Free Radical Research, September 2010; 44(9): 1036–1043

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A novel analytical method to evaluate directly catalase activity of microorganisms and mammalian cells by ESR oximetry

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(Received date: 31 March 2010; In revised form date: 17 May 2010)

Abstract

Electron spin resonance (ESR) oximetry technique was applied for analysis of catalase activity in the present study. Catalase activity was evaluated by measuring oxygen from the reaction between hydrogen peroxide (H_2O_2) and catalase-positive cells. It was demonstrated that the ESR spectra of spin-label probes, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (4-oxo-TEMPO) and 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (4-maleimido-TEMPO) in the presence of H_2O_2 were broadened with the concentrations of catalase. It was possible to make a calibration curve for catalase activity by peak widths of the spectra of each spin-label probe, which are broadened dependently on catalase concentrations. The broadened ESR spectra were also observed when the catalase-positive micro-organisms or the mammalian cells originally from circulating monocytes/macrophages were mixed with TEMPOL and H_2O_2. Meanwhile, catalase-negative micro-organisms caused no broadening change of ESR spectra. The present study indicates that it is possible to evaluate directly the catalase activity of various micro-organisms and mammalian cells by using an ESR oximetry technique.

Keywords: *Catalase activity, ESR, oximetry, bacteria, mammalian cells, analytical method*

Abbreviations: *ESR, electron spin resonance; ROS, reactive oxygen species; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-piperidine 1-oxyl; 4-oxo-TEMPO, 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy; 4-maleimido-TEMPO, 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy; GSH, reduced glutathione; GPx, glutathione peroxidise.*

Introduction

It is well known that aerobically respiring organisms can effectively generate adenosine triphosphate (ATP) for vital activity by utilizing oxygen as a terminal electron acceptor, which is the so called 'oxidative phosphorylation' in electron transfer systems [1,2]. During the aerobic respiration, some of the oxygen molecules, however, are converted to reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical, which cause oxidative damage in tissues or cells [3–5]. It is suggested that the ROS play a critical role in the pathology of some diseases, such as Parkinson's disease, rheumatoid arthritis and ischaemic attacks [6]. Accordingly, organisms living under aerobic conditions are equipped with enzymatic defense mechanisms by which ROS are suppressed. Catalase, one of such enzymes, takes on a part of the defense mechanisms and catalyses a reaction in which H_2O_2 is decomposed into water and oxygen [7–9]. Therefore, measuring the catalase activity of living organisms is a valuable key factor to evaluate the resistance against oxidative stress.

Catalase activity of living organisms including bacteria is usually determined by spectrophotometric

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd.
DOI: 10.3109/10715762.2010.495750

analysis as reported by previous studies [10–14]. In brief, a known concentration of H_2O_2 and a sample containing catalase are mixed and incubated. Then, the remaining H_2O_2 level is determined spectrophotometrically [15]. This method is applied for liquid samples such as serum and plasma, tissue homogenate and cell lysate, whilst it is least suitable for measuring catalase activity directly in cultured cells. We have focused on measuring the generation of oxygen from the reaction between H_2O_2 and cells and have developed a useful analytical method with high sensitivity to evaluate catalase activity of micro-organisms and mammalian cells by using an electron spin resonance (ESR) oximetry technique.

To measure oxygen concentration in aqueous solutions, there are several techniques such as amperometry, fluorescent quenching and ESR oximetry [16–18]. Diepart et al. [19] reported that the ESR oximetry is a more sensitive method for measuring the dissolved oxygen in aqueous than the Clark oxygen electrode (amperometry) and fluorescent quenching assay. ESR oximetry has been developed to measure the oxygen concentration *in vitro* and *in vivo*, especially in tumour tissues. The method is based on the interaction of paramagnetic oxygen with free radicals, causing the variation of the line width of an ESR spectrum [18]. Molecular oxygen is a biradical [20], but it cannot be observed directly by ESR when it is dissolved in aqueous solutions. However, the increased concentration of the biradical molecular oxygen in a solution containing a free radical leads to spin exchange and broadens the spectrum of the free radical. Dissolved oxygen concentration can be quantitatively determined by analysing the magnitude of broadened spectrum [18].

Therefore, the hypothesis was raised that the catalase activity in cultured cells can be directly determined by applying the ESR oximetry technique if oxygen is generated by the reaction between H_2O_2 and cultured cells which contain catalase. The purpose of the present study is to develop and evaluate a novel analytical method for the catalase activity of cultured cells such as fungal, bacterial and mammalian cells. In the present study, authentic spin label probes 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (4-oxo-TEMPO) and 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy (4-maleimido-TEMPO) were used as free radicals.

