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Oxygen Electrode as a New Tool to Evaluate Hydroxyl Radical-Scavenging Ability

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Abstract

An oxygen electrode was applied to determine hydroxyl radical (•OH) levels for the first time. The method is based on the determination of •OH generated by the Fenton reaction using the reaction of •OH with a scavenger and the resulting radical consuming an oxygen molecule stoichiometrically. Thus, the •OH-scavenging abilities of antioxidant reagents, as well as the concentration of •OH, can be determined by the measurement of consumption of dissolved oxygen using an oxygen electrode. A good correlation between the present method and conventional colorimetry was obtained for the estimation of the •OH-scavenging activities of antioxidants. Furthermore, the results correlated with the •OH-scavenging rate constants of the reagents evaluated by a "cupric ion reducing antioxidant capacity (CUPRAC)" assay. We applied the present method to estimate the •OH-scavenging abilities of commercially available alcoholic drinks.

Keywords Oxygen electrode; Oxygen consumption; Hydroxyl radical; Hydroxyl radical-scavenger; Antioxidant; Alcoholic drink

1. Introduction

Reactive oxygen species, including hydroxyl radicals (•OH), are generated in various biological systems and often cause oxidative stress, which is related to aging and diseases such as cancer, cardiovascular disease, Alzheimer's disease, and Parkinson's disease [1]. A number of methods have been developed for the determination of •OH levels. These methods include electron spin resonance (ESR) with spin trapping agents [2,3], high-performance liquid chromatography (HPLC) analysis of products formed by a reaction with •OH [1,4], luminol chemiluminescence [5–8], and colorimetry using suitable color-producing reagents [9,10].

In the present study, we developed a new method for the detection of •OH using an oxygen electrode. Although various kinds of substances are readily determined using an oxygen electrode coupled to enzymes (so-called enzyme electrodes) [11], we were particularly interested in the application of an oxygen electrode to determine biologically important short-lived species such as •OH. We have previously applied an oxygen electrode to analyze lipid peroxidation mechanisms by the measurement of the consumption of dissolved oxygen [12], and the present investigation is an extension of the use of the electrode to determine •OH levels, which also involves oxygen consumption. The present method is based on the determination of •OH generated by the Fenton reaction using a scavenging reaction expressed by the following reaction sequences:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$

$$RH + \cdot OH \rightarrow R\cdot + H_2O \quad (2)$$

$R\cdot + O_2 \rightarrow ROO\cdot$ (3)

where RH shows an ·OH-scavenger. Equation (1) shows the Fenton reaction. The radical species (R·) produced by the reaction of ·OH and its scavenger (Eq. (2)) further reacts with an oxygen molecule to produce a peroxy radical (ROO·) as shown in Eq. (3) [13–15]. In most antioxidant reagents tested in the present study, ROO· was expected to decompose or disproportionate, leading to the oxidation products of the reagents, such as the corresponding carboxylic acids or aldehydes, and not to cause chain reactions accompanied by further utilization of oxygen molecules [13,15]. Hence, we can determine the concentration of ·OH through the consumption of dissolved oxygen using an oxygen electrode. The ·OH-scavenging abilities of various antioxidants determined by the present method compared favorably with those determined by conventional colorimetry, monitoring of the bleaching of *p*-nitrosodimethylaniline [9], and the ·OH-scavenging rate constants evaluated from a "cupric ion reducing antioxidant capacity (CUPRAC)" assay [10].

2. Experimental

2.1. Reagents

The reagents were obtained from the following sources: iron (II) sulfate heptahydrate, dimethyl sulfoxide (DMSO), D-mannitol, D-glucose, L-lysine, and trisodium citrate dihydrate were from Wako (Osaka, Japan); H_2O_2 (35 wt% in water), sodium acetate trihydrate, methanol, ethanol, and 2-propanol were from Ishizu Seiyaku (Osaka, Japan); *N,N*-dimethyl-4-nitrosoaniline was from Aldrich

(Milwaukee, WI, USA); sodium formate was from Kanto Kagaku (Tokyo, Japan). All other reagents were of analytical reagent grade.

