

Relationship between copper biosorption and microbial inhibition of hydroxyl radical formation in a copper(II)-hydrogen peroxide system

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Abstract The microbial retardation of the spin adduct, DMPO-OH, formed in a copper(II)-hydrogen peroxide-DMPO (5,5-dimethyl-1-pyrroline N-oxide) solution was examined in relation to copper biosorption. A hydroxyl radical is formed in the solution through two steps, the reduction of Cu(II) to Cu(I) by H₂O₂ and the Fenton-type reaction of Cu(I) with H₂O₂. The resultant radical is trapped by DMPO to form DMPO-OH. Microbial cells retarded the DMPO-OH in the Cu(II)-H₂O₂-DMPO far more significantly than in the UV-irradiated H₂O₂-DMPO solution. Egg albumin showed a higher DMPO-OH retardation than microbial cells both in the Cu(II)-H₂O₂-DMPO and the UV-irradiated H₂O₂-DMPO solutions. These results indicated that the retardation effect is related to organic matter and not to microbial activity. Microorganisms having higher affinities for copper ion retarded DMPO-OH more significantly. The linear relationship between the amounts of copper biosorption and the inverse of the median inhibitory doses for DMPO-OH indicated that the microbial cells inhibited the reduction of Cu(II) to Cu(I) by H₂O₂, followed by the decrease of hydroxyl radical formation and the retardation of DMPO-OH. These results also suggest that the coupling between microbial cells and Cu(II) ion can be estimated from their ability to retard DMPO-OH.

Key words: copper biosorption, Cu(II) ion, DMPO, DMPO-OH, hydrogen peroxide, hydroxyl radical elimination

Introduction

While an essential element in biosystems, copper in excess amounts causes toxicity. Thus, the removal of copper from environmental resources has received much attention. Biosorption will be one of the most applicable methods for the removal and recovery of copper because of the remarkable ability of microbial cells to adsorb copper ions, suitability for natural circumstances and low cost (Volesky 1990; Ehrlich and Brierley 1990). The evaluation of the coupling between Cu(II) and microbial cells is a valuable approach to screening microorganisms for copper biosorption.

A hydroxyl radical should be produced in copper(II)-hydrogen peroxide (H_2O_2) through the reduction of Cu(II) to Cu(I) by H_2O_2 and the Fenton-type reaction between Cu(I) and H_2O_2 (Hanna and Mason 1992). While studying the interaction between copper and microbial cells, we observed the microbial retardation of a DMPO-OH radical, a spin adduct of the hydroxyl radical formed in a copper(II)-hydrogen peroxide (H_2O_2)-DMPO (5,5-dimethyl-1-pyrroline N-oxide) solution. The DMPO-OH retardation, in other words, the decrease of the hydroxyl radical, is caused by the direct elimination (scavenging) of the radical (Harriwell and Gutteridge 1999) or the inhibition of the Fenton-type reaction (Lopes, Schulman and Hermes-Lima 1999).

In the present study, therefore, the mechanism of the microbial retardation of DMPO-OH in the Cu(II)- H_2O_2 -DMPO system was examined in relation to copper biosorption.

Materials and Methods

Strains, medium and growth conditions

The strains used in this study, *Arthrobacter nicotianae* IAM 12342, *Bacillus subtilis* IAM 1026, *Citrobacter freundii* IAM 12471, *Corynebacterium glutamicum* IAM 12435, *Escherichia coli* IAM 1268, *Micrococcus luteus* IAM 1056, *Pseudomonas stutzeri* IAM

12097 and *Zooglea ramigera* IAM 12136 (see Table 1) were generously donated by the IAM Culture Collection, Center for Cellular and Molecular Research Institute of Molecular and Cellular Biosciences, The University of Tokyo. The medium for growing bacteria contained the following (in grams per liter of deionized water at pH 6.5): meat extract 3 g; polypeptone 5 g; and sodium chloride 5 g. Bacterial cells were grown in 300 ml of the medium with continuous shaking (130 rpm) at 30°C. Cells in the linear growth phase were collected by centrifugation ($18000 \times g$), washed thoroughly with an isotonic sodium solution, and then used for spin trapping hydroxyl experiments. Fresh cells in one culture were used for following experiments. In parallel, water contents of cells were measured and dry weights of cells were estimated. In all results, cell amounts were described as dry weight basis.

