ORIGINAL ARTICLE

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Salivary Alpha Amylase Enzyme as a Stress Parameter: Establishment and Comparison of Laboratory Methods

Stres Parametresi Olarak Tükürük Alfa Amilaz Enzimi: Laboratuvar Yöntemlerinin Kurulumu ve Karşılaştırılması

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ABSTRACT

Aim: Salivary alpha (α)-amylase enzyme is a biomarker used to measure sympathetic nervous system activity. This study aimed to establish three existing methods for measuring salivary α-amylase enzyme activity in the laboratory and to compare these methods in terms of their usability.

Materials and Methods: α-amylase enzyme activity can be measured in ready-made kits by three methods: Starch-Iodine, 2-chloro-4-nitrophenyl maltotrioside (CNPG3) and dinitrosalicylic acid (DNS) methods. In this context, standard curves were created in the laboratory for manual study of these methods and their advantages and disadvantages were presented. The usability of these methods was tested.

Results: The starch-iodine and CNPG3 methods were successfully established. However, although a standard curve was successfully established for the DNS method, this assay was not suitable for the studies as the samples were not readable and had many disadvantages. The test sensitivities and working ranges were appropriate for the starch-iodine and CNPG3 tests, requiring 4,000-fold dilution for the starch-iodine test and 5-fold dilution for the CNPG3 test. A weak but statistically significant positive correlation was observed between the two tests (R2=0.048 for linear regression; $p<0.05$, R2=0.106 for quadratic regression; $p<0.01$).

Conclusion: The CNPG3 and starch-iodine methods were feasible, cost-effective, accessible, and time-efficient. The starch-iodine method is a cheaper but the CNPG3 method is also a practical test with fewer steps. In this respect, it has been decided that the CNPG3 method is the most effective method in studies based on salivary α-amylase enzyme method.

Keywords: α-Amylase enzyme, CNPG3, DNS, starch-iodine, saliva

ÖZ

Amaç: Tükürük alfa (α) amilaz enzimi sempatik sinir sistemi aktivitesini ölçmek için kullanılan bir biyo-belirteçdir. Bu çalışmanın amacı tükürük α-amilaz enzimi aktivitesini ölçebilmek için mevcut olan üç yöntemi laboratuvarda kurmak ve bu yöntemleri kullanılabilirliği açısından birbirleriyle karşılaştırmaktır.

Gereç ve Yöntem: α-amilaz enzim aktivitesi; nişasta-iyodin, 2-chloro-4-nitrophenyl maltotrioside (CNPG3) ve dinitrosalisilik asit (DNS) yöntemleri olmak üzere üç yöntem ile hazır kitlerde ölçülebilmektedir. Bu bağlamda bu yöntemlerin manuel olarak çalışılabilmesi için laboratuvarda standart eğrileri oluşturuldu ve avantaj ve dezavantajları ortaya konuldu. Bu yöntemlerin kullanılabilirliği test edildi.

Bulgular: Nişasta-iyodin ve CNPG3 metodları başarılı bir şekilde kuruldu, fakat DNS metodu için başarılı bir standart eğri oluşturulmasına rağmen numuneler okunamadığından ve birçok yönden dezavantajları olması sebebi ile bu test çalışmalar için uygun bulunmadı. Nişasta iyodin ve CNPG3 testleri için test hassasiyetleri ve bunların çalışma aralıkları uygun bulunmuş olup, nişasta-iyodin testinde 4.000 kat, CNPG3 testinde ise 5 kat seyreltme gerekmiştir. Kurulan iki test arasında zayıf fakat istatistiksel olarak anlamlı pozitif bir ilişki gözlenmiştir (Lineer regresyon için R2=0,048; $p<0,05$, kuadratik regresyon için R2=0,106; $p<0,01$).

Sonuç: CNPG3 ve nişasta-iyodin metodlarının uygulanabilir, uygun maliyetli, kolay ve zaman açısından kısa sürmesi nedeniyle çalışmalarda kullanılabilir oldukları belirlenmiştir. Nişasta-iyodin metodu daha ucuz bir yöntemdir fakat CNPG3 metodu da daha az aşamadan oluşan pratik bir testtir. Bu açıdan tükürük α-amilaz enzimi yöntemi üzerine kurulu çalışmalardaki en etken yöntemin CNPG3 yöntemi olduğuna karar verilmiştir.

