

Simple Spectrophotometric Method for Analysis of Serum Catalase Activity

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ABSTRACT

Introduction: Catalase is a principal constituent of the antioxidant system that attenuates the oxidative stress, which is ubiquitously associated with several types of pathological disorders.

Aim: This paper describes a discontinuous assay for the assessment of catalase activity using the titanium tetrachloride/sulfuric acid reagent.

Materials and Methods: Samples containing catalase are incubated with hydrogen peroxide for three minutes prior to fast mixing of aliquots of the incubation mixtures with titanium tetrachloride/sulfuric acid reagent, which measures remaining hydrogen peroxide (H₂O₂). Absorbance is then read at 405 nm. Dissociation of hydrogen peroxide is proportional to catalase activity in the used sample. One-way analysis of variance (ANOVA) was used to analyse the resulted data. p-value <0.05

was considered to be statistically significant using SPSS Statistics 17.0 software.

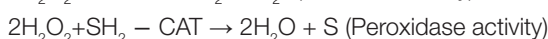
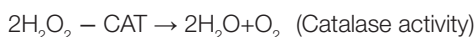
Results: The current method characterizes the use of a correction factor to exclude the interference that arises from the presence of a yellow background colour of serum and/or reducing agents that reduce titanium (IV) to titanium (III). The imprecision of the method was considered by calculating the coefficient of variation, which equals to 3.6% within run and 5.7% between run. The catalase assay measured using the kinetic method produced a good correlation (r=0.9771).

Conclusion: The study explains a simple discontinuous method for catalase activity assessment which can be completed with few steps, and which allows catalase to be measured in the presence of high concentration of other molecules as well as at low levels of H₂O₂.

Keywords: Antioxidant, Hydrogen peroxide, Sulfuric acid, Titanium tetrachloride

INTRODUCTION

Catalase is exceptional when compared to other enzymes of the peroxidase family since it exhibits both catalase activity and peroxidase as shown below. Usual substrates for peroxidase activity (SH₂ in the reaction) consist of ethanol, methanol, formate, nitrite and phenol [1].



Catalase is a principal constituent of the antioxidant system that attenuates the oxidative stress, which is ubiquitously associated with several types of pathological disorders [2]. Catalase is a widely distributed enzyme and is present in all aerobic microorganisms, plant, and animal cells [3]. Many methods have been elucidated for the assessment of catalase activity. Most depend on the measurement of un-reacted hydrogen peroxide after a period of incubation with the catalase enzyme or the volume of oxygen produced by decay of the substrate [4]. The formation of oxygen can be measured by oxygen electrode [5] or low-flow gas meter [6]. The H₂O₂ decay at 240 nm can be measured spectrophotometrically [7,8] or by chemiluminescence [9,10]. Other methods have been used including iodometry [11], titrimetry [12], and polarimetry [13].

The present study reports a simple method which includes the spectrophotometric measurement of hydrogen peroxide with an improved serum catalase assessment. The clinical implication of serum catalase activity is included in assessment of acute pancreatitis. Serum catalase activity is elevated in acute pancreatitis for a significantly longer time than the serum amylase activity and is also documented to be elevated in chronic pancreatitis [14].

Also, the new method is free of the interference that arises from the presence of a strong yellow background found in some types of pathologic sera.

MATERIALS AND METHODS

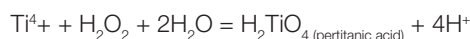
The methodology was based on the article by Hadwan MH and Abed HN, [15].

Principle:

Catalase catalyzes the subsequent reaction:



Catalase activity was measured by incubating the enzyme sample in 1.0 mL substrate (20 μmol per ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37°C for three minutes. The reaction was stopped with titanium sulphate. The method quantifies undecomposed hydrogen peroxide by reacting it with a titanium sulphate reagent to produce pertitanic acid. Absorbance of the yellow pertitanic acid was measured at 405 nm against the blank.