Materials and methods

Reagents

Reagents were purchased from the following sources: catalase (from bovine liver) and reduced glutathione (GSH) from Wako Pure Chemical Industries (Osaka, Japan); TEMPOL, 4-oxo-TEMPO, 4-maleimido-TEMPO and glutathione peroxidase (GPx) from Sigma Aldrich (St. Louis, MO); H_2O_2 from Santoku Chemical Industries (Tokyo, Japan). All other reagents used were of analytical grade.

Micro-organisms and mammalian cells

The stock culture strains of bacteria and the established cell lines were obtained from Institute of Fermentation, Osaka (Osaka, Japan), American Tissue Culture Collection (Manassas, VA), Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan) and/or Riken Cell Bank, RIKEN BioResource Center (Tsukuba, Japan). That is, *Candida albicans* IFO 1269, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* JCM 2152, *Bacillus subtilis* IFO 13722, *Eshcerichia coli* ATCC 25922 and *Aggregatibacter actinomycetemcomitans* JCM 2434, all of which are catalase-positive fungus and bacteria, were used in this study. *Streptococcus mutans* JCM 5705, *Streptococcus salivarius* JCM 5707 and *Enterococcus faecalis* ATCC 29212 were used as catalase-negative bacteria. RAW 264 cells (RCB0535, a mouse macrophage cell line) and THP-1 cells (ATCC TIB-202, human acute monocytic leukaemia cell line) were used as mammalian cells in this study.

All of bacterial strains were cultured on Brain Heart Infusion agar (Becton Dickinson Labware, Franklin Lakes, NJ) and *C. albicans* was cultured on Sabouraud glucose agar (1% pepton, 4% glucose and 1.5% agar). *C. albicans*, *P. aeruginosa*, *B. cereus* and *B. subtilis* were cultured aerobically and *E. coli*, *A. actinomycetemcomitans*, *S. mutans*, *S. salivarius* and *E. faecalis* were cultured anaerobically using Anaero Pack (Mitsubishi Gas Chemical Company, Tokyo, Japan) at 37°C. The bacteria and *C. albicans* were cultured for over 24 h to obtain colonies in stationary phase and given concentrations of bacteria and *C. albicnas* suspensions were prepared in sterile physiological saline. RAW 264 cells were cultured in Eagle minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% foetal bovine serum (ICN Biomedicals, Aurora, OH) and non-essential amino acid (Invitrogen, Carlsbad, CA). THP-1 cells were cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% foetal bovine serum. The cells were cultured and maintained in a plastic culture flask at 37°C under a humidified atmosphere of 95% air and 5% CO_2. The adherent cells of RAW 264 cells and THP-1 cells in the flasks were scraped using a cell scraper and collected by centrifugation. Following two washes with sterile physiological saline, given concentrations of cell suspensions were prepared in sterile physiological saline.

Calibration curve of catalase activity measured by ESR

TEMPOL, 4-oxo-TEMPO and 4-maleimido-TEMPO were dissolved in pure water to prepare 2 mM of each spin-label probe. Catalase was also dissolved in pure water to concentrations of 0.30–62.5 U/mL. One unit of catalase was defined as the amount that catalyses the decomposition of 1 μmol of H_2O_2 per minute at 25°C. The reagents were mixed according to the following protocol; 25 μL of spin-label probe, 100 μL of catalase solution and 125 μL of 500 mM H_2O_2 were placed in a glass tube and then mixed for 5 s. The final concentrations of each reagent were 200 μM for the spin-label probes, 0.12–25 U/mL for catalase and 250 mM for H_2O_2. The mixture was transferred to a quartz cell for ESR spectrometry and the ESR spectrum was recorded on an X-band ESR spectrometer (JES-FA-100, JEOL, Tokyo). The ESR measurement was started 30 s after the addition of H_2O_2. The measurement conditions for ESR were as follows; field sweep, 330.50–340.50 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT for TEMPOL and 4-maleimido-TEMPO and 0.05 mT for 4-oxo-TEMPO; amplitude, 80 for TEMPOL and 4-maleimido-TEMPO and 40 for 4-oxo-TEMPO, sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz: microwave power, 4 mW. The ESR spectra of manganese (Mn^{2+}) were used as an internal standard.

