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SOD-like Activity of Amino Acids and Related Compounds measured by Chemiluminescence Reaction of *Cypridina* Luciferin Analogues*1

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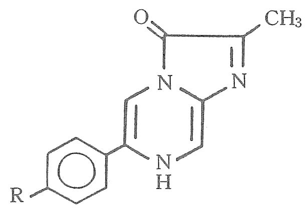
By using the chemiluminescent measuring method of rate constants between superoxide (O_2^-) and antioxidants established in our previous report, rate constants of amino acids and some related sulfur-containing compounds with superoxide were determined by the quenching experiments of chemiluminescence of a *Cypridina* luciferin analogue, 2-methyl-6-phenyl-3, 7-dihydroimidazo[1, 2-*a*]pyrazin-3-one (CLA) in pH 7.0 buffer solutions at 25℃. The results were discussed by comparisons with the known data in the literature. Consequently, we found that the present mesuring method can be applied for the measurements of reaction activities of the antioxidant samples even when it would be fairly unstable.

1 Introduction

Amino acids are suggested as the potential *in vivo* antioxidant against the active oxygen species, especially singlet molecular oxygen ($^1O_2$) and superoxide (O_2^-)[1-4], which play important roles in various biological and chemical processes. For detecting $^1O_2$, direct spectroscopic observation of near-infrared emission at 1.27 μm is one of the best ways, which is the most reliable physical method[1,2]. However, there is no direct spectroscopic way for detecting the latter oxygen species (O_2^-).

Cypridina luciferin analogues, 2-methyl-6-phenyl- and 2-methyl-6-(*p*-methoxyphenyl)-3, 7-



CLA R=H
MCLA R=OMe

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dihydroimidazo [1, 2-*a*] pyrazin-3-ones (CLA and MCLA) were shown to be versatile tools for specific detection of $^1O_2$ and O_2^-[5, 6]. In the previous report, we have reported on rate constants for [CLA] [O_2^-] and [MCLA] [O_2^-] by a quenching experiment of chemiluminescence (CL) by superoxide dismutase (SOD)[7]. In this paper, we describe the application of the chemiluminescent measurement of rate constants of amino acids and related compounds for the SOD-like activity to O_2^- determined by using quenching experiments for chemiluminescence of CLA in pH 7.0 buffer solutions at 25℃.

2 Materials and Methods

SOD (SOD Ⅲ, 3, 520 U/mg) was purchased from Toyobo Co. Ltd, CLA was from Tokyo Chemical Industry, amino acids and the other sulfur-containing samples used were from Wako Pure Chemicals, and hypoxanthine, xanthine oxidase (XOD), and albumin were from Sigma Chemical. Quenching experiments were performed as shown in our former report[7]; A solution containing 10 μl of CLA (final concn 4.4 × 10^{-8} M); (2200-y) μl of 25 mM phosphate buffer (pH 7.1); 500 μl of albumin solution (1 mg/ml in the buffer solution); 50 μl of XOD (200 μl in 1.8 ml of the albumin soln); and y μl of an antioxidant (1.00 × 10^{-3} M) in the buffer solution in a 16 mmϕ quartz cell was placed on a photomultiplier tube (R878, Hamamatsu Photonics) at 25℃ in a dark cell chamber (Aloka Luminometer BLR-102B reconstructed for computer recording). Into the solution was added a 200 μl of 3 mM hypoxanthine in the buffer solution through an injection needle. Total volume of the solution would be 2960 and 2970 μl for 10 and 20 μl of CLA, respectively. CL was measured through the bottom of the quartz cell in the single photon counting technique as usual (Fig. 1).

Reaction rate for O_2^- can be given as follows,

$$d[O_2^-]/dt = E - k_1[O_2^-]^2 - k_2[CLA][O_2^-] - k_3[Q][O_2^-] \qquad (1)$$

where E, k_1, k_2, and k_3 are production rate of O_2^-,

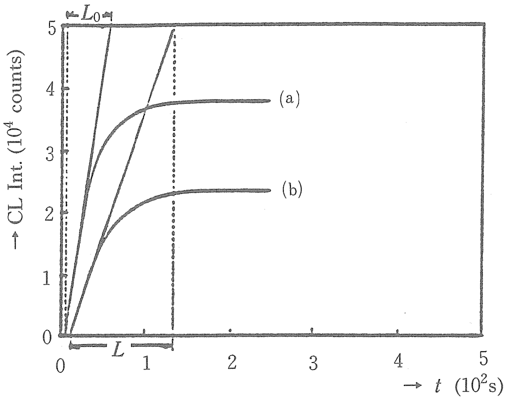

Fig. 1. Chemiluminescence-time curves of CLA (a) with or (b) without a quencher. Measurements were repeated at least three times for each quenching condition.

rate constants of disappearance (2) and of reactions with CLA (3) and with the quencher (Q) (4) of O_2^-, respectively. [CLA], [O_2^-], and [Q] represent concentrations of CLA, of superoxide, and of quenchers used.

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

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

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

At stationary state, the Stern-Volmer equation can be represented as follows,

$$I_0/I = 1 + \{k_3/(k_1[O_2^-] + k_2[CLA])\}[Q] \qquad (5)$$

where I_0/I is ratio of the emission intensity of CLA with O_2^- in the absence and presence of the quencher[7]. The equation 5 indicates that a plot of I_0/I $(=L/L_0$ in Fig. 1) vs [Q] gives a straight line with a slope equal to $k_3/(k_1[O_2^-] + k_2[CLA])$ (Fig. 2). From these values, k_3 for quenching O_2^- with Q can be determined if the values of $k_1[O_2^-]$ and k_2 [CLA] are known. The values* for k_1[4], k_2[7], and [CLA] are known, but those for [O_2^-]

*) $k_1 = 10^2\,M^{-1}s^{-1}$.
$k_2 = 1.08 \times 10^8\,M^{-1}s^{-1}$.