2.2. Alcoholic drinks

Three different kinds of alcoholic drinks were used for the application of the method; shochu (Japanese distilled liquor) (Mugi-koji; Takara-Shuzo, Kyoto, Japan), sake (Gekkeikan; Gekkeikan, Kyoto, Japan), and whiskey (Suntory Old; Suntory, Osaka, Japan).

2.3. Preparation of solutions

The solutions of $FeSO_4$ and H_2O_2 were made up fresh each time using Milli-Q water. The stock solutions of scavengers were prepared in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0).

2.4. Measurement of oxygen consumption

The assay mixture consisting of the required concentrations of H_2O_2 and scavenger dissolved in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0) was put in a cylindrical glass vessel (inner diameter 21 mm, height 40 mm) and then the oxygen electrode (P-type; Biott., Tokyo, Japan) was set and covered with silicon rubber (Fig. 1). The reaction was started by injecting $FeSO_4$ with a microsyringe into the mixture. The total volume of the reaction mixture was 3.5 ml. Oxygen consumption was measured with stirring for about 2 min. The measurements were performed in a thermostat adjusted to 25°C.

2.5. Bleaching of p-nitrosodimethylaniline

Colorimetry using *p*-nitrosodimethylaniline [9] was performed under the same conditions as the oxygen electrode method. The assay mixture consisted of 25 μM *p*-nitrosodimethylaniline, 0.5 mM H_2O_2, and the required concentration of scavenger dissolved in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0). The mixture was put in a conventional quartz cell (light path length 1 cm) for measuring the absorbance and set to a cuvette holder of an Ocean Optics USB2000 miniature fiber-optic spectrometer (Dunedin, FL, USA). A fiber-optic cable was connected to conduct light, and Ocean Optics OOIBase32 software was used to process the data. The reaction was started by adding $FeSO_4$ to a final concentration of 0.3 mM. Measurements were performed at room temperature (about 25°C) with stirring and a decrease in the absorbance at 440 nm of *p*-nitrosodimethylaniline was monitored for 2 min.

3. Results and Discussion

3.1. Detection of hydroxyl radicals by oxygen consumption

Generation of ·OH by the Fenton reaction is known to increase in acidic solutions [16]. To avoid measurements in highly acidic media, assays were performed in nearly neutral media by selecting 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0) as the reaction buffer, in which the rate of oxygen consumption decreased down to 85% of those in pH < 5 but was still very effective. The use of other buffers, such as Hepes–NaOH, Mes–NaOH, and Tris–H_2SO_4, was less effective than that of the phosphate buffer because of smaller rates of oxygen consumption.

Typical traces of oxygen consumption are shown in Fig. 2. In this experiment, we used mannitol, a highly selective ・OH scavenger, which is known not to react with reactive oxygen species except for ・OH [17]. After the reaction was started with the injection of $FeSO_4$, rapid oxygen consumption was observed within 20 s. It should be pointed out that the response time (90% of final signal) of the oxygen electrode was rather long (less than 20 s) (specifications for oxygen electrode; Biott., Tokyo, Japan), and thus monitoring the ・OH-scavenging reaction through the measurement of oxygen consumption required a certain amount of time. The decrease in oxygen concentration correlated with the reaction sequences shown in the Introduction, indicating that R・ (mannitol radical), formed by the reaction of mannitol with ・OH, further reacted with an oxygen molecule to produce ROO・ (the peroxy radical of mannitol). This result was in good accordance with a previous ESR study [13], showing that the mannitol radical reacted with an oxygen molecule stoichiometrically and the resulting peroxyl radical did not participate in further reactions with oxygen molecules. The fact that the level of oxygen consumption became constant after a sufficient reaction time also supported the view that the peroxy radical of mannitol did not take part in further consumption of oxygen molecules. The rates of oxygen consumption, estimated from the reaction traces shown in Fig. 2, as well as the total amounts of oxygen consumed, were enhanced with the increasing concentration of mannitol. Similar experiments were performed by changing the concentrations of H_2O_2 and $FeSO_4$. As shown in Fig. 3, the rates of oxygen consumption increased with increases in the concentrations of the individual components (mannitol, H_2O_2, and $FeSO_4$) involved in the reaction mixture and finally reached constant values. The limited

concentration range of $FeSO_4$ tested in Fig. 3c, compared to other cases (mannitol and H_2O_2 shown in Figs. 3a and 3b, respectively), was due to the formation of iron phosphate in the present assay mixture, giving a milky suspension. The precision of the measurement was also evaluated at the concentration of 1 mM of mannitol shown in Fig. 3a. The results obtained in triplicate gave the mean value of 2.55 $\mu M\ s^{-1}$ with 6.5% relative standard deviation.