The first peak width of the spectrum of each spin-label probe as well as the third peak width of the spectrum of the manganese was analysed using Digital Data Processing (JEOL, Tokyo, Japan). The peak width of the spin-label probe was divided by the peak width of the manganese marker to calculate relative peak width. The relative peak widths for each spin-label probe and the catalase concentrations were plotted to make a calibration curve. The equation of the linear curve was calculated by the method of least squares. The range of the catalase concentration which made the correlation coefficient over 0.98 was regarded as an optimal range for determination.

In order to confirm that the broadening of ESR spectra was caused by dissolved oxygen, argon gas bubbling for 5 min was performed for deoxygenation immediately after mixing of catalase and H_2O_2 (final concentration of 1.0 U/mL for catalase and 250 mM for H_2O_2). The ESR spectra obtained from the deoxygenated reaction mixture were compared to the control spectra obtained from the reaction mixture without argon gas replacement.

Determination of catalase activity of various micro-organisms and mammalian cells

TEMPOL was used for the measurement of catalase activity in cell suspensions (bacterial, fungal and mammalian cells). The RAW 264 cells and THP-1 cells were cooled on ice until assayed. Before the measurement, the cells were warmed in a temperature-controlled bath at 37°C for 3 min. According to the method described above, instead of catalase, each suspension of the micro-organisms and the mammalian cells was mixed with TEMPOL in a glass tube and then H_2O_2 was added. ESR spectra were recorded with the same protocol as described above. The catalase activity was measured in triplicate for each sample and was evaluated using the calibration curve.

Evaluation of potential factors affecting the assay. Since this assay determines catalase activity by means of measurement of oxygen generation, oxygen generation by autolysis of H_2O_2 and normal respiration of cells might affect the results of assay. Therefore, oxygen generation rate from the reaction between catalase and H_2O_2 was compared to the oxygen generation rate by autolysis of H_2O_2 and the oxygen consumption rate by cells. The oxygen generation and consumption were measured using oxygen electrode (Disolved Oxygen Meter 5100, YSI, Tokyo, Japan). To measure oxygen generation from the reaction between catalase and H_2O_2, 250 mM of H_2O_2 and catalase at concentrations adjusted from 0–0.75 U/mL were mixed. Then, the dissolved oxygen in the sample was evaluated after the mixture was stirred for 5 s.

For the analysis of oxygen generation by autolysis of H_2O_2, dissolved oxygen levels in varying concentration of H_2O_2 aqueous solutions (0, 250, 500 and 1000 mM H_2O_2) were compared. The H_2O_2 aqueous solutions were deoxidized by argon gas replacement to evaluate the oxygen generation without the influence of dissolved oxygen. Then, the increase of dissolved oxygen was measured up to 5 min at 25°C.

B. subtilis and THP-1 cells were used for the evaluation of oxygen consumption by normal respiration. The cells were suspended in sterile physiological saline. The cell number was adjusted to 1×10^7 CFU/mL for *B. subtilis* and 1×10^6 cells/mL for THP-1 cells, both of which corresponded to the maximum cell number used in the assay for catalase activity. The dissolved oxygen in each cell suspension was monitored up to 60 min at 25°C.

Besides catalase, it is known that mammalian cells as well as some micro-organisms have a GPx system which can decompose H_2O_2 [21]. In the GPx system, GPx catalyses a reaction where GSH serves as an electron donor to reduce H_2O_2. Although H_2O_2 is decomposed without oxygen generation in this system, the GPx system might affect the assay of catalase activity if it seriously reduces the concentration of H_2O_2. Therefore, the influence of the GPx system was investigated. GPx was dissolved in pure water to be 20 U/mL. One unit of GPx was defined as the amount that catalyses the oxidation of 1.0 μmole of GSH by H_2O_2 per minute at pH 7.0 at 25°C. GSH was also

dissolved in pure water to be 800 μM. The GPx and GSH solutions were mixed with 1 M H_2O_2 so that the final concentrations of each reagent were 10 U/mL for GPx, 200 μM for GSH and 250 mM for H_2O_2. The concentration of GSH (200 μM) was designed by the calculation to find the maximum concentration of GSH in the cell suspension used in the assay for catalase activity. In other words, it was calculated on the assumption that the cell is spherical shape with a diameter of 30 μm and contains 10 mM of GSH per cell [22,23]. The calculation revealed that the 10^6 cells theoretically contain less than 150 nmole of GSH. Therefore, since the concentration of GSH in the cell suspension of 10^6 cells/mL was less than 150 μM, we used 200 μM of GHS in the experiment. Immediately after mixing the reagents, the mixture was incubated at 37°C for 3 min. Then, the concentration of H_2O_2 was determined using a commercially available assay kit (Hydrogen Peroxide Chemiluminescent Detection Kit, Assay Designs, Ann Arbor, MI) and a luminometer (Phelios AB-2350, ATTO, Tokyo, Japan).

