Int J Pharm Bio Sci 2013 Jan; 4(1): (P) 949 - 954

Research Article Analytical Chemistry

International Journal of Pharma and Bio Sciences ISSN 0975-6299

PRECISE METHOD FOR THE ASSESSMENT OF CATALASE-LIKE ACTIVITY IN SEMINAL FLUIDS

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ABSTRACT

In the present study, a sensitive modification of the colorimetric assay for catalase activity is reported. This assay is based on the reaction of undecomposed hydrogen peroxide with ammonium molybdate to give a yellowish color, which has maximum absorbance at 354 nm. Catalase-like activity is calculated from a comparison with the activity of a certified standard enzyme with known unit activity (50 k.units/litre). The seminal plasma catalase activity of a sample of normal individual has been determined. The imprecision of the method was calculated by measuring the coefficient of variation, which equals to 2.61% within a run and 5.35% between run. The catalase assay performed using kinetic method yielded a good correlation (r = 0.98504).

Key word: catalase-like activity; hydrogen peroxide; seminal plasma; ammonium molybdate.

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INTRODUCTION

In the presence of catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) hydrogen peroxide is converted to oxygen and water. This enzyme is broadly distributed in plants [1], animals, and microbes [2]. Catalase-like activity plays an important role in reducing toxic hydrogen peroxide in the cell. It is found in seminal plasma, in dialyzed seminal plasma (> 12 kD), in an ultrafiltrate of seminal plasma (< 5 kD) and in spermatozoa. The catalase-like activity of whole seminal plasma and spermatozoa is used to evaluate the levels of reactive oxygen species production [3,4]. There are several methods are used to assess catalase-like activity. The most common method involves measuring the decrease in absorbance of hydrogen peroxide at λ equal to 240 nm [5]. Other methods have been divided into two categories. The first includes the old assays such as the iodimetric titration method of Dille and Watkins [6], the use of sodium perborate as a source of hydrogen peroxide [7], the determination of undecomposed hydrogen peroxide either with dichromate in acetic acid to give a chromic acetate after heating which is measured colorimetrically at 570-610 nm [8], or with ammonium molybdate to form a yellowish complex measured at 405 nm [9] or 410 nm [10], and different assays employing permanganate titration of undecomposed hydrogen peroxide [11,12,13]. This type of assays has some advantages, such as the availability of laboratory equipment to perform in most laboratories and there is no need for advanced skills to be completed. The disadvantages include the inability to measure the very low levels of catalase and the results do not have high compatibility with each other. The second category of assays includes two methods. The first is based on the enzymatic consumption of hydrogen peroxide using INH-PC [iso-nicotinicacidhydrazide- pyrocatechol] system [14]. The response of the Catalase activity is ascertained by the rate of the reaction involving hydrogen peroxide. The addition of H_2O_2, INH-PC [iso-nicotinicacidhydrazide-pyrocatechol] indicator system formed a chromogenic product with absorbance maxima at 490 nm. Consequently the activity of Catalase is directly measured by the chromogenic response in the formation of the coupled product. The second assay developed amperometric flow injection method for determination of undecomposed hydrogen peroxide [15]. This method based on Catalase enzyme (CAT) immobilization on a glassy carbon electrode (GC) modified with electrochemically deposited gold nanoparticles on a multiwalled carbon nanotubes/chitosan film. This type of assays has some disadvantages such as the unavailability of laboratory equipment to perform in a large number of laboratories and the need for advanced skills to completed. The advantages include the ability to measure the very low levels of catalase and give results with high compatibility.In this study, a simple and precise colorimetric method for rapid assay of Catalase like activity is described.

MATERIALS AND METHODS

Principle

Catalase catalyzes the following reaction:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O+O_2$$

The activity of Catalase was estimated by incubation the enzyme sample in 60 mM sodium-potassium phosphate buffer, pH 7.4, at 37 °C. After stopping the reaction by irreversible

inhibitors, catalase activity was determined by mixing the reaction mixture with ammonium molybdate and measuring the absorbance change at 354 nm. Catalase activity calculated from the comparison with the consequence of a certified standard enzyme with known unit activity (50 k.units/litre).

Reagents

1. Sodium, potassium phosphate buffer (60 mmol/L).
2. H_2O_2 (30 mM) in 60 mmol/L sodium, potassium phosphate buffer.
3. Ammonium molybdate (32.4 mmol/L).
4. Certified catalase of known activity (50 k.units/litre): obtained from Himedia (Product Code: TC037). Its activity was calibrated according to Aebi's method [4].
5. Inhibitor: composed of equal volume of 3 mM KSN and 40 mM-aminotriazole mixed well before using.

Instrument

A spectrophotometers (PG Instruments T80 plus) and (Shimadzu 1800 spectrophotometer) were used in the study.

Procedure

shown in table(1).