To examine the correlation between the rate of oxygen consumption and the rate of ·OH generation, we tried to evaluate the latter rate based on the following reaction kinetics and the reported value of the rate constant, k [18].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

$$d[\cdot OH]/dt = k[Fe^{2+}][H_2O_2],\ k = 76\ M^{-1}\ s^{-1}$$

We estimated the values of d[·OH]/dt in the concentration range below 0.5 mM for $[Fe^{2+}]$ and $[H_2O_2]$, where a linear dependency on oxygen consumption was observed (Figs. 3b and 3c). Using these calculated values, we plotted the relationship and obtained a good correlation (r = 0.90) as shown in Fig. 4. Thus, the present method for measuring the rate of oxygen consumption can be used for the determination of ·OH. The detection limit, expressed as the rate of oxygen consumption, was around 0.5 $\mu M\ s^{-1}$. In the Fenton reaction, oxygen evolution is also known to take place when the concentration of H_2O_2 was much higher than that of Fe^{2+} [19]; however, the present conditions were outside of such oxygen-generating conditions.

3.2. Comparison of the present method with conventional colorimetry

We evaluated the ·OH-scavenging ability of various antioxidant reagents,

comparing the present method with colorimetry. All the antioxidants tested in this work generated similar profiles as in the case of mannitol, under the conditions of $[H_2O_2]$ = 0.5 mM and $[Fe^{2+}]$ = 0.3 mM. That is to say, the rate of oxygen consumption increased with increases in the concentrations of scavengers and reached a plateau. As an index of the scavenging ability of each antioxidant, we determined the 50% inhibitory concentration of each reagent (IC_{50}), which means the concentration giving the half maximum rate of oxygen consumption. To determine IC_{50} values, the concentration of each scavenger was increased up to 100 fold of its minimum concentration where oxygen consumption was detectable. The IC_{50} values of antioxidants tested in the present study are summarized in Table 1, along with those obtained by colorimetry [9] and the reported values of the ·OH-scavenging rate constants [10] discussed below.

In the colorimetric assays, the ·OH-scavenging ability was determined by the bleaching of *p*-nitrosodimethylaniline by monitoring a decrease in absorbance at 440 nm. In this assay, when ·OH reacted completely with the antioxidant tested, change in the absorbance at 440 nm was not induced. Thus, the weaker the scavenging-activity, the larger the change in the absorbance. This was the opposite direction to the case of the oxygen electrode; i.e., the weaker the scavenging-activity, the smaller the change in oxygen consumption. In spite of the large difference in the principle of both methods, there was a good correlation (r = 0.91) between scavenging activities obtained by colorimetry and by the oxygen electrode as shown in Fig. 5a. The activities are expressed as $1/IC_{50}$, with the larger the value, the higher activity it shows. We used sodium salts for acidic scavengers such as formic and acetic acids because large doses of the protonated

forms decreased the pH values of the assay mixture and induced spontaneous bleaching of *p*-nitrosodimethylaniline. However, such small degrees of pH reduction scarcely influenced measurements using the oxygen electrode. Although many antioxidants reacted with ·OH according to the reaction scheme shown in the Introduction, DMSO was an exception [20]. The reaction of DMSO with ·OH is known to produce a methyl radical [20], which might further react with an oxygen molecule to form a peroxy radical because DMSO showed the same pattern of oxygen consumption as those of other scavengers.

Quite recently, the ·OH-scavenging rate constants of various antioxidants were systematically evaluated using CUPRAC assay [10]. We compared these reported values with the present results. As shown in Fig. 5b, a correlation (r = 0.85) was also obtained between $1/IC_{50}$ values evaluated by the oxygen electrode and the rate constants reported by CUPRAC assay.

