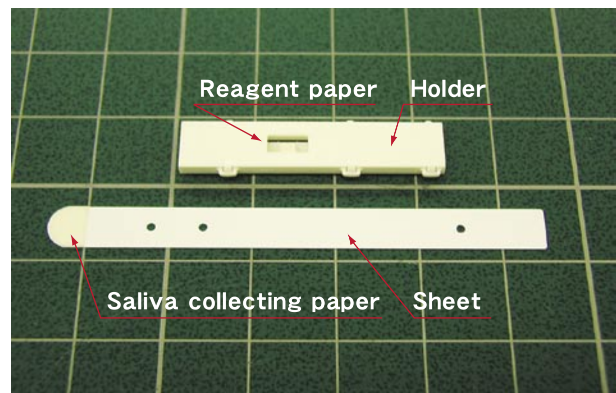

Figure 2. Test strip

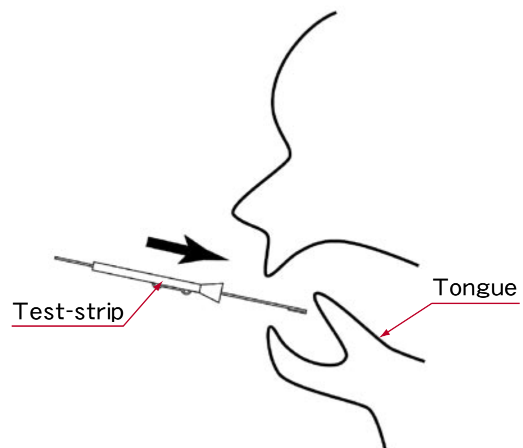

Figure 3. Sampling of saliva

the test paper due to the enzyme reaction is measured using the optical device (**Figure 4c**). The color density after the previously set time from the reaction starting is measured. The amylase activity value converted from the measured value is indicated on the display. In this analyzer, the measuring conditions are set to 30 sec. for collecting saliva, 10 sec. for transcribing, and 20 sec. for the reaction time. The measurement of the salivary amylase activity can be completed in about one minute.

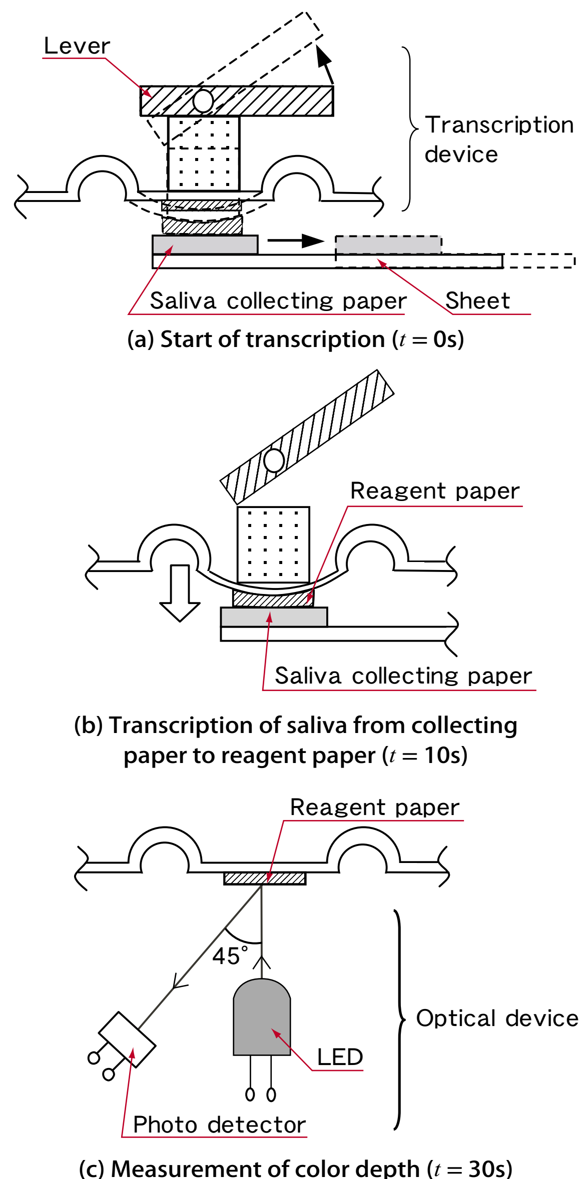

Figure 4. Saliva transcript mechanism and measuring protocol

2.3 Temperature dependence and pH dependence

In order to examine the temperature dependence of the salivary amylase activity, the thermal behavior was evaluated. Currently, the international standard measuring method of the human amylase activity is not specified. In general, a method of using starch as the substrate, a method of using a coupling enzyme, etc., are being adopted. This time, a method using the clinical autoanalyzer (Miracle Ace 919, Nipro Co., Japan) and the enzyme test reagent (Espa AMY liquid2, Nipro Co., Japan) in which the substrate was Gal-G2-CNP for analyzing the salivary amylase activity was adopted. All the saliva collected from three healthy men was used as the saliva sample. First of all, the salivary amylase activity was measured at 37°C, then measured at temperatures of 10, 20, and 30°C for the same saliva samples (n=60) using a spectrophotometer with a built-in constant temperature cell holder. The temperature dependence was obtained by assuming the amylase activity at 37°C, which is close to the human body temperature, to be 100% and calculating the relative activity at other temperatures.