Reagents:

1. Titanium reagent: 0.1% (v/v) TiCl₄ in 20% (v/v) H₂SO₄.
2. Sodium, potassium phosphate buffer (50 mM, pH 7.4): this buffer is prepared by dissolving 1.1 gm of Na₂HPO₄ and 0.27 gm of KH₂PO₄ in 100 mL distilled water [15].
3. H₂O₂ (20 mM) in 50 mmol/L sodium, potassium phosphate buffer: this solution is freshly diluted and standardized daily using a molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm [16].

Instrument: Shimadzu 1800 spectrophotometer was used in the study.

Procedure: shown in [Table/Fig-1].

Calculation: Therate constant of a first-order reaction (k) equation is used to determine catalase activity:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * \left[\log \frac{S^o}{S-M} \right] * \frac{Vt}{Vs} \quad \text{--- (1)}$$

t: time.

S^o: absorbance of standard tube.

S: absorbance of test tube.

M: absorbance of control test (correction factor).

Vt: total volume of reagents in test tube.

Vs: volume of serum.

*The present method uses a correction factor (Control-test) to eliminate the interference that arises from the presence of a strong yellow background colour in the sample containing catalase enzyme. The absorbance of test tube in procedure belong to two types of compounds, undecomposed hydrogen peroxide and interferences that found in the sample. The absorbance of (Control-test) tube in procedure belongs to interference compounds found in the sample only. By subtracting the absorbance of (control-test) tube from the absorbance of test tube; we exclude the interference of a strong yellow background colour. Consequently; the resulting

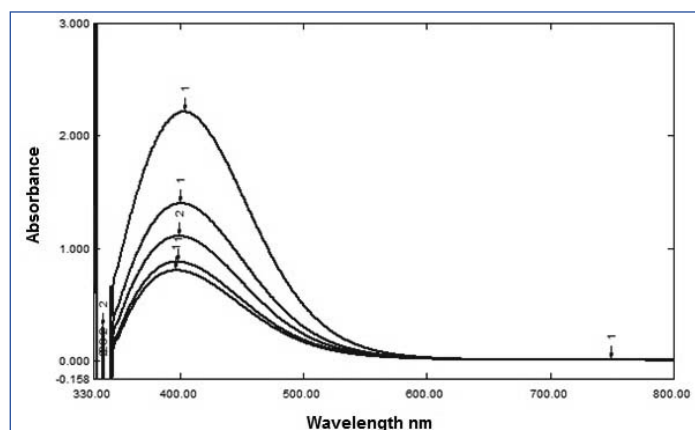
Reagents	Test	Control-test*	Standard	Blank
Serum	100 µL	100 µL	-	-
Distilled water	-	1000 µL	100 µL	1100 µL
Hydrogen peroxide	1000 µL	-	1000 µL	-
Titanium reagent	3000 µL	3000 µL	3000 µL	3000 µL

[Table/Fig-1]: Illustrates the procedure used for measurement of catalase activity. After that, the tubes were kept at room temperature. Changes in absorbance were recorded at 405 nm against the reagent blank.

absorbance was related to un-reacted hydrogen peroxide in enzymatic reaction.

RESULTS

An absorbance spectrum of the colourimetric product was measured from 330 nm to 800 nm and revealed a peak at 405 nm



[Table/Fig-2]: Absorption spectra obtained for the colourimetric products of the present assay.

[Table/Fig-2], suggesting the optimum colourimetric wavelength for the yellow reaction product is 405 nm.