Table 1
procedure

Reagents	Test	Control-test	Standard	Control- Standard	Blank
Sample	20µl	20µl	------		-----
Standard	------		20µl	20µl	-----
Inhibitor	------	500 µl	------	500 µl	500 µl
Substrate	2ml	2ml	2ml	2ml	
Mix with vortex and incubate at 37 °C for 3 min, after that, add:					
Inhibitor	500 µl	------	500 µl	------	500 µl
Ammonium molybdate	1ml	1ml	1ml	1ml	1ml
Mix by vortex then read the absorbance at 354 nm against reagent blank.					

Calculation

The following equation is used to determine catalase activity:

$$\text{Catalase Activity of test kU/L} = \frac{\text{A control test - A.test}}{\text{A control STD - A.STD}} * \text{Catalase Activity} \textit{of} \quad \textit{STD}$$

RESULTS AND DISCUSSION

Results obtained for sample of seminal plasma by this method were compared with that obtained by the method of Aebi [5]. Identical sample, buffer, and substrate concentrations were used in both methods.The results of present assay proved a good precision (Table 2) and a good correlation with the Aebi's Method (Table 3).

Table 2
Precision of the Assay Procedure

	No.	Mean (±SD) k.U/liter	CV%
Within-run	20	58.99±1.545	2.61%
Between-run	20	56.318±3.016	5.35%

Table 3
Statistical Analysis of the Values Obtained for Catalase by Aebi's Method and Present Method.

No. of Samples	20
Mean of Aebi's Method	57.1
Mean of the present method	58.999
Mean of both methods	58.049
Regression coefficient B	0.9851
Regression coefficient A	0.0149
Correlation coefficient	0.9850

This method is essentially described by Goth [9] and Korolyok [10],in which the decomposition of peroxide is estimated spectrophotometrically by a complex reaction with ammonium molybdate at 405 nm and 410 nm respectively. The present method introduced some modifications to increase confidence, sensitivity, and precision.The present assay has number of characteristics distinguish them from other methods. It is utilized a certified standard enzyme with known unit activity as an alternative of necessitation to calibrate precise H_2O_2 concentration to 30 mM in a complex and tedious process. Calibration of precise H_2O_2 concentration is not necessary in the present assay because catalase concentration in sample is achieved by comparing with standard catalase enzyme. The second characteristic includes measurement of absorbance at a wavelength equal to 354 nm, which makes the results more credible, accurate, and convenient. In previous studies, Korolyok [10] used 410 nm while, Goth [9] chose 405 nm instead of 410 nm wavelength. Goth attributed the cause of this selection to the accessible with spectrophotometers and with filter photometers. Perhaps that choice was suitable before two decades. Nowadays, with the huge development in spectrophotometric techniques, investigators cannot accept this explanation of choosing these wavelengths.The selection of wavelengths other than 354 nm (such as 405 nm or 410 nm) includes considerable disadvantages. It requires the use of high concentration of hydrogen peroxide because of the disappearance of the value of the absorbance at ≥ 390 nm from the spectrum when low concentration of hydrogen peroxide is used, while it remains stable at peak near 354 nm, as shown in Figure (1)

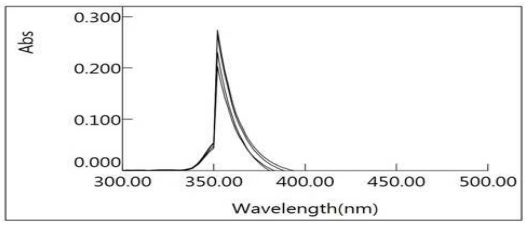

Figure 1
Spectrum of low concentrations of hydrogen peroxid prepared in 60 Mm phosphate buffer after its reaction with 32.4 mM ammonium molybdate

The using of low low concentrations of hydrogen peroxid prepared is necessary to determine micro levels of catalase [16].The second disadvantage is the inexpedient interference between closely spaced concentrations of the enzyme at wavelengths 405 nm and 410 nm, which makes previous assays unable to distinguish or differentiate between them, while the present method can easily distinguish between these concentrations, as shown in figure (2).

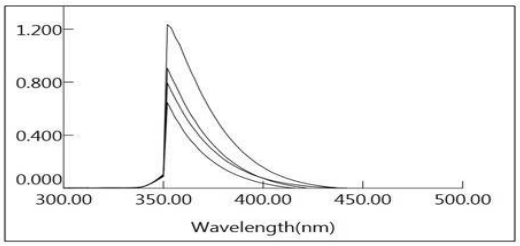

Figure 2
spectrum of closely spaced concentrations of hydrogen peroxid prepared in 60 Mm phosphate buffer after its reaction with 32.4 mM ammonium molybdate

CONCLUSION

One type of interference was observed in earlier assays, which due to the unsuitable wavelength selection. The current assay presents a number of advantages more than existing methodologies. These advantages include, less quantity of sample is required (0.02 ml as compared to 0.2 ml), more precision and accuracy, instrumentals and apparatus are not complicated and available in most laboratories, and the assay is free from interference.

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