Chemicals

5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was obtained from DOJINDO, Ltd., Kumamoto, Japan. During manufacture, purity greater than 99 % was obtained by repeated vacuum distillation and evaluated by gas chromatography. Other chemicals (guaranteed reagents) used in this study were obtained from Nacalai Tesque, Inc., and Wako Pure Chemical Industries, Ltd. The phosphate buffer (0.1 M) was treated with Chelex 100 (Sigma-Aldrich Co.) and used for the experiments

Electron paramagnetic resonance measurements

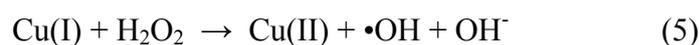
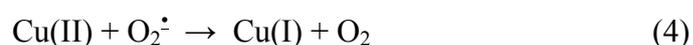
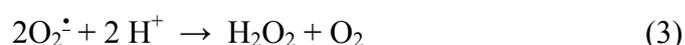
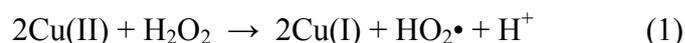
Copper(II) nitrate (final concentration 2.5×10^{-4} M), DMPO (final concentration 0.1 M), H_2O_2 (final concentration 0.1 M), fresh microbial cells (0 - 0.5 g dry cells/l in saline), and phosphate buffer (final concentration 0.01 M, pH 7.4) were mixed, and the mix was aspirated into a capillary tube. Fifty minutes after mixing, its ESR spectrum was recorded using an X-band ESR spectrometer (JES TE-100, JEOL Ltd., Tokyo, controlled by WIN-RAD ESR data analyzer, Radical Research Inc., Tokyo) under the following conditions: microwave power, 5 mW; microwave frequency, 9.42 GHz; magnetic field, 335.3 mT; field amplitude, ± 5 mT;

field modulation, 100 kHz; modulation width, 0.079 mT; sweep time, 1 min; and response time, 0.1 sec. A mixture solution of fresh microbial cells (final concentration 0 – 2 g dry cells/l in saline), H₂O₂ (final concentration 0.01 M) and DMPO (final concentration 0.01 M) in a capillary tube was irradiated with a glass fiber-type UV irradiator (Radical Research RUVF-203S; Hg-Xe lamp, wavelength, 365 nm; power, 200 W) for 2 s. Ten minutes after the irradiation, its ESR spectrum was recorded. The concentrations of H₂O₂ and DMPO for the UV irradiation experiments were adjusted to 0.01 M to obtain almost identical ESR signal intensities for DMPO-OH as those in the Cu(II)-H₂O₂-DMPO system.

Results and discussion

Generation of the DMPO-OH radical in the Cu(II)-H₂O₂-DMPO system

When Cu(NO₃)₂ (2.5 × 10⁻⁴ M), DMPO (0.1 M), and H₂O₂ (0.1 M) were mixed, a large ESR signal of DMPO-OH with four lines (intensity ratio, 1:2:2:1) was observed (Fig. 1a). From its g-value = 2.0066, and its hyperfine coupling constants, a_N = 1.49 mT and a_H = 1.49 mT, the signal was identified to be a DMPO-OH radical. The concentration of DMPO-OH in the first two minutes was about 3 × 10⁻⁵ M. According to Hanna and Mason (1992), the reaction process in the Cu(II)-H₂O₂-DMPO solution, an analog to the Fenton reaction, is as follows:



Therefore, DMPO-OH was formed in the Cu(II)-H₂O₂-DMPO solution through the reduction of Cu(II) to Cu(I) by H₂O₂, the Fenton-type reaction between Cu(I) and H₂O₂, and the spin-trapping reaction of DMPO. The spin-trapping reaction is so fast and complete (Madden and Taniguchi 2001) that the amounts of DMPO-OH can be directly related to those of the

hydroxyl radical formed in the solution. In the following experiments, a mixture solution containing 2.5×10^{-4} M $\text{Cu}(\text{NO}_3)_2$, 0.1 M DMPO and 0.1 M H_2O_2 was used as the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution.