Next, the pH dependence of the salivary amylase activity was determined. In order to adjust the pHs of the saliva samples to specific values, a HCl solution or a NaOH solution diluted with 1% BSA (bovine serum albumin: It stabilizes the enzyme) was added to each

collected sample, and their pHs were adjusted to 4.4-9.1. BSA was used so that the amount of the total protein in the saliva sample may not be diluted by the pH adjustment. A 4.5 μL sample of samples, which had been pH controlled, was dropped on the test paper (4 x 4 x 0.25 mm), and the salivary amylase activity after 30 sec. was measured using the portable analyzer (n=35). The pH of the test paper was measured using a micro pH electrode (9810BN, Orion Research Inc., MA, USA) at the same time. The dimensions of the pH sensing part of this pH electrode were 0.9 mm diameter and 1.1 mm length, therefore, the pH measurement of the sample using several μL is possible. The pH dependence was obtained by assuming the amylase activity at pH=6.5 to be 100% and calculating the relative activity at the other pHs.

2.4 Calibration curve

With this prototype, the enzyme activity is not directly measured but the optically measured color density of the reagent is converted to the enzyme activity. Since there is no definite basis for the output of this measuring instrument, it is necessary to calibrate the output of this prototype in order to know the relation between the output and the standard enzyme activity. The calibration curve based on the above-mentioned enzyme test reagent and the clinical autoanalyzer was then prepared. The entire saliva collected from 7 subjects (5 males and 2 females) was used as the sample. The salivary amylase activity at 37°C of this sample was measured using the enzyme test reagent and the clinical autoanalyzer. At the same time, the same sample was measured using the portable analyzer, and the calibration curve of the portable analyzer based on the clinical autoanalyzer was prepared. The measurement of the salivary amylase activity was repeatedly carried out seven times for each saliva sample, and the five results, which excluded the maximum value and the minimum value of the seven results, were used. Based on the result of this calibration curve, the measuring range and the measuring accuracy of the portable analyzer were determined.

3 RESULTS AND DISCUSSION

3.1 Temperature dependence and pH dependence

A decrease in the salivary amylase activity due to the decrease in the temperature was observed, and it was confirmed that the activity is mostly dependent on the temperature (**Figure 5**). The following equation was obtained as the temperature characteristic equation of the amylase activity under the condition of a standard measuring temperature (R^2=0.99).

$$\%AMY = 0.048T^2 + 0.59T + 12.1 \quad (2)$$

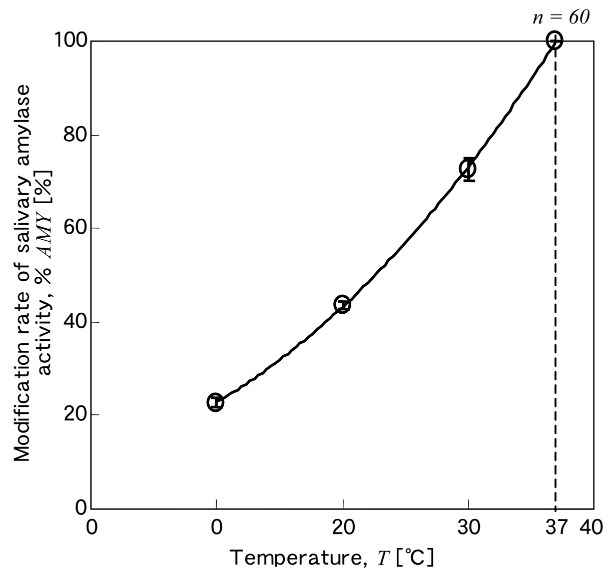

Figure 5. Temperature dependence of salivary amylase activity (%AMY = 0.048T² + 0.59T + 12.1)

A temperature correction function is installed in the portable analyzer in order to correct this temperature dependence. A digital thermometer (DS18S20, Maxim Integrated Products, Inc., CA, USA) is installed in the main unit, and the salivary amylase activity at 37°C is calculated using the ambient temperature detected by this thermometer and the temperature correction equation obtained from equation (2).