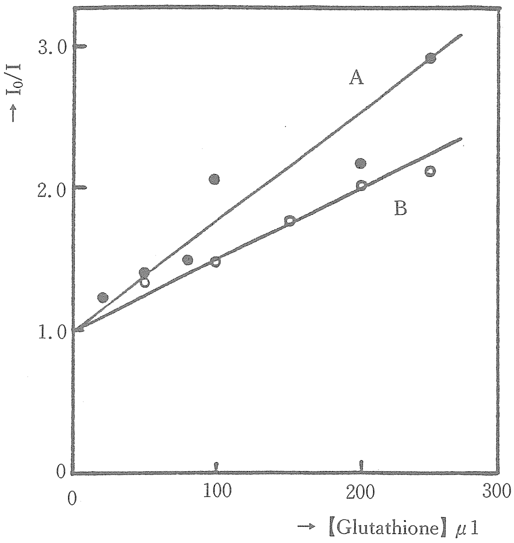

Fig. 2. Stern-Volmer I_0/I-【glutathione】 plots for CL of CLA; The amount of CLA was (A) 10μl and (B) 20μl (final concn 4.4×10^{-8} M and 8.8×10^{-8} M, respectively).
【Glutathione】 means amounts of glutathione (μl of 1.07×10^{-3} M solution) added. The final concentration was 4.00×10^{-5} M at 【Glutathione】 =100 μl.

and $k_1[O_2^-]$ are unknown. In order to eliminate the unknown terms, the I_0/I was plotted for two different concentration of CLA, and from the slopes (A and B) of two straight lines plotted, the each value of k_3 was calculated as shown in equation 8.

$$k_3/(k_1[O_2^-] + k_2[CLA]_1) = A \quad (6)$$

$$k_3/(k_1[O_2^-] + k_2[CLA]_2) = B \quad (7)$$

Therefore,

$$k_3 = k_2([CLA]_1 - [CLA]_2)/(1/A - 1/B) \quad (8)$$

3 Results and Discussion

Results are shown in Tables 1 and 2. The tables show that SOD-like activities can be measured conveniently by the quenching experiment for chemiluminescence of CLA. Neutral amino acids had rate constants ranging from 10^2 to 10^4 $M^{-1}s^{-1}$ and both acidic and basic ones had larger constants. Thiols showed much larger constants. Disulfides such as cystine and oxidized glutathione also showed larger constants than neutral ones did in spite of Asada's report[8]. According to the organic chemical knowledge[4, 9], the oxidation of thiols and disulfides with superoxide proceeds as follows to give sulfinates and sulfonates,

$$RSH \xrightarrow{O_2^-} RSSR \xrightarrow{O_2^-} RSO_2^- + RSO_3^-$$

It is obvious that disulfides should have finite rate constants with superoxide.

Table 1. Rate constants of amino acids with

Amino acid	Rate constant $k_3/10^3\,M^{-1}s^{-1}$	Amino acid	Rate constant $k_3/10^3\,M^{-1}s^{-1}$
Gly	3.27	L-Pro	0.92
L-Ala	0.74	L-HPro	0.54
L-Val	0.65	L-Asp	7.87
L-Leu	1.24	L-Asn	24.0
L-Ileu	0.05	L-Glu	5.70
L-Phe	1.86	L-Gln	4.24
L-Tyr	16.2	L-Lys	9.04
L-Try	11.9	L-Arg	6.02
L-Ser	6.53	L-His	64.5
L-Thr	5.24		

Table 2. Rate constants of sulfur-containing compounds.

Sample	Rate constant $k_3/10^4\ M^{-1}s^{-1}$ Our results	Lit.[a]
$HSCH_2CH(NH_2)COOH$ (L-Cysteine: CySH)	28.4	270
$Me_2C(SH)CH(NH_2)COOH$ (DL-Penicillamine)	12.4	
$HS(CH_2)_2CH(NH_2)COOH$(DL-Homocysteine)	59.3	46
Glu-NH(CH_2SH)CHCO-Gly (Glutathione: GSH)	66.5	67
$MeS(CH_2)_2CH(NH_2)COOH$ (L-Methionine)	6.42	0
$Me_2S^+(CH_2)_2COOH\ Br^-$ (Propiothetin)	0.184	
CyS-SCy (L-Cystine)	2.87	0
GS-SG (Glutathione oxidized)	3.02	0
$H_2N(CH_2)_2SO_3H$(Taurine)	0.00	
HOOC-CH(NH_2)CH_2-SO_3H (L-Cysteic acid)	0.0271	

a) See ref. 8.

Asada et al. also reported rate constants of some other sulfur compounds with O_2^- by the measurement of O. D. change of epinephrine[8], in which rate constants for cysteine is significantly larger than those of the other thiols. It is difficult to understand that cysteine alone has such a large rate constant than penicillamine, homocysteine, and glutathione do.

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ウミホタルルシフェリン誘導体での化学発光測定法による アミノ酸およびその関連物質のSOD様活性

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先に著者らが開発したウミホタルルシフェリン誘導体（CLA類）とスーパーオキシド（SO）との反応速度定数の測定法を応用して，アミノ酸およびその関連物質のスーパーオキシド・ジスムターゼ（SOD）様抗酸化活性を測定した。活性はCLA類の化学発光に対する消光速度の測定からSOとの反応速度定数の形で表示し，既知の速度定数と比較検討した。その結果，極く微量の試料でまた短時間に，SOとの反応活性を測定することができ，そのため不安定な試料の測定も可能であることが判明した。