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Fluorimetry as a Simple and Sensitive Method for Determination of Catalase Activity

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Abstract

***Background*:** Catalase enzyme plays an important role in the anti-oxidation defense of body so it is important to measure its activity. Nowadays catalase activity measurement is performed by expensive imported kits in various scientific fields. The purpose of this study was to design a sensitive fluorimetry method for measuring catalase activity with improved sensitivity, accuracy and speed.

***Materials and Methods*:** In this study, the reaction of hydrogen peroxide with peroxidase (as a reaction accelerator) was used in fluorimetry for catalase activity measuring in serum samples in order to increase the sensitivity of the assay. The sensitivity and intra- and inter-assay accuracy, verification test, recovery and parallelism tests, comparison method and correlation and coherence investigation methods were also performed. In order to increase the accuracy and speed of reading, the assay was performed in microplates and reading was done in fluorimetry plates.

***Results*:** The percentage of intra- and inter-assay variation coefficients were measured 3.8-6.6 % and 4.1-7.3%, respectively. Comparison of the results of mentioned method for 50 serum samples with common colorimetric method showed a good correlation (0.917). In assessing the accuracy, the recovery percent was obtained 91% to 107%. The test sensitivity was measured 0.02 IU/ml.

***Conclusion*:** The fluorimetry method by microplate reading has a sufficient precision, accuracy and efficiency for catalase activity measuring as well as speed of measurement. Thus it can be an alternative method to conventional imported colorimetric methods.

Introduction

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) is an anti-oxidant enzyme that catalysis the breaks down of hydrogen peroxide (H_2O_2) into water and oxygen without causing free radicals [1]. This enzyme is known as a defense to oxidative stress [2]. Catalase is also applied as an early indicator of environmental inorganic pollution [3], as bacterial detector in food industry [4], and even for the detection of life on the moon [5]. Catalase exists in all aerobic cytochrome-containing mammalian cells. Catalase activity varies greatly from tissue to tissue; the highest activity is found in liver and kidney, whereas the lowest activity is seen in the connective tissue [6]. In eukaryotic cells, catalase is accumulated in organelles called peroxisome [7, 8]. Hydrogen peroxide production in eukaryotic cells is the final result of the reaction of superoxide dismutase (SOD) and various oxidases. Hydrogen peroxide accumulation may cause cellular damage through oxidation of proteins, DNA and lipids that finally lead to mutagenesis and cell death. The role of hydrogen peroxide in oxidative stress-related pathologies, such as inflammation, cancer, diabetes, cardiovascular disease, anemia, Parkinson's disease and Alzheimer's disease is fully studied [9]. The majority of conventional methods for measuring catalase activity use colorimetric reactions. In these reactions, the remaining hydrogen peroxide level (produced by catalase) reacts with chromogen. The amount of formed color is inversely related to enzyme activity [10, 11]. Up to now spectrophotometric [12, 13], Gasometry [14], luminometry [15] and fluorimetry [16] methods have been used to measure catalase activity. The sensitivity of emission methods such as fluorimetry is higher than absorption methods such as colorimetric method. The aim of this study was to design and optimize the sensitive fluorimetry method to quick and easy assay of catalase activity.

Materials and Methods

Analytical scale (Sartorius, Germany), 96-well ELISA plate reader (Sunrise, Tacan A-5082, Austria), 96-well fluorimetry plate reader (Flaster BMG, Austria) acidometer machine (Mettler MP-220, Switzerland) were applied in this study.

Chemicals and Enzymes: All chemicals and high purity enzymes include sodium phosphate, disodium phosphate, hydrogen peroxide, catalase, Resazurin (Fluorogen), peroxidase were purchased from Sigma or Merck. Black 96-wells microplates for fluorimetry were obtained from Nunc C, Denmark. In order to compare the results with commonly used methods, catalase assay kit was purchased from Randox, UK. Distilled water with <0.01 µs/ml conductivity was used.

Reaction Buffer: 100 mM phosphate buffer, pH 7.4 and 100 mM Tris buffer, pH 7.8 were prepared. The buffers were stored at 2-8℃ until use.

Hydrogen peroxide Solution: To prepare 10 mM hydrogen peroxide solution (molecular mass of 34, 30% solutions with 1.11 g/ml density), 102 µl of the mentioned mixture was resolved in phosphate buffer up to 100 ml. The buffer was stored at 2-8 ℃ until use. Fresh solutions with concentrations of 10 to 100 mM of mentioned hydrogen peroxide were prepared.