3.3. Application of the present method to evaluate the hydroxyl radical-scavenging abilities of alcoholic drinks

We applied the oxygen electrode method to evaluate the ·OH-scavenging abilities of three different kinds of commercially available alcoholic drinks; shochu (Japanese distilled liquor), sake, and whiskey, and the results were compared with those obtained by conventional colorimetry (Table 1). The ethanol concentrations in shochu, sake, and whiskey were 25, 13, and 43%(v/v), which corresponded to 4.30 M, 2.24 M, and 7.40 M, respectively. The IC_{50} values of these alcoholic drinks, converted to ethanol concentrations, were almost the same as that of ethanol itself, indicating that the ·OH-scavenging activities of the alcoholic drinks are

determined only by ethanol. We initially supposed that the IC_{50} value of sake might be much lower than those of shochu and whiskey, because sake is made from fermented rice and is non-distilled, and thus sugar components contained in sake could work as an additional ·OH-scavenger. However, the total amount of sugars is around 5%, which is significantly lower than that of ethanol [21]. Hence, the effect of sake was similar to those of shochu and whiskey as shown in Table 1.

4. Conclusions

The present study demonstrated that the oxygen electrode can be used for the determination of ·OH levels, through which we can evaluate the ·OH-scavenging activities of various antioxidant reagents. This study extended the use of oxygen electrode into a new field for the detection of biologically important short-lived species such as ·OH.

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Table 1 The ・OH-scavenging abilities of various antioxidants (expressed as IC_{50} values) evaluated by oxygen (O_2) electrode and colorimetry using *p*-nitrosodimethylaniline and the ・OH-scavenging rate constants (k) reported by CUPRAC assay [10]

	Antioxidant	O_2 Electrode IC_{50} (mM)	Colorimetry IC_{50} (mM)	CUPRAC assay $k \times 10^9$ (M^{-1} s^{-1})
1	DMSO	0.017	0.1	4.24
2	Formate	0.035	0.17	1.89
3	Ethanol	0.05	0.27	0.069
4	Glucose	0.06	0.3	0.51
5	2-Propanol	0.07	0.25	0.046
6	Methanol	0.08	1.75	0.037
7	Mannitol	0.12	0.25	0.6
8	Citrate	0.15	6	1.48
9	L-Lysine	0.6	0.45	0.249
10	Acetate	0.6	5	— [a]
Alcoholic drinks [b]				
	Shochu	0.055	0.31	
	Sake	0.06	0.35	
	Whiskey	0.05	0.3	

[a] Not reported.
[b] IC_{50} values are expressed as the concentration of ethanol.

Figure Legends

Fig. 1. Experimental setup for monitoring ·OH-scavenging reaction using an oxygen electrode.

Fig. 2. Dynamic response of the oxygen electrode caused by the reaction of ·OH with mannitol. The concentration of mannitol was changed between 0 and 5 mM with the same concentrations of H_2O_2 (0.5 mM) and $FeSO_4$ (0.3 mM) in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.0). The reaction was started by the addition of $FeSO_4$. The total volume of the reaction mixture was 3.5 ml.

Fig. 3. Plots of the rates of oxygen consumption as a function of the concentrations of (a) mannitol, (b) H_2O_2, and (c) $FeSO_4$. Except for changing the concentration of reactant indicated in each figure, the concentrations of other reactants were kept constant as mannitol (10 mM), H_2O_2 (0.5 mM), and $FeSO_4$ (0.3 mM).

Fig. 4.. Relationship between the rates of oxygen consumption and the rates of ·OH generation. The plots were made under the constant concentration of 10 mM mannitol and changing the concentrations of H_2O_2 (0.1–0.5 mM) and $FeSO_4$ (0.1–0.5 mM).

Fig. 5. Plots of $1/IC_{50}$ values of antioxidants estimated by oxygen electrode against (a) $1/IC_{50}$ values obtained by colorimetry using *p*-nitrosodimethylaniline

and (b) the ·OH-scavenging rate constants (*k*) reported by CUPRAC assay [10]. The numbers shown in figures correspond to the numbers of the antioxidants used in Table 1.

Biographies

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