Results

The peak width of TMPOL, 4-maleimido-TEMPO and 4-oxo-TEMPO was 0.17, 0.18 and 0.06 mT, respectively. However, when the spin-label probes were mixed with catalase and H_2O_2, the ESR spectra were broadened dependently on the catalase concentrations. The peak width of spin-label probes was broadened up to 0.9 mT by the oxygen generated by the reaction between catalase and H_2O_2, while the peak width of Mn^{2+} marker was relatively stable and was 0.11 mT with the inter-measurement error of 0.01 mT. Representative spectra of TEMPOL mixed with different concentrations of catalase are shown in Figure 1A. Broadened ESR spectrum induced by catalase and H_2O_2 was recovered to the original shape by deoxygenation by argon gas replacement (Figure 1B). All of the spin-label probes used in this study showed high correlation between the broadened relative peak width and catalase concentration. TEMPOL and 4-maleimido-TEMPO had a similar optimal range of the calibration curve and high correlation coefficients. The optimal range with TEMPOL was from 0.5–10.0 U/mL (Figure 2) and that with 4-maleimido-TEMPO was from 0.25–12.5 U/mL. The correlation coefficient for TEMPOL was 0.9962 (Figure 2) and that for 4-maleimido-TEMPO was 0.9959. In the case of 4-oxo-TEMPO, which had the highest peak and sharpest spectrum among the spin-label probes used in this study, optimal range was relatively narrow (0.25–2.25 U/mL) and there was a slightly lower correlation coefficient (r = 0.9872) compared to those for other two spin-label probes.

The broadened ESR spectra were also observed when the catalase-positive micro-organisms or the mammalian cells were mixed with TEMPOL and H_2O_2. The broadened spectra were similar to those caused by catalase. On the other hand, the catalase-negative bacteria did not cause the broadened ESR spectra. Representative catalase activities from catalase-positive bacteria and *C. albicans*, catalase-negative bacteria and mammalian cells are shown in Figure 3. The slope values (catalase concentration divided by the number of cells) in the graph indicate the catalase activity per cell. The number of the cells