Anahtar Kelimeler: α-Amilaz enzimi, CNPG3, DNS, nişasta-iyodin, tükürük

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INTRODUCTION

Saliva is an easily obtainable fluid in our body and its importance is increasing gradually[1-3]. Salivary alpha (α) -amylase enzyme is an enzyme produced by salivary glands, released due to activation of the autonomic nervous system and breaks down starch[4]. It is also an essential parameter in the investigation of stress physiology[5,6]. Three methods can theoretically measure salivary α-amylase enzyme activity. The starch-iodine method is based on the breakdown of starch by α-amylase enzyme. The starch that α-amylase enzyme cannot break down is stained with iodine and determined by reading in a spectrophotometer[7]. The dinitrosalicylic acid (DNS) method is based on determining the amount of sugar reduced[8]. In the substrate method, 2-chloro-4-nitrophenyl maltotrioside (CNPG3) acts as a substrate and shows the enzyme's activity by forming a yellow color when bound to the enzyme[9-11].

While different methods can measure α-amylase, these methods are mainly studied with ready-made kits and no studies comparing them with each other have been found. Therefore, in this study, three methods measuring α-amylase enzyme activity were established in the laboratory and compared with each other in terms of cost, time, and practicality.

This study aimed to establish these tests in the laboratory, to determine the most appropriate test and then use it to investigate stress physiology. In addition, knowledge on this subject will be gained.

MATERIALS AND METHODS

Experimental Studies

Establishment of the Starch-Iodine Method

The starch-iodine method aims to detect the α-amylase enzyme's linkage between glucoses. It breaks down and interacts with the starch and iodine, creating a blue-violet color. This allows the activity of α-amylase to be measured.

Starch consists of two groups called amylose and amylopectin. Amylose linear is a molecule. Glucose molecules form a helix lined up one after the other, and form a double helix. Two amylose molecules are wrapped together in a double helix and can form a blue-violet color. Iodine molecules can enter these helices and form a blue-violet color. Amylopectin has a shape branching from the center like a bush[7]. It was decided that the standard starch-iodine solution, which was most suitable for the study, was the ready liquid 1% solution, and the ready solution was used in the studies.

Protocol for Establishing the Standard Curve in the Starch-Iodine Method

In the formation of the standard curve, α-amylase was diluted several times. It was decided that the most appropriate concentration was 1st Standard: 30 U/mL, 2nd Standard: 3 U/mL, 3rd Standard: 1.5 U/mL, 4th Standard: 0.6 U/mL, 5th Standard: 0.3 U/mL, 6th Standard: 0.15 U/mL and 7th Standard: 0.06 U/mL. Samples were diluted to a specific range to read the samples. When the samples were not read at the end of these trial-and-error procedures, they were re-diluted and re-run in the test. For this purpose, buffer solution (PBS) was used for dilution.

Protocol of the Starch-Iodine Test

The starch-iodine method was used to determine α-amylase activation in saliva samples. The working protocol of this method is summarized below;

- 40 µL of starch solution was pipetted into each well.
- 40 µL saliva (α-amylase) was added to all wells (shaken gently for 5-10 seconds).
- Incubated in an oven (50 degrees celsius) for 30 minutes.
- 20 µL HCl solution was added to all wells.
- 100 µL iodine solution was added to all wells.
- Read at 580 nm in a spectrophotometer with plate reader (Figure 1)[12].

Establishment of CNPG3 Method

The primary purpose of the CNPG3 method is to function by binding to the enzyme as a substrate. The enzyme binds to its substrate and creates a yellow color. Thus, the activity of α-amylase can be measured[11,13].

CNPG3 chromogen is a commercially available substrate[14]. It is directly soluble in PBS and forms a light yellow color. A

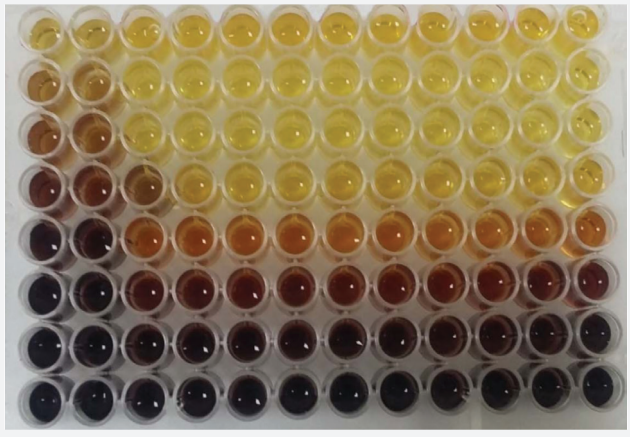

Figure 1. The starch-iodine method demonstrates the color changes caused by different concentrations of α-amylase. In the starch-iodine test, the color changes from yellow to dark brown. The enzyme's activity is measured by reading this color in a spectrophotometer (580 nm)

standard curve was created for the application of the assay. The α-amylase enzyme was diluted at different ratios to form this standard curve.