The present method uses a correction factor (Control-test) to remove the interference that arises from the presence of a yellow background colour of serum and/or reducing agents. Sugars and amino acids are the main constituents of the serum. Also, they have the reduction ability to interfere with the reagents that are used for assessment of catalase activity [17]. Possible interferences of compounds that could disturb catalase activity assessment were measured using the methods documented by Hadwan MH and Abed HN, [15]. To determine the effect of interferences that might disturb the catalase measurement, seven volumetric flasks were taken, to each one was added one ml

of catalase known activity (500 kU/L) obtained from Himedia (Product Code: TC037). Its activity was calibrated according to Aebi's method [18] and nine ml of 55.55 µmol/L of one interference only that dissolved in phosphate buffer (50 mM, pH 7.4). The final activity equals to 50 kU/L of catalase with 50 µmol/L of interference. Catalase enzyme activity was measured by the present method (with and without correction factor). [Table/Fig-3] shows the effects of several interferences on the catalase activity. Catalase enzyme activity was not affected significantly by a considerable amount of each interference compound when assayed by the present method. However, interferences affected catalase enzyme

Substance	Concentration of substance	Observed Catalase activity			
		Method without correction factor	Recovery %	Present method	Recovery %
-	0	48.87	-	49.3	-
Glucose	50	48.72	99.6	49.1	99.5
Cysteine	50	48.1	98.4	49.2	99.7
Histidine	50	46.51	95.1	48.89	99.1
Lysine	50	46.53	95.2	49.0	99.3
Arginine	50	48.1	98.4	49.0	99.3
Methionine	50	48.3	98.8	48.93	99.2

[Table/Fig-3]: Shows the effects of various interferences on the catalase assay.

activity when method was used without a correction factor. The result established from [Table/Fig-3] proves that the correction factor was necessary to get a precise result.

Results achieved for serum by the present method were compared with that produced by the method of Aebi H [18]. Matching sample

Variables	n	Mean (±SD): k.U/liter	CV %*
Within-run	20	111.5±4.1	3.6 %
Between-run	20	108.8±6.3	5.7 %

[Table/Fig-4]: Precision of the assay procedure.
* CV%: the percentage of ratio of the standard deviation to the mean.

Method	n (20)
Mean of Aebi's Method	116
Mean of the present method	112
Mean of both methods	114.5
Regression coefficient B	0.9781
Regression coefficient A	0.0219
Correlation coefficient	0.9769

[Table/Fig-5]: Statistical analysis of the values obtained for catalase by Aebi's method and present method.

and buffer were used in both methods. The results of the present assay showed a good precision [Table/Fig-4] and a good correlation with the Aebi's method [Table/Fig-5].

Present in assay	Equivalents of hydrogen peroxide	Calculated activity kU/L	Observed activity ^a kU/L	Recovery %
Enzymatic sample	-	-	100	-
Enzymatic sample + hydrogen peroxide	10	110	108	98.10 %
Enzymatic sample + hydrogen peroxide	25	125	122	97.6 %
Enzymatic sample + hydrogen peroxide	50	150	146	97.33 %
Enzymatic sample + hydrogen peroxide	100	200	195	97.5 %
Enzymatic sample + hydrogen peroxide	200	300	291	97.0 %

[Table/Fig-6]: Analytical recovery of hydrogen peroxide added to the reaction solution after the end of enzymatic reactions.
^a mean of triplicate determinations

Accuracy of the present assay protocol was determined by recovery of hydrogen peroxide added to the reaction solution after the end of enzymatic reaction detailed in the [Table/Fig-6]. The high accuracy of the present assay protocol was belonging to high molar extinction coefficient and correction factor.

The result concluded from [Table/Fig-6] proves that the linearity of the following method reaches to about 300 kU.

DISCUSSION

This following article elucidates a new method for measuring catalase activity in serum. The method quantifies undecomposed hydrogen peroxide by reacting it with a titanium sulphate reagent to produce pertitanic acid. Absorbance of the yellow pertitanic acid was measured at 405 nm against the blank (as shown in [Table/Fig-2]).