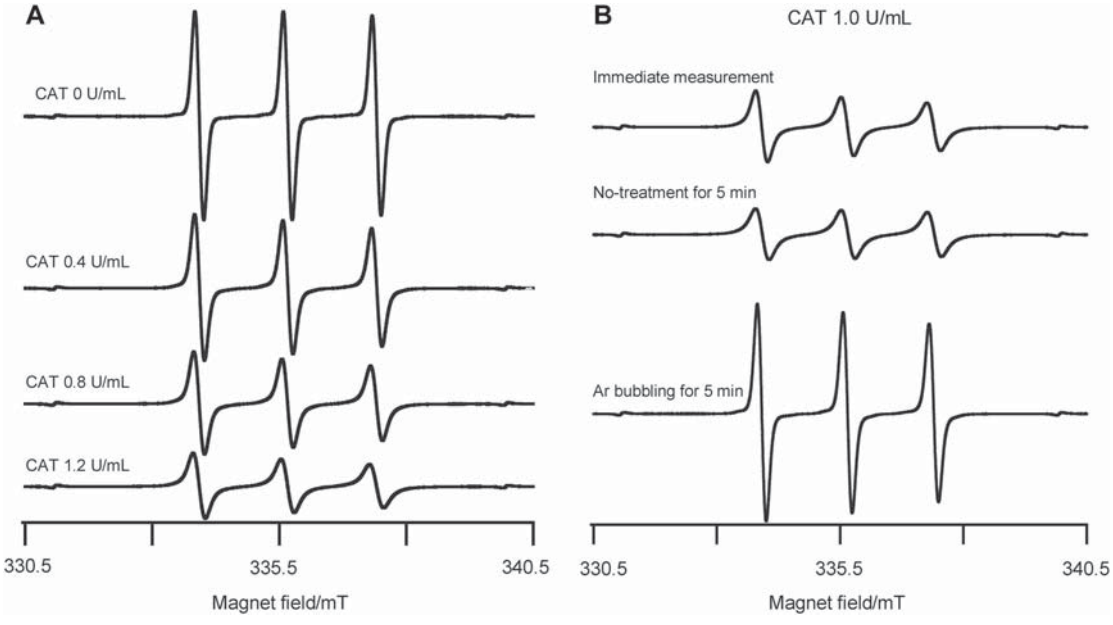

Figure 1. Representative ESR spectra of TEMPOL obtained by the reaction between H_2O_2 and catalase (CAT). When the TEMPOL was mixed with 250 mM H_2O_2 and various concentrations of CAT, the spectra were broadened with the concentrations of CAT (A). The broadened ESR spectrum caused by 1.0 U/ml CAT was recovered toward the original shape by argon (Ar) gas bubbling for 5 min (B).

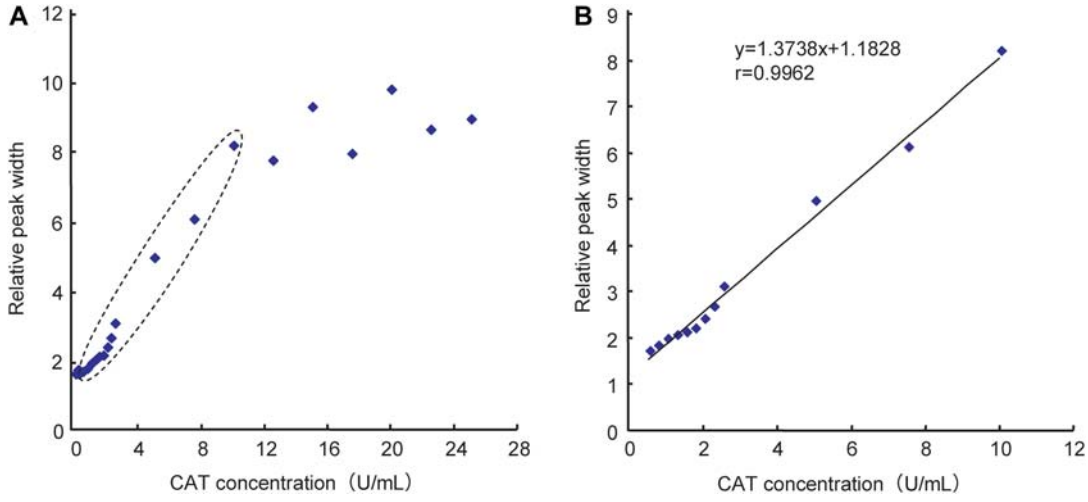

Figure 2. Calibration curve using TEMPOL. The linear relation between the relative peak width and catalase (CAT) concentration was observed only in the area circled by dots (A). The equation of the calibration curve showed high correlation coefficient (r = 0.9962) (B). Each value represents the mean of triplicate determinations.

showed a high correlation with the catalase activity (Figure 3). The catalase activities per cell for catalase positive-micro-organisms were as follows; *C. albicans*, 3.3×10^{-8} U/cell; *P. aeruginosa*, 5.3×10^{-8} U/cell; *B. subtilis*, 2.4×10^{-7} U/cell; *B. cereus*, 8.0×10^{-8} U/cell; *E. coli*, 3.2×10^{-9} U/cell; *A. actinomycetemcomitans*, 6.4×10^{-10} U/cell (Figure 4). Meanwhile, *S. mutans*, *S. salivarius* and *E. faecalis* showed approximately zero slope, meaning they had no catalase activity, even at the cell concentrations of 10^9 CFU/mL order. It was also possible to evaluate the catalase activity of the mammalian cells, which showed relatively high activity per cell. That is, 9.0×10^{-7} U/cell for RAW 264 cells and 7.8×10^{-7} U/cell for THP-1 cells (Figure 4).

When catalase was mixed with 250 mM H_2O_2, the concentration of dissolved oxygen increased with the catalase concentration (Figure 5A). The increased amount of dissolved oxygen induced by catalase at a concentration of 0.75 U/mL was 23.4 mg/L. Catalase, even at a concentration of 0.06 U/mL, increased dissolved oxygen to 2.2 mg/L. On the other hand, the oxygen generation by autolysis of H_2O_2 increased with the concentrations of H_2O_2, but the amounts were negligibly small, especially when 250 mM H_2O_2 was applied (Figure 5B), compared with those generated by the reaction between catalase and H_2O_2 (Figure 5A). The total amounts of dissolved oxygen generated for 5 min from 0, 250, 500 and 1000 mM H_2O_2 were 0.02, 0.10, 0.19 and 0.42 mg/L, respectively. In addition, only slight consumption of oxygen by cells was observed, even when the dissolved oxygen was monitored up to 60 min (Figure 5C). That is, 10^7 CFU/mL of *B. subtilis* consumed 0.6 mg/L of oxygen per hour.