Effect of microbial cells on hydroxyl radicals formed in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ system

In the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution, the ESR signal intensity of DMPO-OH gradually decreased with time. When microbial cells, such as *Arthrobacter nicotianae*, were present in the solution, the ESR signal intensity of DMPO-OH decreased more rapidly than that in the solution without the cells (Fig. 1). Large amounts of the cells, such as 0.5 g dry cells/l, diminished the DMPO-OH signal completely. These results suggested that microbial cells retarded the DMPO-OH in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution. As described in the previous section, the amounts of DMPO-OH are directly related to those of the hydroxyl radical formed. Microbial cells, therefore, inhibited hydroxyl radical formation in the solution. The inhibition of hydroxyl radical formation varied with different species of microorganisms. In the UV-irradiated $\text{H}_2\text{O}_2\text{-DMPO}$ (UV- $\text{H}_2\text{O}_2\text{-DMPO}$) solution, DMPO-OH was also retarded by microbial cells. Figure 2 indicates the effect of the microbial cell concentration on the ESR signal intensity of DMPO-OH in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ and the UV- $\text{H}_2\text{O}_2\text{-DMPO}$ solutions. As shown in Fig. 2, the retardation of DMPO-OH by microbial cells in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution was much larger than that in the UV- $\text{H}_2\text{O}_2\text{-DMPO}$ solution. Egg albumin showed a higher DMPO-OH retardation than microbial cells both in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ and the UV- $\text{H}_2\text{O}_2\text{-DMPO}$ solutions. These results indicated that the retardation effect is related to organic matter and not to microbial activity. Furthermore, the DMPO-OH retardation was higher in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution than in the UV- $\text{H}_2\text{O}_2\text{-DMPO}$ solution in similar manner as microbial cells. In the UV- $\text{H}_2\text{O}_2\text{-DMPO}$ solution, hydroxyl radical interacted with organic matter directly. On the other hand, in the $\text{Cu}(\text{II})\text{-H}_2\text{O}_2\text{-DMPO}$ solution, organic matter coupled with the $\text{Cu}(\text{II})$ ion (Nakajima 2002) in addition to having a direct interaction with the hydroxyl radical.

The median inhibitory doses for DMPO-OH formation, ID_{50} (g dry cells/l), being

defined as the cell concentrations when the ESR signal intensity of DMPO-OH is half of that without microbial cells, are listed in Table 1 with the amounts of copper biosorption by various microbial cells (Nakajima 2002). As shown in Table 1, ID₅₀ in the Cu(II)-H₂O₂-DMPO solution was about one fifth to one tenth of that in the UV-H₂O₂-DMPO solution. No clear relationship between the ID₅₀ in the Cu(II)-H₂O₂-DMPO and that in the UV-H₂O₂-DMPO solutions was observed. These results indicated that the retardation of DMPO-OH in the Cu(II)-H₂O₂-DMPO solution was not caused by the direct elimination of the radical, as mentioned above. Table 1 also indicates that microbial cells that have taken up larger amounts of copper have relatively smaller ID₅₀ values for DMPO-OH in the Cu(II)-H₂O₂-DMPO solution, suggesting that microbial cells with higher copper-coupling abilities heavily retarded DMPO-OH.

In the Cu(II)-H₂O₂-DMPO solution, the initial concentration of Cu(II) ion was 2.5×10^{-4} M, and that in [H₂O₂] = [DMPO] = 0.1 M. As long as larger amounts of H₂O₂ and DMPO than of Cu(II) ion are present in the solution, the amounts of the hydroxyl radical and DMPO-OH should be mainly related to the concentration of free Cu(II) ion as follows:

$$[\text{DMPO-OH}] = C_1[\bullet\text{OH}] \approx C_2[\text{Cu(II)}] \quad (7)$$

where C₁ and C₂ are suitable constants. The free Cu(II) ion, which is able to react with H₂O₂, should be estimated as follows:

$$[\text{Cu(II)}] = [\text{Cu(II)}]_0 - mQ_0 \quad (8)$$

where [Cu(II)]₀ is the initial Cu(II) concentration (M), m, the amount of microbial cells in the solution (g dry cells/l), and Q₀, the amount of copper coupled with microbial cells. As the ID₅₀ values (g dry cells/l) are defined as the cell concentrations when the ESR signal intensity of DMPO-OH is half of that without microbial cells, the amount of microbial cells, m, at [Cu(II)] = [Cu(II)]₀/2 becomes ID₅₀. Thus, Equation (8) could be rewritten as follows.