Standard solution of catalase with a specific activity: The solutions containing 0-500 IU/ml of purchased enzyme activity with 10 IU/ml activity in buffer were prepared. one IU/ml catalase activity was defined as an amount of the enzyme which is able to hydrolysis one µmol of hydrogen peroxide per minute at a 25℃ and pH 7.

Peroxidase solution with a specific activity: The solutions containing 0.1-1 IU/ml of purchased enzyme activity with 100 IU/ml activity in buffer were prepared. One IU/ml peroxidase activity was defined as an amount of the enzyme which is able to convert one µmol of pirogalol to purpurogalin per minute at a 25℃ and pH 7.

Flourogen substrate solution: The solutions with concentrations of 50 to 250 mM were prepared using dimethyl sulfoxide solvent and the reaction buffer. This solution is sensitive to light so it is stored in dark or aluminum foil-wrapped containers.

Sample: Fifty 5ml sera samples were obtained from normal volunteer people who had been referred to Taleghani hospital to check their health status. To separate the sera, the blood samples were put in conventional tubes. After 10 min incubation at room temperature the centrifugation was conducted for 15 minutes at 3500 rpm.

Optimization of the method: The optimum conditions (time, concentration of hydrogen peroxide and concentration of flourogen) were determined as described below on five specimens based on recovered test method. To measure catalase activity, 50 µl of serum sample and 50 µl of hydrogen peroxide solution were added to each well of fluorescence-specific 96-wells microplate (Nunc C, Denmark) and incubated for 15 min at 37℃. Then 50 µl peroxidase and 50 µl of Resazurin flourogen was added to the wells and fluorimetry reading was performed with excitation at 560 nm and emission at 590 nm. The standard curve was plotted by using the fluorescence signal on the vertical axis and catalase activity on the horizontal axis. In each microplate standards with 0, 0.5, 1, 2, 3 and 4 IU/ml activity were used. Plotting the logarithm of the average fluorescence signal of standards on vertical axis and standards activities on the horizontal axis, the standard curve was plotted. Catalase activity was measured using the regression of the mentioned curve. Distilled deionized water was considered as the zero standards. The related activity to the average signal of zero standard with 10 times repeat plus twice of its standard deviation, was applied as the basis of threshold determination of the method. Serial dilution of a serum sample was used for confirmation. Distilled deionized water was used for diluting. In order to assess the accuracy of the test, inter- and intra-assay of the samples with low, medium and high concentration with eight replicates were used. Catalase activity measurement was used as the basis of the recovery test after adding different standards to 6 random samples with three times repeat. The adding of zero standards was used as the control. Catalase activity measurement in 6 samples with low, medium and high concentration after continuous dilutions with three time repeats was used as the basis of the parallel test. The results of this method were analyzed beside the results of the colorimetric method in 50 samples. The obtained data were analyzed by SPSS software. Quantitative variables were expressed as mean±standard deviation. Pearson regression test was used to evaluate the correlation between the two methods. As mentioned before, the percent of variation coefficient was used for describe the accuracy of the two methods.

Results

In different conditions of time and hydrogen peroxide and flourogen concentration, catalase activity recover in five samples showed that 15 minutes, 50 µl of hydrogen peroxide and 50 µl of flourogen can be considered as optimal conditions. According to the concentration of the average signal of zero standard with 10 times repeat plus twice of its standard deviation, the limit of detection was determined 0.02 IU/ml. The percent of intra-assay variation coefficient of samples with low, medium and high concentrations were determined 6.6, 3.8 and 5.7, respectively. These coefficients for inter-assay were 7.3, 4.1 and 6.2, respectively (Tables 1 and 2). As shown in Table 3, the recoveries of 6 studied samples were obtained 91-107 %. After continuous dilution up to 1:32, the ratio of the measured concentration to the expected concentration was determined from 0.83 to 1.45, respectively (Table 4). Regression analysis of the present fluorimetry results in comparison with conventional colorimetric method, showed an acceptable correlation (0.917) (Figure 1).