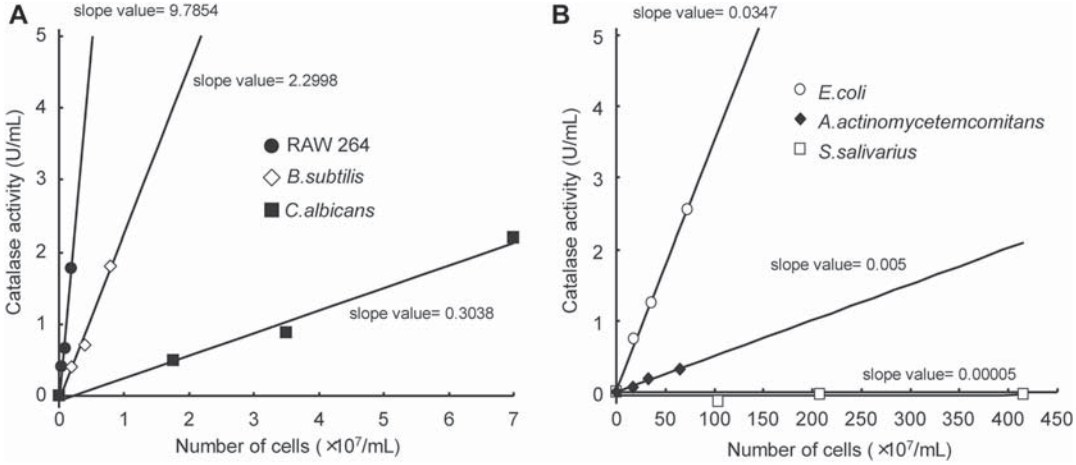

Figure 3. Catalase activity of *C. albiacans* cells, the representative bacterial cells and RAW 264 cells. *B. subtilis* (aerobic bacteria) and RAW 264 cells (mammalian cells) showed large slope values (A). *C. albicans* (aerobic fungus) showed a moderate slope value (A). *E. coli* (facultative anaerobe) *and A. actinomycetemcomitans* (facultative anaerobe) showed small slope values (B). *S. salivarius* (facultative anaerobe), which was catalase-negative bacteria, showed almost zero slope (B). Each value represents the mean of triplicate determinations.

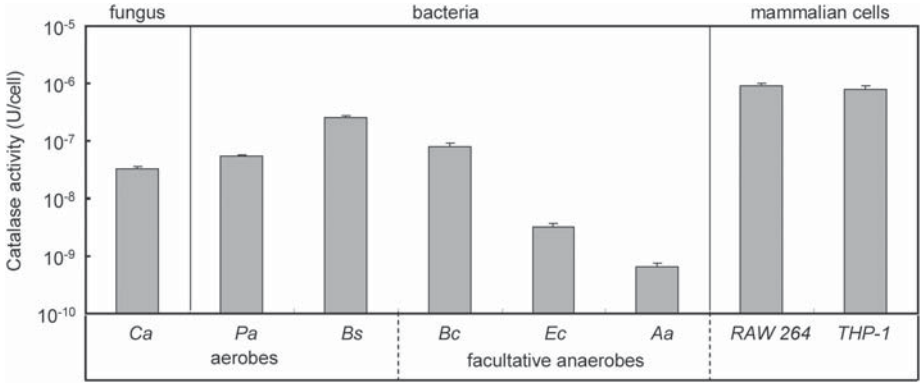

Figure 4. Comparison of the catalase activity (U/cell) of catalase-positive cells. The mammalian cells had higher catalase activity than the micro-organisms. The facultative anaerobes seemed to have catalase activity lower than that in the aerobes. Each value represents the mean ± SD of triplicate determinations. *Ca*: *C. albicans*, *Pa*: *P. aeruginosa*, *Bs*: *B. subtilis*, *Bc*: *B. cereus*, *Ec*: *E. coli*, *Aa*: *A. actinomicetemcomitans*.

Similarly, 10^6 cells/mL of THP-1 cells consumed 1.3 mg/mL of oxygen per hour. The oxygen consumption rate of cells used in the assay was fairly low compared with the oxygen generation rate of the reaction between catalase and 250 mM H_2O_2.