$$Q_0 = [\text{Cu(II)}]_0/2\text{ID}_{50} \quad (9)$$

As shown in Fig. 3, Q₀, the amounts of copper biosorption, is almost linearly related to the inverse of the ID₅₀ values, except for *Bacillus subtilis* (IAM 1026). Therefore, the assumption in Equation (7) holds for most microbial cells. These results indicated that the microbial retardation of DMPO-OH in the Cu(II)-H₂O₂-DMPO solution is mainly related to the

coupling of microbial cells with the Cu(II) ion. As described in the previous section, the amount of DMPO-OH is directly related to that of the hydroxyl radical formation. The inhibition of hydroxyl radical formation should be related to the reaction containing Cu(II), which is shown in Equation (1) and explained as the reduction of Cu(II) to Cu(I) in the previous section.

For *Bacillus subtilis* (IAM 1026), the amount of copper biosorption was not linearly related to the inverse of the ID₅₀ values. Previous results of ESR analysis indicated that the interaction between Cu(II) and cell proteins was quite similar among the bacteria tested (Nakajima 2002). The characteristics of copper biosorption are not, therefore, the main cause for the lack of linearity. The most likely reason for the lack of linearity of *Bacillus subtilis* (IAM 1026) appears to be the higher catalase activity of *B. subtilis* (Holt 1986). Catalase might decompose hydrogen peroxide, which results in a reduction in the formation of the hydroxyl radical. Further studies will be undertaken to clarify the peculiar behavior of *B. subtilis*.

Conclusion

The hydroxyl radical formation in the Cu(II)-H₂O₂-DMPO solution was heavily inhibited by microbial cells. Egg albumin inhibited the hydroxyl radical formation much more significantly than microbial cells, which indicates that inhibition is caused by organic matter, not by microbial activity. The microbial inhibition of hydroxyl radical formation appears to be caused by the inhibition of the reduction of Cu(II) to Cu(I) with H₂O₂, the first step in a series of reactions leading to hydroxyl radical formation, but not by the direct elimination of the hydroxyl radical. As the amounts of copper biosorption are linearly related to the inverse of the ID₅₀ values, the microbial retardation of DMPO-OH in the Cu(II)-H₂O₂-DMPO solution can be used to evaluate the coupling between microbial cells and Cu(II) ion.

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Table 1. Median inhibitory dose for DMPO-OH and the amounts of copper biosorption by bacteria.

Species	ID ₅₀ (g dry cells/l) ^{a)}		Cu adsorbed ^{b)} ($\mu\text{mol/g dry cells}$) Q ₀
	Cu(II)	UV	
<i>Arthrobacter nicotianae</i> IAM 12342	0.13 \pm 0.02	2.1 \pm 0.3	603 \pm 5
<i>Bacillus subtilis</i> IAM 1026	0.11 \pm 0.02	2.3 \pm 0.3	327 \pm 5
<i>Citrobacter freundii</i> IAM 12471	0.31 \pm 0.04	2.3 \pm 0.3	212 \pm 3
<i>Corynebacterium glutamicum</i> IAM 12435	0.94 \pm 0.10	5.5 \pm 0.6	98 \pm 3
<i>Escherichia coli</i> IAM 1268	0.27 \pm 0.04	1.7 \pm 0.2	287 \pm 6
<i>Micrococcus luteus</i> IAM 1056	0.15 \pm 0.02	2.5 \pm 0.3	529 \pm 3
<i>Pseudomonas stutzeri</i> IAM 12097	0.17 \pm 0.02	2.5 \pm 0.3	361 \pm 6
<i>Zooglea ramigera</i> IAM 12136	0.18 \pm 0.02	3.0 \pm 0.4	362 \pm 4
Egg albumin	0.06 \pm 0.01	0.75 \pm 0.12	ND

a) Median inhibitory dose, ID₅₀ (g dry cells/l), represents the estimated value \pm standard error of the cell concentration when the ESR signal intensity of DMPO-OH is the half of that for without microbial cells. b) Experimental conditions on copper biosorption were described previously (Nakajima 2002). Sorption amounts represent as mean \pm standard deviation of triplicate on one culture. “ND” means “not determinable”.

