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Measurement of Catalase Activity Using Catalase Inhibitors

Kazunori Takemoto[1], Ami Ozaki[2], Masayuki Yagi[3], Hideya Ando[2]

[1]Medical Science Education Center, Okayama University of Science, Okayama, Japan
[2]Department of Applied Chemistry and Biotechnology, Okayama University of Science, Okayama, Japan
[3]Rosette Co., Ltd., 3-26-10, Higashi-Shinagawa, Tokyo, Japan
Email: k-takemoto@ous.ac.jp

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Abstract

Catalase is an enzyme that scavenges hydrogen peroxide in the body and has the role of protecting the organism from oxidative stress. Since catalase activity is associated with various diseases, including diabetes, skin diseases like vitiligo, renal failure, and heart failure, it is important to measure its activity. However, it has been difficult to accurately evaluate catalase activity alone, because there are other substances *in vivo*, such as iron ions, that decompose hydrogen peroxide in addition to catalase. To solve this problem, we conducted a study to develop a method to correctly measure catalase activity from samples containing impurities with hydrogen peroxide removal activity. In this study, catalase inhibitors were added to bovine catalase solution, ferric chloride solution, cell lysates of control cells and experimentally generated catalase knockdown cells (CAT KD), and these mixtures were reacted with hydrogen peroxide to determine the percentage of hydrogen peroxide remaining in the reaction solution after a certain time. The catalase inhibitors used, 3-amino-1H-1,2,4-triazole (3-AT) and sodium azide (NaN_3), inhibited the removal of hydrogen peroxide by bovine catalase at a high rate in *in-vitro* experiments. However, these catalase inhibitors did not inhibit hydrogen peroxide removal in the Fenton reaction of iron ion and hydrogen peroxide in *in-vitro* experiments. On the other hand, hydrogen peroxide removal by cell lysate was inhibited by the addition of 3-AT or NaN_3. The inhibitory effect was equivalent or superior to that of CAT KD cells, in which catalase was experimentally knocked down. These results suggested that 3-AT and NaN_3 specifically inhibit hydrogen peroxide removal of catalase. Through these studies, we found that when cell lysate with a catalase inhibitor was mixed with hydrogen peroxide, hydrogen peroxide that was not removed by catalase inhibition remained in the test tube after a certain time, and this residual hydrogen

peroxide reflected the hydrogen peroxide removal activity of catalase. By measuring this unremoved hydrogen peroxide, it was possible to evaluate catalase activity from samples containing impurities that have hydrogen peroxide removal properties.

Keywords

Catalase, Hydrogen Peroxide, Inhibitor, Measurement Method

1. Introduction

Catalase (EC 1.11.1.6.) is an enzyme found in the cells of humans and other animals, plants, and microorganisms. Intracellularly, it accumulates in peroxisomes and is responsible for the detoxification of hydrogen peroxide, a reactive oxygen species, into water and oxygen [1]. In mice, impaired function of the islets of Langerhans has been reported due to decreased catalase activity [2]. Furthermore, tissue deposition of carbonylated proteins [3] and impaired erythrocyte membrane function [4] due to decreased catalase activity have been reported. Reduced catalase activity in humans has been associated with the development of diabetes [5], renal and cardiac failure [6] [7], and vitiligo of the skin [8]. The diseases associated with catalase deficiency are varied [9]. Therefore, it is important to assess the activity of catalase, which is closely related to hydrogen peroxide levels.