In order to eliminate the effect of the pH of the saliva on the measured value, the test paper is impregnated with the buffer, which has had its buffer capacity adjusted to pH=6.5 using MES. As a result of the measurement, it has been found that the salivary amylase activity indicated a maximum value at pH=6.5 which decreased when it changed to acidic or alkaline (**Figure 6**). The following equation was obtained as a pH characteristic equation (R^2 = 0.96).

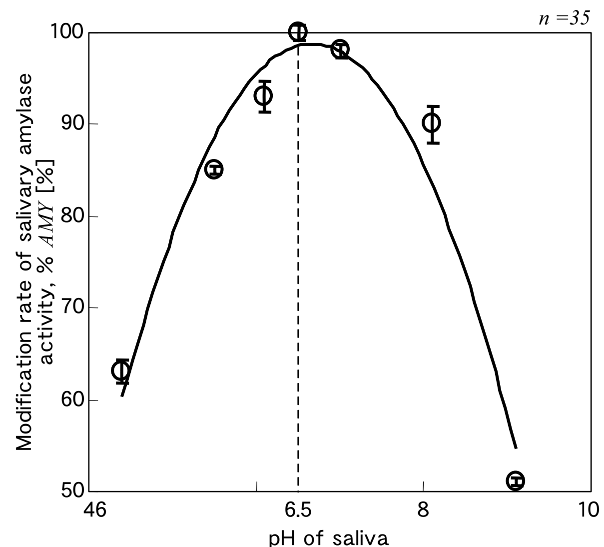

Figure 6. pH dependence of salivary amylase activity (%AMY = -0.075pH² + 0.99pH - 2.3)

$$\%AMY = -0.075\text{pH}^2 + 0.99\text{pH} - 2.3 \quad (3)$$

A pH correction function is also included in the portable analyzer. Usually, it is set to indicate the value at pH=6.5. When the pH in the mouth of the subject is expected to have a big difference from neutral, the pH in the mouth should be measured using a commercially available pH test paper. By inputting the measured pH value into the

analyzer, the salivary amylase activity at pH=6.5 is calculated using the correction equation obtained from equation (3), and then the effect of pH can be eliminated.

3.2 Calibration curve

Figure 7 shows the calibration curve of the portable analyzer based on the analysis result using the clinical autoanalyzer. An excellent calibration curve of R^2 = 0.99 was obtained in the salivary amylase activity range of 10-140 kU/L. As for the measuring accuracy, it exceeded the standard criterion of a portable simplified measuring instrument, R^2 = 0.95, and was confirmed to be sufficient. As for the measuring range, it can be easily used within the assumed range of practical use, and it is also possible to change the measuring range by adjusting the reagent and the measuring conditions.

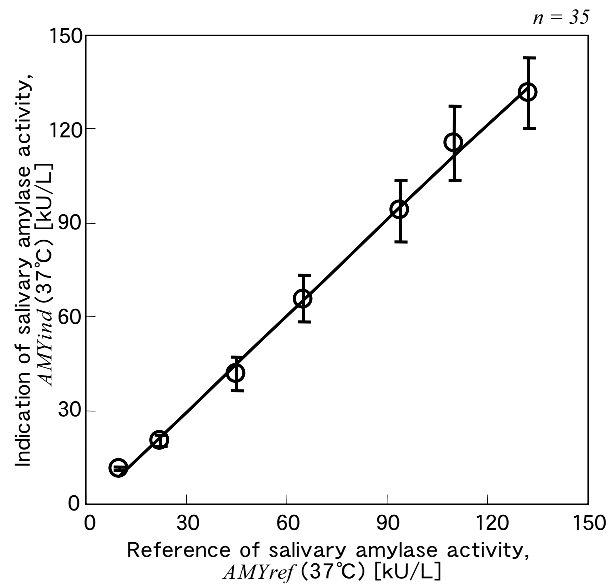

Figure 7. Calibration curve of portable salivary amylase activity analyzer (y = 0.97x - 1.71)

4 CONCLUSION

In order to achieve monitoring of the sympathetic nervous activity in daily life, a dry chemistry-type portable salivary amylase activity analyzer was experimentally manufactured. The measuring technology of the salivary amylase activity using colorimetry was developed. Functions to correct the ambient temperature and the pH of saliva, which influence the activity, are installed in the prototype. As for the calibration curve, which indicated the performance of the portable analyzer, an excellent result of R^2 =0.99 was obtained in the salivary amylase activity range of 10-140 kU/L. The measuring range is sufficient for the assumed range of practical use, and the accuracy exceeding that of a conventional simplified measuring device was achieved. The technology, which allowed the analysis of the salivary amylase activity in about one minute including the saliva collection, has been established.

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