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New spectrophotometric assay for assessments of catalase activity in biological samples

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ABSTRACT

A novel, simple, and accurate colorimetric assay was established for assessments of catalase activity in biological fluids and tissues. H_2O_2 dissociation rates are directly proportional to catalase activity, and the principle of the present assay is based on reactions of ammonium metavanadate with H_2O_2 under acidic conditions. The resulting reduction of vanadium (V) to vanadium (III) produces a red–orange peroxovanadium complex with absorbance maxima at 452 nm. Biological samples containing catalase were incubated with 50-mM phosphate buffer solution containing 10-mM H_2O_2 as a substrate for two min. Subsequently, ammonium metavanadate in sulfuric acid was used as an indicator reagent and was added to reaction mixtures to determine remaining H_2O_2 concentrations.

The precision of the present novel assay was indicated by coefficients of variation of 4.09% within runs and 2.56% between runs. Moreover, in experiments with homogenized red blood cell solutions, peroxovanate and dichromate assays of catalase activities were highly correlated (r = 0.993). In further experiments, we demonstrated application of the peroxovanadate method to assessments of catalase activity in bacterial and liver homogenates. The present method is accurate, simple, rapid, and inexpensive and can be used for routine clinical measurements and scientific investigations.

Introduction

Glutathione peroxidase and catalase are key antioxidant enzymes that mitigate reactive oxygen species and free radicals, and protect lipids, DNA, and proteins from oxidative modification [1]. Although these enzymes compete as scavengers of H_2O_2, their relative contributions to H_2O_2 detoxification remain unclear [2]. Catalase is ubiquitously expressed and degrades H_2O_2 into oxygen and water [3], and protects against H_2O_2 that is produced by host immune cells to attack pathogens [4].

Numerous methods have been devised to assess catalase activity [5–20], and the most popular of these involves UV spectrophotometric determinations of H_2O_2 at 240 nm. However, because various proteins and DNA absorb UV light, this spectrophotometric method is not appropriate for assessments of catalase activity in protein containing biological solutions [8]. In addition, release of molecular oxygen gas from catalase reactions hampers spectrophotometric measurements, leading to low H_2O_2 sensitivity and failure to assess physiological levels of H_2O_2 (below 1.0 mM). Conversely, physiologically high substrate levels can inhibit catalase, further hampering assessments of activity [8,9]. Other methods assess changes in intact H_2O_2 concentrations or oxygen release from the decay of substrate. Oxygen production can be measured accurately using low-flow gas meters [10] or oxygen electrodes [11]. Other applicable methods employ iodometry [12], titrimetry [13], chemiluminiscence [14,15], polarimetry [16], and spectrophotometry [17,18]. Among these, recent assessments of catalase activity were performed using an iso-nicotinic acid hydrazide-pyrocatechol system to monitor catalytic consumption of H_2O_2 [19] according to the formation of a chromogenic complex with an absorption maxima at 490 nm. Another modern application was developed using a flow injection unit with an amperometric sensor that monitors unreacted H_2O_2 [20], but this method requires specific laboratory instruments.

Herein, we report a novel optimized spectrophotometric method for determining catalase activities in biological samples. To this end, we used a novel reagent to estimate catalase activities, and show the absence of interference from fats, amino acids, proteins, and sugars in biological samples.

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Materials and methods

Chemicals

All chemical reagents were purchased from commercial sources and were of analytical grade.

Principle

The principle of the method involves the reaction of ammonium metavanadate with H_2O_2 under acidic conditions, and depends on the reduction of vanadium (V) to vanadium (III) by H_2O_2. Although H_2O_2 is considered a strong oxidant, it can act as a reductant under certain redox conditions. Accordingly, reduction of vanadium (V) leads to the formation of a red–orange peroxovanadium complex, which has a maximum absorbance at 452 nm [21,22]. The reaction between vanadium and H_2O_2 is shown in the following equation (1) [23]:

$$H_2O_2 + NH_4VO_3 + H_2SO_4 \rightarrow NH_4[VO(O_2)SO_4] + 2H_2O \quad (1)$$

Catalase enzyme activity was determined by monitoring absorption of the red–orange peroxovanadium complex at 452 nm.

Reagents

Sulfuric acid solution (0.5 M) was prepared by appropriate dilution of concentrated sulfuric acid in 200 ml of distilled water. Ammonium metavanadate solution (0.01 M) contained 0.2925 g of ammonium metavanadate in 200 ml of 0.5-M sulfuric acid. Phosphate buffer (50 mM; pH 7.0) was prepared by mixing solutions a and b at a ratio of 1:1.5. Solution (a) was prepared by dissolving of 6.81 g of KH_2PO_4 in one liter of distilled water, and solution (b) was prepared by dissolving a 8.90 g of $Na_2HPO_4.2H_2O$ in one liter of distilled water. Fresh H_2O_2 (10 mM) solutions were prepared by mixing 0.1134 ml of 30% H_2O_2 with 100 ml of phosphate buffer, and the solution was adjusted to 10-mM using the molar extinction coefficient of H_2O_2 at 240 nm (43.6 M^{-1} cm^{-1}). Catalase standard solution was prepared by dissolving 20 mg of catalase enzyme powder (HiMedia; Product code: TC037, New Delhi, India) in 100 ml of 50-mM phosphate buffer solution (pH 7.0). Catalase was diluted to 5 U mL^{-1} and final catalase activity was adjusted using the dichromate method as described by Sinha [20] and modified by Hadwan [26].