To measure catalase activity, residual hydrogen peroxide after the reaction in which hydrogen peroxide is decomposed and removed by catalase is often detected by a colorimetric or fluorescence reaction [10] [11]. These detection methods are easy to operate and enable measurement of catalase activity in a short time. Another method is to check the oxygen bubbles generated when catalase reacts with hydrogen peroxide [12], but in this case, it is difficult to measure in a small quantity. On the other hand, there are other substances besides catalase that detoxify hydrogen peroxide. Glutathione peroxidase is a known antioxidant enzyme that removes hydrogen peroxide and lipid peroxide using reduced glutathione [13]. Furthermore, it is well known that iron ions (Fe^{2+}) react with hydrogen peroxide in a Fenton reaction [14]. Because there are multiple substances that remove hydrogen peroxide in this way, the decomposition of hydrogen peroxide is also referred to as a "catalase-like reaction". The general method of measuring catalase is based on the principle of measuring the concentration of residual hydrogen peroxide in the reaction solution of cell lysate and hydrogen peroxide by colorimetric or fluorescence intensity. Therefore, if a substance that removes hydrogen peroxide is mixed in other than catalase, as in the case of biological samples used for general measurements, what is actually being measured is "catalase-like activity," and it is difficult to evaluate only catalase activity strictly. As far as we could find, there is no known method to measure only catalase activity in cell lysates,

and the catalase activity actually measured is the hydrogen peroxide removal activity by various substances, including catalase.

The hydrogen peroxide decomposition reaction by catalase consists of a two-step reaction as follows [9] [15]:

Equation (1):

$$\text{Enzyme}\left[\text{Porphyrin Fe}\left(\text{III}\right)\right]+\text{H}_2\text{O}_2 \rightarrow \text{Compound I}\left[\text{Porphyrin Fe}\left(\text{IV}\right)=\text{O}\right]+\text{H}_2\text{O}$$

Equation (2):

$$\text{Compound I}\left[\text{Porphyrin Fe}\left(\text{IV}\right)=\text{O}\right]+\text{H}_2\text{O}_2 \rightarrow \text{Enzyme}\left[\text{Porphyrin Fe}\left(\text{III}\right)\right]+\text{H}_2\text{O}+\text{O}_2$$

In the reaction Equation (1), Fe(III) is oxidized to Fe(IV) by withdrawing a proton from hydrogen peroxide to become Compound I. In the reaction Equation (2), Fe(IV) is returned to Fe(III) by the reduction of Compound I by hydrogen peroxide, different from Equation (1), and at this time, oxygen molecules and water are simultaneously generated. In this two-step reaction, sodium azide (NaN_3) and 3-amino-1H-1,2,4-triazole (3-AT) selectively inhibit the hydrogen peroxide scavenging reaction of catalase [16] [17] NaN_3 inhibits the reaction of heme iron [Porphyrin Fe(III)] in the above reaction Equation (1), and 3-AT is thought to inhibit the reaction of Compound I [Porphyrin Fe(IV) = O] in the above reaction Equation (2).

Therefore, to solve the problem that catalase activity cannot be accurately evaluated only by measuring hydrogen peroxide removal activity, a new study was conducted to evaluate catalase activity from samples contaminated with various substances with hydrogen peroxide removal activity using 3-AT or NaN_3, which have catalase inhibitory effects. We propose a new method to evaluate only catalase activity from samples contaminated with various substances having hydrogen peroxide removal activity.

2. Materials and Methods

2.1. Reagents

Bovine catalase, hydrogen peroxide, NaN_3, 3-AT, iron(II) chloride tetrahydrate, and RIPA Buffer were purchased from Fujifilm Wako Pure Chemicals Co. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to measure proteins in cell lysates. OxiSelect™ Hydrogen Peroxide Assay Kit (Cell Bio Labs., Inc.) was used to measure hydrogen peroxide, and the fluorescence reaction in which the fluorescent substrate ADHP (10-acetyl-3,7-dihydroxyphenoxazine) generates resorufin in the presence of peroxidase was used. The fluorescence reaction was utilized.