To examine if the GPx system affects the assay of catalase activity through reduction of H_2O_2, H_2O_2 concentration was monitored in the presence of 10 U/mL of GPx, 200 μM GSH and 250 mM H_2O_2 (Figure 6). The determination of H_2O_2 concentrations revealed that GPx and GSH at the concentration of 200 μM hardly affected the concentration of H_2O_2 (Figure 6).

Discussion

ESR oximetry is a technique that can detect oxygen with high sensitivity and a relatively short time for analysis [19,24–27]. In more detail; oxygen level is quantitatively determined by analysing the ESR spectra broadened by oxygen molecule. In the present study, it is demonstrated that oxygen generated by the reaction between H_2O_2 and catalase causes ESR spectrum broadening, as evidenced by the fact that deoxygenation of the reaction mixture by argon gas replacement recovered the broadened ESR spectrum to the original shape of the spectrum (Figure 2). We also confirmed by amperometry that the concentration of dissolved oxygen in H_2O_2 was increased with the concentrations of catalase (Figure 5A). Also as shown in Figure 2, relative peak width of ESR spectrum was linearly increased by the addition of catalase in a concentration-dependent manner. These results tempted us to apply this technique to determine catalase activity in cultured cells (bacterial, fungal and mammalian cells). As is the case with the reaction between H_2O_2 and catalase, the ESR spectra were broadened by the addition of H_2O_2 to cell suspensions, suggesting that oxygen was generated by the reaction between H_2O_2 and catalase in the cells. This was also supported by the fact that no broadened ESR spectra were observed when H_2O_2 was added to catalase-negative bacteria suspensions.

The present study is the first to evaluate the catalase activity of various micro-organisms and mammalian cells using an ESR oximetry technique. The optimal ranges of the calibration curve of TEMPOL (Figure 2) and 4-maleimido-TEMPO, which could detect 0.25–0.5 U/mL of catalase activity, showed that this analytical method has high sensitivity. In addition, the

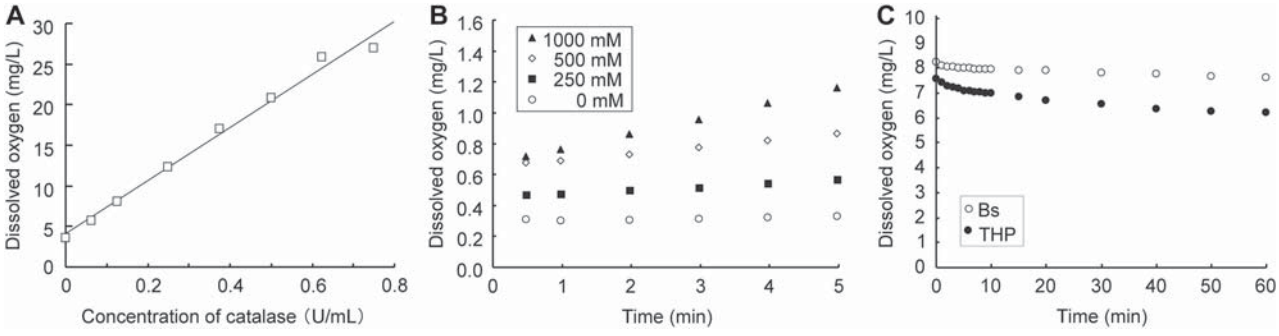

Figure 5. Comparison of oxygen generation and consumption. Dissolved oxygen in 250 mM H_2O_2 increased remarkably with the concentration of catalase (A). On the other hand, the increased amount of dissolved oxygen by autolysis of H_2O_2 was limited (B). Furthermore, dissolved oxygen in physiological saline was consumed by cells, but the consumption was also limited (C). *Bs*: *B. subtilis*. Each value represents the mean of triplicate determinations.