Table 1. Results of intra-assay variation coefficient study of catalase activity measurement using fluorimetry and microplate readings with eight repeats

Variation coefficient %	Rpeats number	Mean±SD
6.6	8	1.20±0.0792
3.8	8	2.40±0.0912
5.7	8	3.60±0.2052

Table 2. Results of inter-assay variation coefficient study of catalase activity measurement using fluorimetry and microplate readings with eight repeats

Variation coefficient %	Repeats number	Mean±SD
7.3	8	1.50±0.1095
4.1	8	2.50±0.1025
6.2	8	3.55±0.2201

Table 3. Results of recovery test of catalase activity measurement using fluorimetry and microplate reading

Recovery %	Sample Conc.	Expected	Measured	Standard
91	2	1.25	1.14	0.5
93	2	1.50	1.39	1
109	2	2	2.10	2
96	2	2.50	2.40	3
107	2	3	3.21	4

Table 4. Results of parallel test in catalase assay using fluorimetry and microplate reading

Percent ratio	Measured (Iu/ml)	Expected (Iu/ml)	Dilution
100	3.96	3.96	1
105	2.07	1.98	2
91	0.90	0.99	4
94	0.46	0.49	8
92	0.23	0.25	16
84	0.10	0.12	32

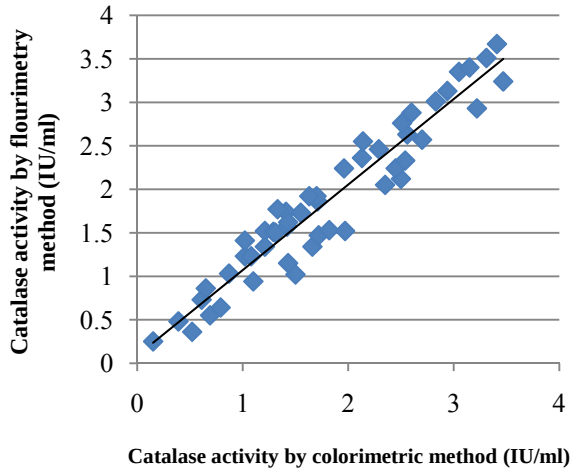

Figure 1. Comparison of the results of catalase activity assay using fluorimetry and microplate readings with the results of conventional colorimetric method in 50 sera samples

Discussion

Hydrogen peroxide is a normal metabolite in living cells [17]. However, hydrogen peroxide removal is important for cells because the excess amount of hydrogen peroxide can oxidize cellular components. Catalase is involved in the breakdown of hydrogen peroxide in cells. Studies on erythrocytes [18-20], hepatocytes [21] and cultured fibroblasts [22] showed that catalase is effective in rather high concentrations of hydrogen peroxide. In 1964, Lente et al. conducted a study to determine catalase activity by a colorimetric method using titanium sulfate. In this method a yellow complex of titanium sulfate and hydrogen peroxide was formed, but its sustainability was about 10 minutes [23]. In 2003, in order to determination of catalase activity by a derived fluorimetry method from a fluorescent substrate a study was done. In this method, hydrogen peroxide in hydrogen peroxide -tetracycline-EU (III) system is consumed by catalase. The detection limit for catalase was calculated 1 IU/ml that is less than our method [24]. In this study, considering the importance of catalase activity measurement, the enzyme activity in human serum samples was assayed by the sensitive fluorimetry method. In fact, in this study, the all of three approaches in the improvement of the enzyme activity measurement that have been reported separately, were used after the reaction condition optimization to finally achieve the method includes all of the mentioned benefits. In the first approach, most of the reported methods and available kits apply the colorimetric methods and different chromogen that often have low solubility. But in the present method, the fluorescent signals resulting from the flourogen oxidation system was used to increase the sensitivity and despite insoluble chromogens, it had a higher solubility in water and produced more signal. In the second approach, although the reaction of catalase function product and flourogen is occurred spontaneously, to improve the efficiency and reduce the time, peroxidase enzyme (HRP) was used to accelerate the final reaction rate. In this manner, the total test time was reduced at least 5 minutes. Finally, as the third step in this study to enhance the accuracy of the method, 96 wells microplate reading was used. By applying of this change, in addition to improving the accuracy and the results reproducibility, the time was also reduced. In addition of increasing the measurement speed, fluorimetry method using microplate reading have a higher efficiency, accuracy, correctness and sensitivity to measure catalase activity so it may be a good alternative method to the conventional imported colorimetric methods.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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