2.2. *In Vitro* Catalase Inhibition by NaN_3 and 3-AT

Bovine catalase (1.0 Units/mL) dissolved in PBS was mixed with hydrogen peroxide (final concentration 50 μM) and allowed to react for 30 minutes at room

temperature, and the hydrogen peroxide concentration in the mixture was measured. 3-AT (25, 50 mM) or NaN_3 (0.1, 0.5 mM) prepared in PBS was added to the same reaction solution as a catalase inhibitor, and the reaction was carried out at room temperature for 30 minutes, and the hydrogen peroxide concentration was measured. The hydrogen peroxide concentration was set to be measured with the OxiSelect™ hydrogen peroxide assay kit, and the same concentration of hydrogen peroxide was used in subsequent experiments. The OxiSelect™ Hydrogen Peroxide Assay Kit's fluorescence reaction was used to measure the hydrogen peroxide concentration, and the Micro Plate Reader MTP-900 (CORONA ELECTRIC) was used to measure the fluorescence. From the measured hydrogen peroxide concentration, the hydrogen peroxide residual rate was determined and compared.

2.3. Decomposition of Hydrogen Peroxide by Iron Ions and Its Inhibition

Hydrogen peroxide solution prepared with PBS and iron(II) chloride tetrahydrate (final concentration 0-200 μM) prepared with pure water were mixed and allowed to react for 30 minutes at room temperature, and the residual hydrogen peroxide concentration in the reaction solution was measured using the fluorescence reaction of the OxiSelect™ hydrogen peroxide assay. The residual hydrogen peroxide concentration was also measured after a similar reaction by adding NaN_3 or 3-AT to the same reaction solution.

2.4. Preparation of Low Catalase Activity Cells by siRNA

Human dermal fibroblasts were cultured in Dulbecco's Modified Eagle Medium (SIGMA) containing 1% Antibiotic Antimycotic Solution (SIGMA), 10% fetal bovine serum (SIGMA). Cells were maintained at 37˚C, 5% CO_2. For siRNA transfection, cells were seeded in 6 well plates (20×10^4/well). 24 hours after seeding, Control siRNA-A: sc-37007, or Catalase siRNA(h): sc-45330 (Santa Cruz Biotechnology, Inc.) was transfected into cells using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific), respectively. Dilution of siRNA and Lipofectamine RNAiMAX Reagent for transfection and other methods followed the standard protocol for Lipofectamine RNAiMAX Reagent. 48 hours after siRNA transfection, cells were detached and harvested with 0.25% Trypsin-EDTA (Gibco) and washed with PBS. RIPA buffer with protease inhibitor cocktail (Sigma) was added to the cell pellet. Lysis was achieved by pipetting, and the cell lysate was obtained by centrifugation of the lysed solution. Cell lysates were stored frozen at −20˚C until used in subsequent experiments after total protein concentration was determined by the BCA method.

2.5. Western Blotting Analysis

The efficiency of siRNA transfection was evaluated by Western blotting. Proteins extracted from each cell were electrophoresed (15 μg/Lane) and transferred to

nitrocellulose membranes. Primary antibodies were Catalase (H-9) sc-271803 and β-actin (C4) sc-47778, respectively, and secondary antibody was m-IgG$_k$ BP-HRP sc-516102 (Santa Cruz Biotechnology, Inc.). The protein bands were confirmed by detecting the luminescence emitted by Clarity™ Western ECL Substrate (Bio Rad) using ChemiDoc™ XRS+ with Image Lab™ Software (Bio Rad).

2.6. Hydrogen Peroxide Removing by Cell Lysate and Its Inhibitory Effect by Catalase Inhibitor

Hydrogen peroxide (final concentration 50 μM) was added to cell lysate (total protein content prepared in PBS to a final concentration of 0.02 μg/μL) in which RIPA Buffer was added to the cell pellet with protease inhibitor cocktail (Sigma), and the reaction was carried out at room temperature. The percentage of hydrogen peroxide residue was determined. 3-AT (final concentration 50 mM) or NaN_3 (final concentration 0.5 mM) prepared in PBS as a catalase inhibitor was added to the cell lysate, and hydrogen peroxide was added to the lysate.

2.7. Calculation of Catalase Activity

In the reaction of cell lysate and hydrogen peroxide, the amount of hydrogen peroxide remaining after the addition of the catalase inhibitor was defined as unremoved hydrogen peroxide, which was converted to known bovine catalase activity.

