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Antioxidative Activity with Superoxide of Oxygen Stress Relievers, Chlorophyll Derivatives*1

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Using the chemiluminescent measuring method for rate constants between superoxide (O_2^-) and antioxidants established in our previous report, the reaction constants between O_2^- and metallo-chlorophyllins or chlorins e_6, the oxidative stress relief activity of which at ischemia-reperfusion was inspected, were measured by the quenching experiments of chemiluminescence of a *Cypridina* luciferin analogue, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA), in 25 mM buffer solutions (pH 7.0) at 25°C. The results were discussed by making comparisons with the known data in the literature. Consequently, we found that the present measuring method can be applied for the measurement of reaction activities of the antioxidative samples even when fairly unstable. The results prove that the oxidative stress relievers have strong antioxidative activity against O_2^- in aqueous solutions.

1 Introduction

Most living things are considered to possess well arranged antioxidant systems in order to protect themselves against the toxicity of oxygen (active oxygen species), although the full details are under being clarified. Antioxidative activities and mechanisms are well established for major antioxidants known to be highly active even in trace amounts, such as ascorbic acid, tocopherols, and superoxide dismutase (SOD).[1] In living bodies, however, there are many antioxidative compounds whose activity per mole is not high but the total activity is comparable to principal antioxidative compounds, because of their abundance. They could have some important roles in the organisms, such as anticancer, anti-inflammation, and oxidative stress relief at ischemia-reperfusion. We describe herein the rate measurements of such oxidative stress relievers supposed to be antioxidants, chlorophyll derivatives,[2] with superoxide (O_2^-), using a highly sensitive *Cypridina* chemiluminsecence method developed in our laboratories.[3]

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2 Materials and Methods

Quenching experiments were performed as shown in our former report.[3] 2-Methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) and salcomine were purchased from Tokyo Chemical Industry. The chlorophyllins and chlorins e_6 used were from Tama Biochem. Co., Tokyo. SOD (bovine) was supplied by Toyobo Enzyme Laboratory, Tsuruga. Hypoxanthine, xanthine oxidase (XOD), and bovine albumin were from Sigma Chemical Co. Cytochrome C (horse heart muscle) and vitamin C were from Wako Chem., Osaka. Reaction rate constant k_3 for O_2^- can be shown as Equation 1 described in the previous reports[3,4] for reactions (2-4). At a stationary state, Eq. 1 is derived from the Stern-Volmer treatment for two different concentrations of CLA,

$$k_3 = k_2([CLA]_1 - [CLA]_2) / (1/A - 1/B) \quad (1)$$

$$2O_2^- \xrightarrow{k_1} O_2 + O_2^{2-} \quad (2)$$

$$CLA + O_2^- \xrightarrow{k_2} P\ (\text{Oxyluciferin}) + h\nu \quad (3)$$

$$Q + O_2^- \xrightarrow{k_3} O_2 + O_2^{2-} + \text{Product(s)} \quad (4)$$

where k_1,[5] k_2 (1.08×10^8 $M^{-1}s^{-1}$),[3] and k_3 are rate constants of the disappearance (2) and reactions with CLA (3) and with a quencher (Q) (4) of O_2^-, respectively. [CLA], [O_2^-], and [Q] represent concentrations of CLA, superoxide, and quenchers used, respectively. A and B represent slopes for $[CLA]_1$ and $[CLA]_2$.

3 Results and Discussion

The results are shown in Table 1. The table includes results for similar compounds as references and shows that antioxidative activities for the chlorophyll analogous compounds can be conveniently measured by the quenching experiment of chemiluminescence (CL) of CLA, except Fe-chlorin e_6 Na_3 and Fe- and Ni-chlorophyllins Na_3. Strong fluorescence from these compounds inhibited the quenching experiment. The chlorophyll analogues showed reaction rate constants ranging from 10^5 to 10^6 $M^{-1}s^{-1}$. These values are as large as those for cytochrome C and ascorbic acid (vitamin C),[6] which suggests that these compounds are antioxidants as strong as the representative natural antioxidants. Nakamura and his co-workers showed that the chlorophyll derivatives act as oxidative stress relievers for patients with SOD-mimic activity in cancer bearing hosts and suggested that the compounds are antioxidative against superoxide.[2,7]

The present results experimentally proved this supposition. However, the reaction rate constants are not parallel to the relative activity of the 5,5-dimethyl-1-pyrroline-1-oxide (DMPO)-OOH adduct given by Nakamura et al.[2] as shown in Table 1. In contrast to the literature, chlorin e_6, having no metal ion, showed a larger constant than the other ones. This can be explained as follows: *in vivo* oxidative stress by superoxide occurs on the cell membranes of the phospholipids or inside the cell and the water-soluble ascorbic acid traps radicals soluble in water but cannot trap peroxy radicals in a membrane,[8] whereas the present quenching experiments were performed in aqueous solutions. SOD shows different strength of activity depending on the internal organs acted upon.[9]

Salcomine, [Co(salen)$_2$], which has a structure resembling to the chlorophyll derivatives, showed a very large rate constant, 1.89×10^8 $M^{-1}s^{-1}$. This compound and its homologues could be excellent, oxidative stress relievers if they are not strongly toxic, as shown in the literature.[10] SOD showed a rate constant, 2.2×10^9 $M^{-1}s^{-1}$ to superoxide. This value is as same as that of SOD used as a reference (2×10^9 $M^{-1}s^{-1}$) for determination of the rate constant of CLA to superoxide.[3] Therefore this proves that the rate constant (k_2) of CLA is reasonable.

Table 1. Rate Constants of Chorophyllins with O_2^-.

Substrates	$k_3/10^5\ M^{-1}s^{-1}$	$IC_{50}/\mu g \cdot ml^{-1}$ [a]
SOD (bovine)	22000	0.28
Fe-chlorin e_6 Na_3	----[b]	2.1
Fe-chlorophyllin Na_3	----[b]	6.4
Co-chlorophyllin Na_3	23.9	9.5
Ni-chlorophyllin Na_3	----[b]	93
Cu-chlorophyllin Na_3	8.28	500
Cu-chlorin e_6 Na_3	21.9	
Zn-chlorin e_6 Na_3	3.47	850
Mg-chlorophyllin Na_3	37.5	1400
Chlorin e_6 Na_3	53.6	>2500
Cytochrome C (horse heart muscle)	42.8	
Salcomine	1890	
Vitamin C	29.9	
Vitamin B_{12}	0	

a) See ref. 2. b) Fluorescence inhibited the quenching experiment.

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葉緑素関連物質（酸素障害緩和剤）のスーパーオキシドに対する抗酸化活性

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ウミホタルルシフェリン誘導体（2-メチル-6-フェニル-3,7-ジヒドロイミダゾ[1,2-*a*]ピラジン-3-オン）（CLA）とO_2^-による化学発光に対する消光反応を利用して，脳血栓や心筋梗塞，臓器移植の際などの虚血ー再灌流時における酸素障害に対して抗酸素障害活性を示す葉緑素関連物質（クロロフィリンおよびクロリンe_6の金属錯体）とスーパーオキシド（O_2^-）との反応速度定数（抗酸化活性）を，25 mM 緩衝液（pH 7.0）中，25℃において初めて測定した。物質によっては，アスコルビン酸とO_2^-との反応速度（$10^6\ M^{-1}s^{-1}$）に匹敵する速度定数をもつことを見いだした。本研究により，酸素障害がスーパーオキシドによって起きている障害である可能性が実験的に証明されたことになる。