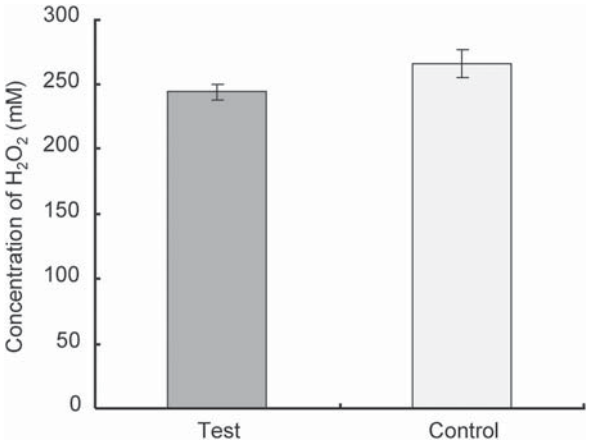

Figure 6. Concentration of H_2O_2 in the mixture of GPx and H_2O_2. In test sample, 250 mM H_2O_2 was mixed with 200 μM of GSH and 10 U/mL of GPx and in control sample, 250 mM H_2O_2 was mixed with pure water instead of GSH and GPx. GSH and GPx at the concentrations used in this experiment did not seem to reduce the concentration of H_2O_2 in the assay for catalase activity. Each value represents the mean ± SD of triplicate determinations.

small standard deviation of triplicate measurements (Figure 4) indicates that the method is highly reproducible. The other advantage of the method is that a small amount of assay volume is enough to be analysed. For instance, 100 μL of 10^7–10^8 cells/mL for micro-organisms and of 10^6 cells/mL for mammalian cells were used for each assay. So far, catalase activity was expressed in U/mg protein of samples containing cell lysate in the previous studies [10–14]. In the case of bacterial, fungal and plant cells, all of which have a rigid cell wall, special techniques are required to obtain cell lysate, such as enzymatic digestion of cell wall and physical destruction of cell wall using a Braun disintegrator [28,29]. Thus, we can say that it is also advantageous to skip these complicated procedures in the catalase assay of cells with a cell wall.

We also demonstrated that the oxygen generation by autolysis of 250 mM H_2O_2 and normal respiration of cells hardly affected the assay of calatase activity. The increased amount of dissolved oxygen by autolysis of H_2O_2 was fairly small compared with that by the reaction between catalase and H_2O_2, even though the concentration of H_2O_2 was 1000 mM. In the present study, we used 250 mM H_2O_2 for the substrate of the assay to minimize the influence of the increase in dissolved oxygen. Oxygen consumption by normal respiration of cells is quite low compared to the oxygen generation by the reaction between catalase and H_2O_2. In addition, since the assay took only ~ 3 min per measurement, it was considered that respiration of cells did not or only slightly reduced the amount of oxygen generated in the assay system. The GPx system, another possible factor affecting the assay, also did not or only slightly affected the assay for catalase activity, since it did not reduce the concentration of H_2O_2 under the condition applied in this catalase assay. It is reasonable that 200 μM GSH with 10 U/mL of GPx cannot reduce the concentration of H_2O_2 (250 mM) because the concentration of GSH is much less than that of H_2O_2. Catalase directly catalyses the reaction where H_2O_2 are decomposed into oxygen and water, while GPx catalyses the reaction where GSH serves as an electron donor to H_2O_2. Therefore, it is considered that the amount of H_2O_2 decomposed by the GPx system depends on the concentration of GSH. Thus, 200 μM GSH that corresponds to the concentration in the cell suspension used for the catalase assay is too low to decompose 250 mM H_2O_2. These findings suggest that the potential factors examined in this study do not affect the novel assay for catalase activity. Although conventional measurements of catalase activity were also performed by means of evaluation of oxygen or H_2O_2, possible factors affecting the results such as the influences of oxygen generation by autolysis of H_2O_2 and decomposition of H_2O_2 by GPx system have not been paid attention. Thus, if the method for catalase activity is based on the measurement of H_2O_2 and/or O_2, at least these factors are evaluated for each method.

In this study, bacteria in the stationary phase were used because it is suggested that the catalase activity of bacteria in stationary phase is higher than that in the exponential phase [12]. Among the catalase-positive bacteria used in this study, aerobes showed a tendency to have higher catalase activity than facultative anaerobes. *B. subtilis* showed the highest catalase activity followed by *B. cereus*, *P. aeruginosa*, *E. coli* and *A. actinomycetemcomitans*. Although *B. cereus* belongs to facultative anaerobes, it is well cultured in aerobic conditions. In the present study, as the bacteria was cultured in aerobic conditions, we postulate that high catalase activity was observed in the bacteria. Besides micro-organisms, mammalian cells used in this study showed high catalase activity. These cells are originally from circulating monocytes/macrophages, so that they are exposed to oxygen in the living body. Thus, the method can also be a novel tool for studying the relation between oxidative stress and stress-resistance at the cellular level.

Acknowledgements

We would like to give our thanks to Professor Toshihiko Ozawa (Yokohama College of Pharmacy) for very insightful discussion.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 23 June 2010.