2.8. Statistical Evaluation

The measurement results obtained were presented as mean ± SD. Data were also evaluated using the Student's T-test, where $p < 0.05$ was judged to be significantly different.

3. Results

3.1. 3-AT and NaN_3 Inhibited the Hydrogen Peroxide Removing Reaction by Bovine Catalase *in Vitro*

Bovine catalase diluted in PBS was reacted with hydrogen peroxide *in vitro* for 30 minutes at room temperature, and the percentage of hydrogen peroxide remaining was determined and used as a control. The residual rate of hydrogen peroxide was also determined by adding 3-AT or NaN_3 as a catalase inhibitor to the same reaction and comparing it with the control. Hydrogen peroxide was removed at a high rate in the control. On the other hand, in the reaction solution to which 3-AT was added, the residual rate of hydrogen peroxide increased significantly as the concentration of 3-AT increased, confirming that the 50 mM 3-AT solution inhibited the hydrogen peroxide reaction of bovine catalase at a high rate (**Figure 1(a)**). In the reaction solution with NaN_3, a significant increase in hydrogen peroxide residual was also observed as the concentration of NaN_3 increased, confirming that the 0.5 mM NaN_3 solution inhibited the hydrogen peroxide removal reaction of bovine catalase at a high rate (**Figure 1(b)**).