The titanium tetrachloride/sulfuric acid reagent can be represented as a "stop bath" for enzymatic reaction that is regulated by catalase enzyme. When the enzyme solution is mixed with sulfuric acid, its molecules are denatured; any hydrogen peroxide which has not been decomposed by the catalase will react with the titanium to produce a peroxotitanium complex (pertitanic acid), which has a maximum absorbance at 405 nm.

Despite the discovery of the titanium sulfate method for H_2O_2 estimation by Eisenberg [19] several decades ago, it wasn't appealing to use in the estimation of serum catalase activity because there are two types of interferences. The interferences include a yellow background colour of serum and/or reducing agents that reduce titanium (IV) to titanium (III). The only exception is the work that is done by Baudhuin and his assistants [20,21] to measure catalase activity in some tissues. Baudhuin and his coworkers did not explain how to remove interferences and did not compare their own method with other methods. The present method uses a correction factor (Control-test) to remove the interference that arises from the presence of a yellow background colour of serum and/or reducing agents. Sugars and amino acids are the main constituents of the serum. Also, they have the reduction ability to interfere with the reagents that are used for assessment of catalase activity [17]. [Table/Fig-3] shows the effects of several interferences on the catalase activity. Catalase enzyme activity was not affected significantly by a considerable amount of each interference compound when assayed by the present method. However, interferences affected catalase enzyme activity when method was used without a correction factor. The result established from [Table/Fig-3] proves that the correction factor was necessary to get a precise result.

The present method has introduced several advantages when compared with Aebi's method. Catalase activity can be considered by measuring the rate of dissociation of hydrogen peroxide at 240 nm (Aebi's method). There are practical problems with this method, which is related to using very high and un-physiological concentrations of H_2O_2 (5-50 mM) for achieving acceptable initial absorbance (the absorbance of H_2O_2 at 240 nm is only $43.6 M^{-1} cm^{-1}$). These high concentrations of hydrogen peroxide cause direct, but variable auto-inactivation of catalase by transformation of the active enzyme - H_2O_2 complex I to the inactive complex II [18]. Additionally, many cellular constituents such as proteins absorb intensely at 240 nm so that low activities of catalase often have to be assessed by the continuous method in contrast to a higher background absorbance.

The present method is more accurate (as shown in [Table/Fig-4]) and sensitive than Aebi's method (as shown in [Table/Fig-5,6]) because of the high molar extinction coefficient for H_2TiO_4 ($\epsilon = 689 M^{-1} cm^{-1}$ at 407 nm) [22] compared with the molar extinction coefficient for H_2O_2 at 240 nm ($\epsilon = 43.6 M^{-1} cm^{-1}$) [16]. Sensitivity is often described in terms of the molar extinction coefficient (ϵ , M^{-1}

cm^{-1}) of the measured product. The degree of the sensitivity is very significant in the spectrophotometric determination of an analyte [23].

On the other hand, the current method was preferable when compared with some colourimetric methods such as Hadwan MH and Almashhedy LA, and Ou P and Wolff SP, [7,8]. Hadwan MH and Almashhedy LA, presented simple method to measure catalase activity [7]. They used a certified standard enzyme with identified unit activity as an alternative of necessitation to standardize exact hydrogen peroxide concentration to 30 mM in a difficult process. The disadvantage of this method consists of the difficulty to obtain a certified catalase enzyme with identified activity. Ou P and Wolff SP, introduced a simple method to measure catalase activity. Ou P and Wolff SP, did not clarify how to exclude interferences and did not compare their own method they used with other procedures [8].

LIMITATION

The results of the present study were not compared with that obtained by commercial kits.

CONCLUSION

The study explains a simple discontinuous method for catalase activity assessment which can be completed with few steps, and which allows catalase to be measured in the presence of high concentrations of other molecules as well as at low levels of H_2O_2 . The titanium tetrachloride/sulfuric acid reagent is a sensitive assay for H_2O_2 which thus facilitates measurement of catalase at low concentrations of substrate confirming that auto-inactivation of catalase is reduced during the steps of measurement.

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