Standard Curve Generation Protocol in CNPG3 Method

In the formation of the standard curve, several dilutions were made and it was decided that the most appropriate dilutions were 1st Standard: 30 U/mL, 2nd Standard: 15 U/mL, 3rd Standard: 7.5 U/mL, 4th Standard: 6 U/mL, 5th Standard: 3.75 U/mL, and 6th Standard: 3 U/mL. Samples were diluted to a specific range so that the samples could be read. When all samples were not read, the samples were re-diluted at different ratios and read. Dilutions were made with PBS.

Protocol of the CNPG3 Test

CNPG3 method was used to determine α-amylase activation in saliva samples. The working protocol of this method is summarized below;

- 175 µL of PBS solution was pipetted into each well.
- 5 µL saliva (α-amylase) was added to all wells (shaken gently for 5-10 seconds).
- Incubated for -1 hour (at 37 degrees celsius).
- 20 µL of CNPG3 solution was added to all wells.
- Read at 405 nm in a spectrophotometer with plate reader (Figure 2)[14-16].

Setting up the DNS Method

The DNS method is used to measure α-amylase activity[17] and is based on measuring the amount of reducing sugar. The DNS method is used to estimate the concentration of reducing sugars in a sample. Reducing sugars contain the free carbonyl group, which can reduce most reagents. All monosaccharides and some disaccharides are reducing sugars[8]. When 3,5-DNS reacts with reduced sugars, orange colored 3-amino-5 nitrosalicylic acid is formed.

Standard Curve Generation Protocol in DNS Method

In forming the standard curve, several dilutions were made and it was decided that the most appropriate dilutions were 1st Standard: 30 U/mL, 2nd Standard: 3 U/mL, 3rd Standard: 0.3 U/mL, and 4th Standard: 0.03 U/mL. The appropriate standard curve was created for the test, but although the samples were diluted, no color intensity was obtained in the range of the standard curve. There is also a boiling step in the DNS test. It seems easy for a few samples but impractical when many must be read. Although boiling occurs in sealed tubes, water can get into the tubes.

Protocol of the DNS Test

The DNS method was used to determine α-amylase activation in saliva samples. The study protocol of this method is summarized below;

- 0.9 mL substrate and 0.1 mL enzyme were added to each tube.
- Incubated at 37 degrees for 5 minutes.
- 1 mL of DNS was added.
- Boiled for 10 minutes and allowed to cool.
- It was read at 540 nm in a spectrophotometer with plate reader, (Figure 3)[18,19].

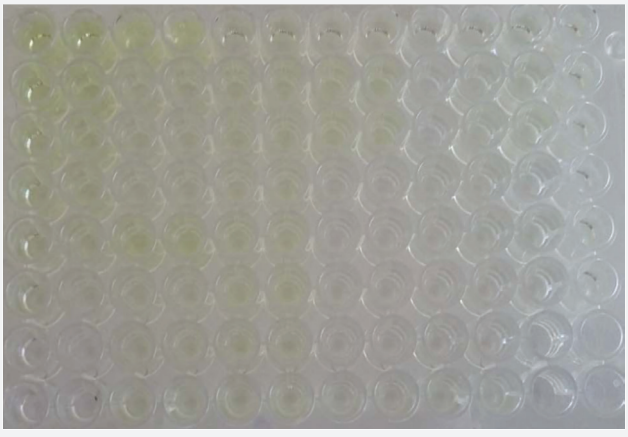

Figure 2. CNPG3 method demonstrating the color changes caused by using different concentrations of α-amylase. In the CNPG3 assay, a light yellow color is formed with the addition of CNPG3 and α-amylase activity is determined by reading in a spectrophotometer (405 nm)