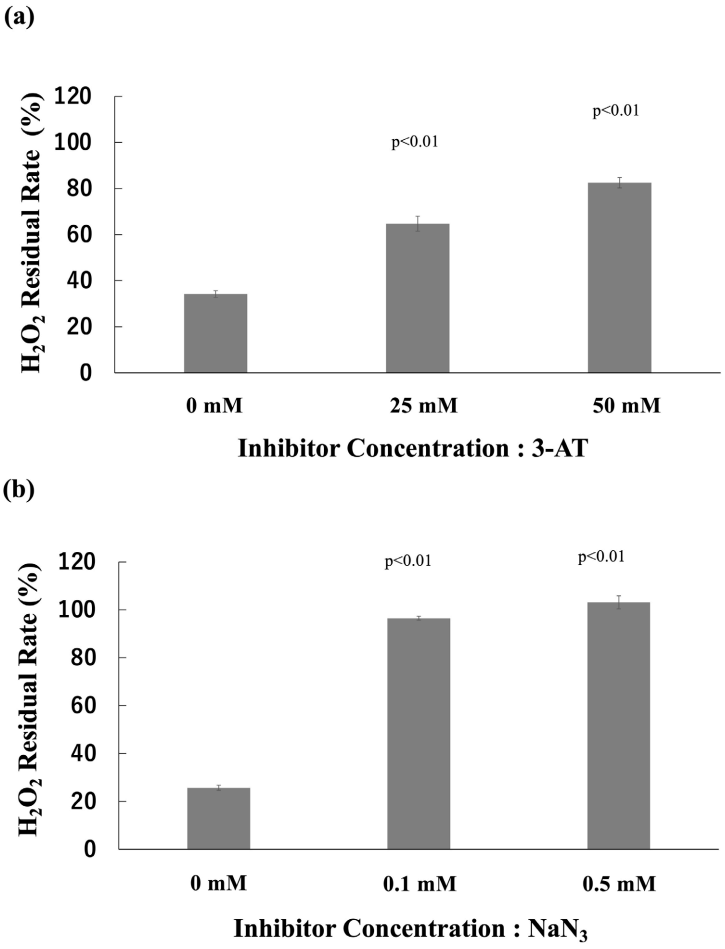

Figure 1. (a) 3-AT inhibited the hydrogen peroxide scavenging reaction of catalase. Hydrogen peroxide (final concentration 50 μM) was added to a mixture of bovine catalase (1.0 Units/mL) and 3-AT (25,50 mM) and allowed to react for 30 minutes at room temperature. The percentage of hydrogen peroxide remaining in the mixture was determined and expressed as mean ± SD (n = 3). Statistical analysis was performed by Student's T-test and compared with the percentage of hydrogen peroxide remaining at 3-AT 0 mM. $p < 0.01$: There was a significant difference of $p < 0.01$ between the 0 mM group and the 3-AT 25, 50 mM group. (b) NaN_3 inhibited hydrogen peroxide scavenging of catalase. Hydrogen peroxide (final concentration 50 μM) was added to a mixture of bovine catalase (1.0 Units/mL) and NaN_3 (0.1, 0.5 mM) and allowed to react for 30 minutes at room temperature. The percentage of hydrogen peroxide residues in the mixture was measured and expressed as mean ± SD (n = 3). Statistical analysis was performed by Student's T-test. $p < 0.01$: There was a significant difference of $p < 0.01$ between the 0 mM group and the NaN_3 0.1, 0.5 mM group.

3.2. 3-AT and NaN_3 Did Not Affect the Fenton Reaction with Iron Ions and Hydrogen Peroxide *in Vitro*

To determine whether 3-AT and NaN_3 inhibit the Fenton reaction between iron ions (Fe^{2+}) and hydrogen peroxide, the residual hydrogen peroxide was measured after the reaction of hydrogen peroxide with aqueous iron(II) chloride tetrahydrate solutions (0 - 200 μM) for 30 minutes *in vitro* at room temperature. The hydrogen peroxide residual percentage was also determined when the catalase

inhibitor 3-AT or NaN_3 was added to the same reaction, and compared to the hydrogen peroxide residual percentage when only iron(II) chloride tetrahydrate solution was added. As the concentration of the iron(II) chloride tetrahydrate solution increases, the hydrogen peroxides residual rate in the reaction solution decreased, and the iron ions reacted more with hydrogen peroxide *in vitro*. The percentage of hydrogen peroxide remaining in the reaction solution with 3-AT or NaN_3 was comparable to that of iron(II) chloride tetrahydrate solution alone, indicating that hydrogen peroxide reacted with iron(II) at a high rate. The reaction of hydrogen peroxide with iron(II) chloride tetrahydrate solution was not affected by these catalase inhibitors (Figure 2).

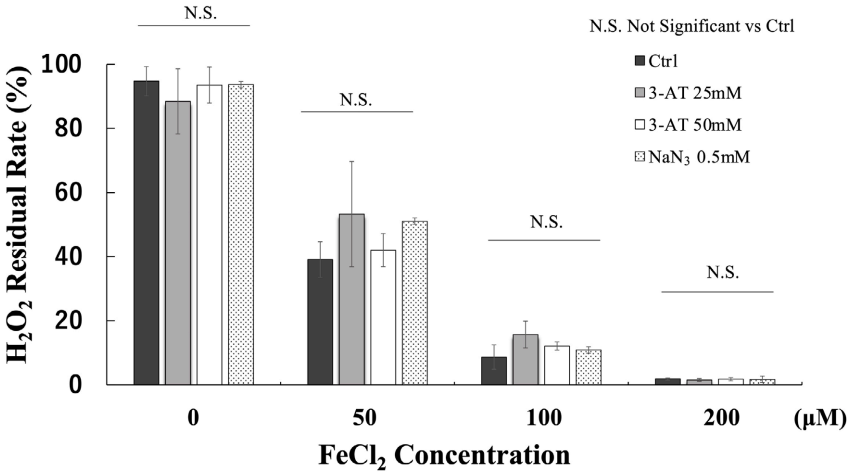

Figure 2. 3-AT and NaN_3 did not affect the reaction between iron ions and hydrogen peroxide. Hydrogen peroxide (final concentration 50 μM) was added to a mixture of aqueous iron(II) chloride tetrahydrate solution and 3-AT (25, 50 mM) or NaN_3 (0.5 mM) and allowed to react for 30 minutes at room temperature. Hydrogen peroxide residuals were expressed as mean ± SD. Statistical analysis was performed by Student's T-test to compare the percentage of hydrogen peroxide residues among inhibitors at each concentration of iron(II) chloride tetrahydrate. Closed bar indicates Ctrl, Shaded bar indicates 3-AT (25 mM), Open bar indicates 3-AT (50 mM), and Dot bar indicates NaN_3 (0.5 mM).