Blood samples

Erythrocyte lysates were prepared using 4-ml aliquots of whole blood from a researcher at the Advanced Biochemistry Laboratory (Chemistry Dept., College of Science, University of Babylon, Iraq) following peripheral venous puncture. Heparinized test tubes were used to prevent coagulation and whole blood samples were centrifuged at 400 × *g* for 10 min. Subsequently, plasma and buffy coat were discarded after centrifugation and 500-µl aliquots of the resulting RBCs were washed three times in 5-ml aliquots of 0.9% NaCl solution and were centrifuged at 400 × *g* for 10 min after each wash. Two-ml aliquots of ice cold double distilled water were then transferred into test tubes containing 500 µl of erythrocyte mixtures (five-fold dilution), and were vortexed for 5 s and incubated for 15 min at 4 °C in the dark. Finally, the resulting 2.5 ml of five-fold, re-suspended stock hemolysates in phosphate buffer solution (0.05 M) were diluted to a dilution factor of 500. The resulting hemolysate solutions were used as a source of catalase activity.

Tissue preparations

Four-week-old broiler chickens were purchased from a local market at Hilla city, Iraq. Male albino mice and rats were purchased from the central animal house at the College of Science, University of Babylon, Iraq. Immediately before measurements of tissue catalase activities, rats were sacrificed and kidney and liver tissues were surgically excised. Livers were immediately washed in 0.9% (w/v) NaCl solution to remove contaminating blood and were then homogenized using a glass homogenizer and ice cold 1.15% (w/v) potassium chloride solution. Homogenate solutions were then filtered and diluted (at a ratio of 1:500) with 0.05 M phosphate buffer for analyses of catalase activity, which were performed immediately.

Table 1
Procedure for assessments of catalase activity.

Reagents	Test	Standard	Blank
Sample	1000 µl	–	–
Distilled water	–	1000 µl	3000 µl
Hydrogen peroxide	2000 µl	2000 µl	–
After mixing, test tubes were incubated for 2 min at 37 °C and the following reagent was added thereafter:			
Ammonium metavanadate reagent	2000 µl	2000 µl	2000 µl
After mixing, test tubes were incubated for 10 min at 25 °C and absorbance was recorded at 452 nm against a reagent blank.			

Procedure

Enzyme activity procedure was elucidated in (Table 1). The rate constant (k) of the first-order reaction equation for catalase activity was calculated using the following formula:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * log\frac{S^0}{S} \quad (2)$$

where t is time, S^0 is the absorbance of the standard solution, and S is the absorbance of the sample.

Results and discussion

In the present method, the ammonium metavanadate/sulfuric acid reagent acted as a catalase reaction stopper by denaturing the protein. Subsequently, H_2O_2 molecules reacted with ammonium metavanadate to form the peroxovanadium complex (equation (1)) and wavelength scans from 200 to 700 nm revealed absorption maxima at 452 nm (Fig. 1A). Catalase activity is directly proportional to the rate of H_2O_2 catalysis, and decreases in absorbance of the reduced vanadium complex were proportional to catalase activity (Fig. 1B). Catalase standard solution was prepared by dissolving 20 mg of catalase enzyme powder (HiMedia; Product code: TC037, New Delhi, India) in 100 ml of 50-mM phosphate buffer solution (pH 7.0). Catalase was diluted to 8 U mL^{-1} and final catalase activity was adjusted using the dichromate method as described by Sinha [20] and modified by Hadwan [26].

The present observations of the colored peroxovanadium complex showed that it has high stability at room temperature. In agreement, Nogueira et al. [21], reported no significant changes in the absorbance of peroxovanadium at 450 nm for 180 h at 25 °C.

Probable interferences of chemicals that could affect catalase activity measurements were assessed using the methods described by Hadwan and Abid [24]. Briefly, 9-ml solutions of various chemicals (Table 2) in 50 mM phosphate buffer (pH 7.4) were mixed with 1 ml catalase solutions of known activity (5 U/mL). Subsequently, catalase activity was determined using the dichromate method described by Sinha [20] and modified by Hadwan [25] and deviations from the final activity of 0.5 U mL^{-1} were recorded (Table 2).

Catalase activities in homogenized diluted red blood cell solutions were assessed using the present novel method and were compared with those determined using the dichromate method described by Sinha [20] and modified by Hadwan [25]. Matching samples and buffers were used