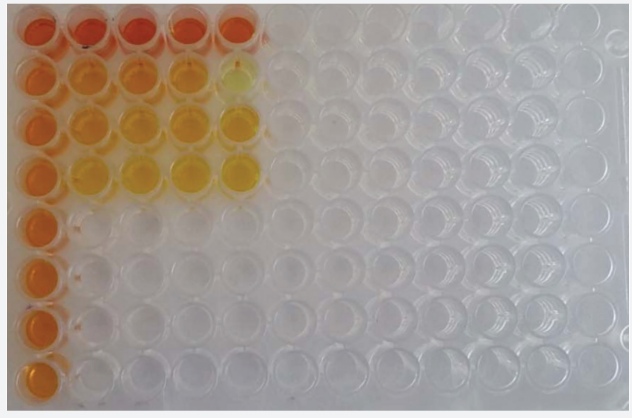

Figure 3. Demonstration of the color changes caused by using different concentrations of α-amylase by DNS method. In the DNS assay, yellow color is formed with the addition of DNS and α-amylase activity is determined by reading in a spectrophotometer (540 nm)

Statistical Analysis

MINITAB (USA) statistical program was used for data analysis. Data are presented as mean±standard error. A 4-parameter logistic curve was used to construct standard curves (Gen 5, BioTek Synergy, USA). The relationship between the Starch-iodine test and CNPG3 test was demonstrated by the Pearson correlation.

RESULTS

Starch-Iodine Standard Curve

Standard curves of α-amylase obtained by starch-iodine assay were run in 5 tests and average standard curve values were obtained (Figure 4).

Standard curve showing the changes that occurred. Color changes caused by changes in α-amylase concentrations (units/mL) were measured spectrophotometrically at a wavelength of 580 nm, and the standard curve graph above was obtained. Values are presented as the mean and standard error values obtained in 5 tests. The standard curve was linear between 0.06 U/mL and 1.5 U/mL.

CNPG3 Standard Curve

The α-amylase standard curves obtained with the CNPG3 assay were run in 13 assays and average standard curve values were obtained (Figure 5).

DNS Test Standard Curve

Standard curve showing changes in density. Standard curve, 3-30 U/mL between the concentration difference of approximately 0.300 units in the optical density. Change has occurred (Figure 6).

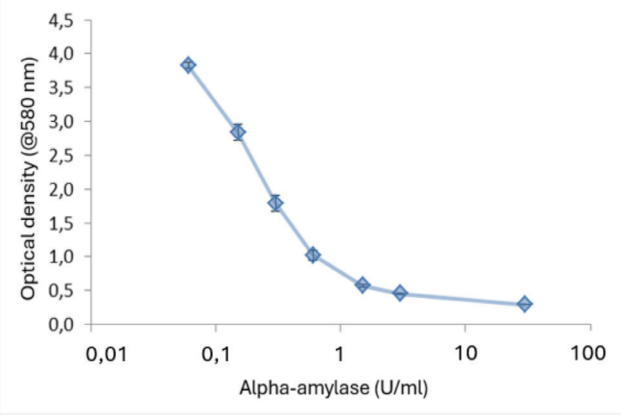

Figure 4. Optical density at increasing α-amylase concentrations according to the starch-iodine method

DISCUSSION

In the present study, the measurement methods of salivary α-amylase activity were investigated and the methods of measuring salivary α-amylase activity were compared for the first time. α-amylase starch-iodine compared to measure the enzyme activity in saliva method, CNPG3 method and DNS method are discussed.

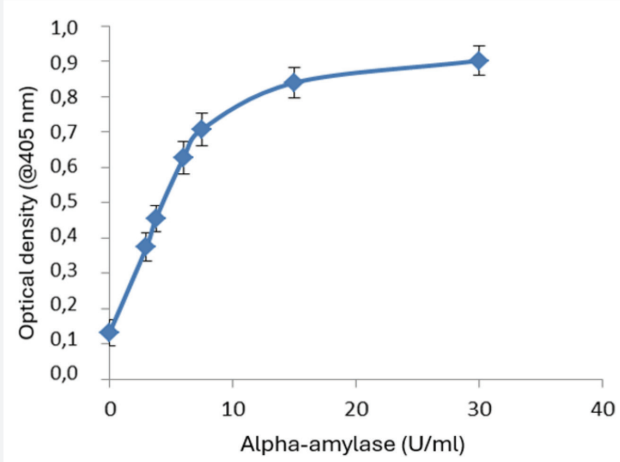

Figure 5. CNPG3 optical response at increasing α-amylase concentrations according to the chromogen substrate method. A standard curve showing changes in density. Measurements made at 405 nm is presented as the mean of 13 standard curves (±SEM). The curve was linear in the range 0-7.5 U/mL