3.3. Hydrogen Peroxide Removal Inhibited by 3-AT and NaN_3 Was Equal to or Better than Hydrogen Peroxide Removal in CAT KD Cells

To confirm that hydrogen peroxide removing by cell lysate is a function of catalase, hydrogen peroxide was added to the cell lysate of human dermal fibroblasts (control cells) and CAT KD cells (cells in which catalase was knocked down by siRNA introduction into human dermal fibroblasts) (Figure 3(a)). The residual hydrogen peroxide rate was determined after a 30-minute reaction at room temperature. The same experiment was also performed by adding catalase inhibitor to cell lysates obtained from control cells or CAT KD cells. The lysate from control cells showed a high rate of hydrogen peroxide removing. The addition of 3-AT or NaN_3 as catalase inhibitors to the reaction inhibited hydrogen peroxide removing, and CAT KD cell lysates did not remove hydrogen peroxide well. These results

indicate that hydrogen peroxide added to the cell lysate is removed by catalase. Furthermore, the addition of catalase inhibitors inhibited the removal of hydrogen peroxide as much as or more than catalase knockdown (Figure 3(b)). In the reaction of cell lysate with hydrogen peroxide, the amount of hydrogen peroxide remaining after addition of 3-AT or NaN_3 was considered as un-removed hydrogen peroxide, which was converted to known bovine catalase activity, which was 0.58 ± 0.06 Unit/ml for control cells and 0.05 ± 0.02 Unit/ml for control cells and 0.05 ± 0.02 Unit/ml for CAT KD cells. These results indicated that the amount of un-removed hydrogen peroxide by the addition catalase inhibitor reflected the catalase activity.

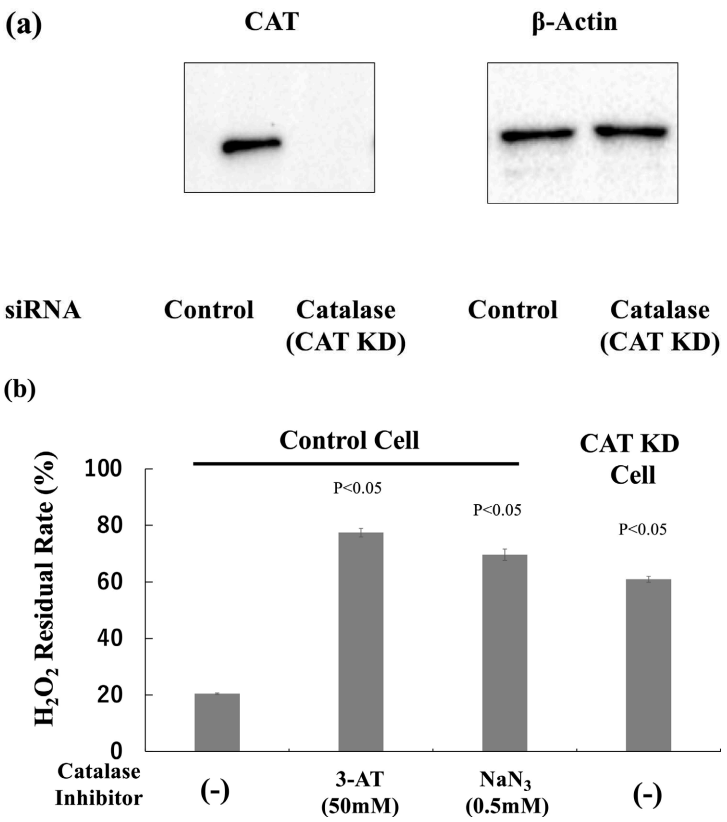

Figure 3. (a) Expression of Catalase in low-catalase-active cells created by siRNA transfection. Knockdown of catalase was confirmed by Western blotting from low-catalase human dermal fibroblasts (CAT KD) created by siRNA Catalase transfection. (b) 3-AT and NaN_3 inhibited hydrogen peroxide removing by cell lysate as much as or more than CAT KD cells. *In vitro*, hydrogen peroxide was added to cell lysate and CAT KD cells, and the residual hydrogen peroxide rate after the reaction was measured. At the same time, hydrogen peroxide was added to a mixture of cell lysate and catalase inhibitor (3-AT, NaN_3), and the residual hydrogen peroxide rate after the reaction was measured. $p < 0.05$: Significant difference was observed between the control cell CAT Inhibitor (−).

4. Discussion

In this study, we have provided a new method to evaluate catalase activity from cell lysates containing a mixture of several hydrogen peroxide scavengers. Currently,

many companies offer measurement kits for catalase activity, and most of them use a method in which a sample is reacted with hydrogen peroxide, and the residual hydrogen peroxide in the reaction solution is measured by colorimetric or fluorescence analysis. However, it has been difficult to accurately determine catalase activity alone when using living cells or cell extracts as samples, because they are affected by hydrogen peroxide degradation by substances other than catalase. To solve this problem, we devised a new method for measuring catalase activity using catalase inhibitors.

First, we confirmed the removing of hydrogen peroxide by catalase using a commercial aqueous solution of bovine catalase powder. Bovine catalase removed hydrogen peroxide at a high rate, and the residual hydrogen peroxide in the reaction solution was significantly reduced. However, when 3-AT was added to the same reaction solution, the residual hydrogen peroxide rate increased due to the catalase inhibitory effect. Similarly, the addition of NaN_3 also increased the percentage of hydrogen peroxide residue due to the catalase inhibitory effect. These results indicate that the added inhibitors prevented catalase from scavenging hydrogen peroxide. 3-AT and NaN_3 have been reported to inhibit catalase in various previous studies [16] [17].

Iron ion is a possible scavenger of hydrogen peroxide, and the reaction of iron ion with hydrogen peroxide *in vitro* resulted in a decrease in residual hydrogen peroxide in the reaction solution (**Figure 2**). The addition of NaN_3 or 3-AT to the reaction of iron ions with hydrogen peroxide left a low percentage of hydrogen peroxide remaining, indicating that the Fenton reaction is not inhibited by these catalase inhibitors. It is important to note that NaN_3 and 3-AT do not inhibit the reaction of hydrogen peroxide with iron ions because the inhibitor-based catalase assay method developed in this study requires specific inhibition of catalase in the cell lysate.

Hydrogen peroxide was removed when hydrogen peroxide was added to cell lysate, but its remotion was highly inhibited when hydrogen peroxide was added to cell lysate containing a catalase inhibitor (**Figure 3(b)**). This indicates that hydrogen peroxide scavenging by cell lysate is due to the action of catalase; the residual hydrogen peroxide rate was lower in cell lysate from CAT KD cells than in control cell lysate with a catalase inhibitor. This was thought to be due to the fact that catalase could not be completely eliminated by siRNA knockdown, and the slight amount of residual catalase removed hydrogen peroxide (**Figure 3(b)**). Therefore, catalase inhibition by 3-AT and NaN_3 is more potent than catalase gene knockdown by siRNA in inhibiting hydrogen peroxide removing, and the residual hydrogen peroxide rate after reaction of catalase inhibitor with hydrogen peroxide in CAT KD cell lysate was similar to that in control cell lysate. The residual hydrogen peroxide rate in CAT KD cell lysate reacted with hydrogen peroxide was similar to that in the same experiment using control cell lysate (data not shown).