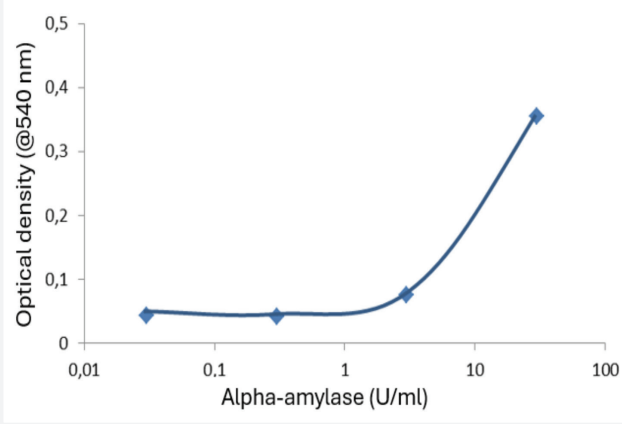

Figure 6. The standard curve in the DNS test shows changes in optical density with increasing α-amylase concentrations. In the standard curve, there was a change of approximately 0.300 units in optical density in response to the concentration difference between 3-30 U/mL

Determination of α-amylase by Starch-Iodine Method

The starch-iodine test was successfully established, and the dynamic range of the standard curve was found to be between 0.05-3 U/mL and its sensitivity was 0.05 U/mL. Additionally, the optical density range of the test is approximately 0 to 4,000, which shows a dynamic change. In other words, it is a test susceptible to α-amylase activity. In this form, the test can detect α-amylase when diluted approximately 4,000x in saliva samples. However, this level of dilution relatively increases the workload and causes a loss of time. On the other hand, it seems to be a preferable method because the incubation period is very short, and the materials are easy and cheap to obtain.

However, the necessity of separate dilutions for each saliva sample has a slight negative impact on the practical applicability of the test. The starch-iodine test is a test that is cheap, easy to set up and apply, and whose materials are readily available. However, it requires many dilutions and relatively increases the workload. On the other hand, the most crucial feature of the test is that the optical density values vary between 0 and 4,000. Therefore, it is a dynamic test, which is an essential reason for preference.

Determination of α-amylase by CNPG3 Method

The CNPG3 assay was successfully established, and the dynamic range of the standard curve was found to be between 3-15.75 U/mL with a sensitivity of 0.100 U/mL. Furthermore, the optical density range of the assay also shows a dynamic range of approximately 0.1 to 0.8. As such, the assay can detect α-amylase in saliva samples at approximately 5x dilution. The single reconstitution minimizes the workload and wasted time, but the large amount of saliva used and the length of the incubation period have a negative impact. The CNPG3 assay is an easy to set up and implement method that reduces the workload but is relatively expensive.

Determination of α-amylase by DNS Method

The DNS method is known as a method used for the determination of urea and sugar in the biochemical field. DNS material was prepared under laboratory conditions, and a standard curve was created. It was determined that the dynamic range of the DNS standard curve was between 0.3-0.03 U/mL and its sensitivity was 0.4 U/mL. Additionally, the optical density range of the test varies slightly between approximately 0.1 and 0.4. For this reason, the probability of being preferred is very low, and the fact that the test consists of many stages is considered a negative factor. The presence of a problematic step, such as boiling, and the fact that the samples are not within the optical range of the standard curve are also noted as essential shortcomings. Additionally, although the test works as intended, it is not suitable for practically examining large numbers of samples.

Study Limitations

In our study, there may be some limitations in revealing the advantages and disadvantages of the methods established in the laboratory.

CONCLUSION

The starch-iodine and CNPG3 chromogen tests were successfully developed. The standard curve of the DNS test was successfully established, but the samples were not compatible with the optical density. The sensitivities and working ranges of the established tests were appropriate; 4000-fold dilution was required for the starch-iodine test and 5-fold dilution was required for the CNPG3 test. Both tests appear to be inexpensive, easy to perform, and of short duration. Although the starch-iodine test is 4-5 times cheaper than the CNPG3 test, the CNPG3 test is a more practical test with fewer steps.

Ethics

Ethics Committee Approval and Informed Consent: Since this study was a laboratory study, ethical committee approval and informed consent were not required.

Authorship Contributions

Concept: Ö.B., Design: Ö.B., Data Collection or Processing: Ö.B., S.Y., Analysis or Interpretation: Ö.B., S.Y., Literature Search: Ö.B., S.Y., Writing: Ö.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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