Glutathione is known to detoxify hydrogen peroxide. A study of 3-AT treatment of keratinocytes for catalase inhibition reported increased activity of glutathione

peroxidase and glutathione reductase [18], which is consistent with the results of the 3-AT, meaning that the hydrogen peroxide scavenging pathway by glutathione peroxidase and glutathione reductase is not inhibited by 3-AT. These results suggest that 3-AT and NaN_3 specifically inhibit catalase.

Since a certain amount of hydrogen peroxide scavenging is observed even when catalase is inhibited, it is clear that the hydrogen peroxide scavenging effect of the cell lysate is a combined effect of various substances including catalase. The difference in residual hydrogen peroxide between catalase inhibition and non-inhibition in this reaction system can be used to determine the amount of un-removed hydrogen peroxide due to catalase inhibition (**Figure 4**). Since the un-removed hydrogen peroxide reflects the amount of catalase inhibition, hydrogen peroxide scavenging by substances other than catalase can be disregarded. We considered that the amount of un-removed hydrogen peroxide which was added to the catalase inhibitor could be evaluated comparatively as catalase activity.

The method provided in this study enables us to evaluate the contribution ratio of catalase to the hydrogen peroxide removal and can be applied to the search for catalase activators. In the future, if we relate this method to the evaluation of catalase activity in living cells, we will be able to evaluate the relationship between catalase and other substances that scavenge hydrogen peroxide (e.g., glutathione).

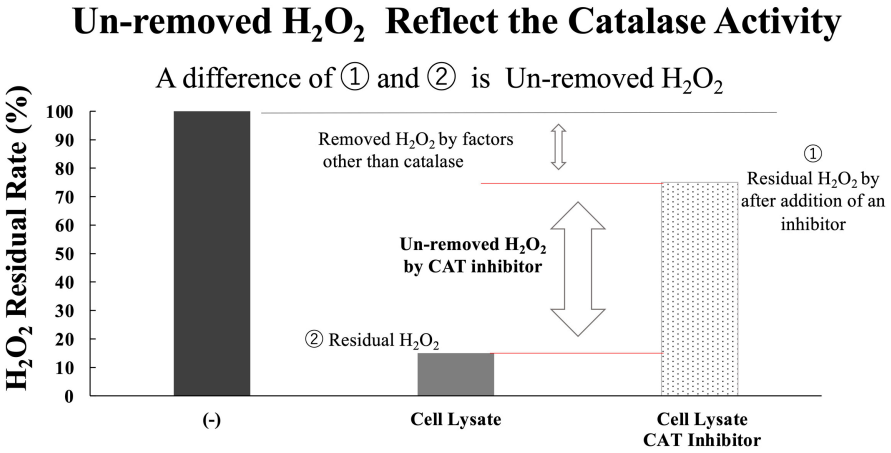

Figure 4. Evaluation method of catalase activity. When hydrogen peroxide is added to a solution that does not contain cell lysate (e.g. PBS), hydrogen peroxide is not removed (closed bar), but when hydrogen peroxide is added to cell lysate, hydrogen peroxide is removed to some extent over time and a small amount of hydrogen peroxide remains (② shade bar in **Figure 4**). When hydrogen peroxide is added to cell lysate containing a catalase inhibitor, hydrogen peroxide is removed by substances other than catalase, but catalase is inhibited, so a large amount of hydrogen peroxide remains (**Figure 4**, ① dot bar). The difference between ① and ② indicates un-removed hydrogen peroxide due to catalase inhibition, which reflects the inhibited catalase activity. Therefore, un-removed hydrogen peroxide exhibits catalase activity.

5. Conclusion

We have developed an accurate method to evaluate catalase activity from cell

lysates containing impurities with hydrogen peroxide scavenging activity using catalase inhibitors. In this study, 3-AT or NaN_3, which specifically inhibits catalase, was added to the subject samples and reacted with hydrogen peroxide. The results showed that the difference in the amount of hydrogen peroxide remaining in the samples with or without the inhibitors is the true amount of hydrogen peroxide removed by catalase, which reflects the catalase activity.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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