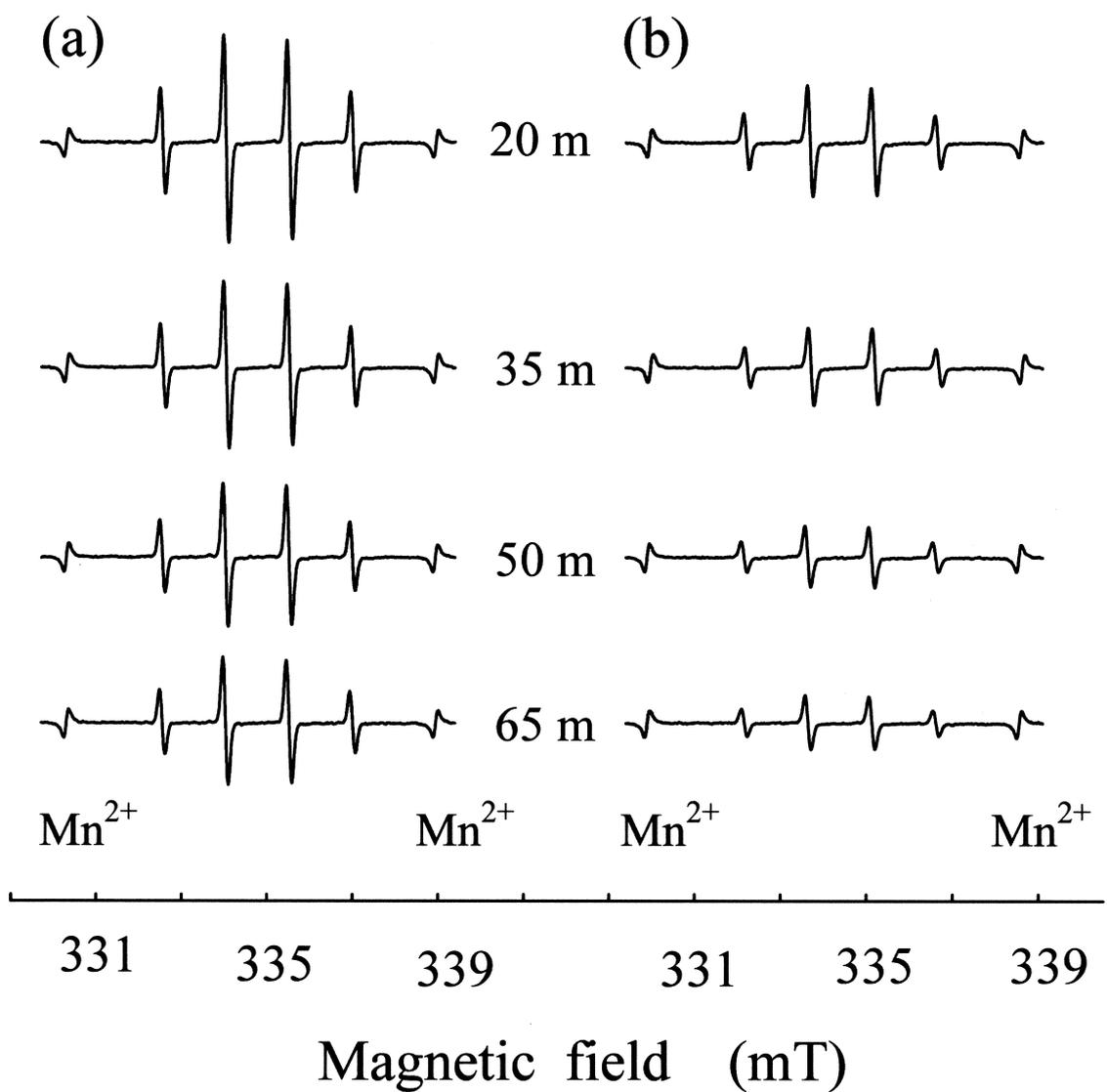


Fig. 1. The ESR spectra of the DMPO-OH signal in the Cu(II)-H₂O₂-DMPO system without (a) and with *Arthrobacter* cells (b) at each time after the preparation of the mixture.

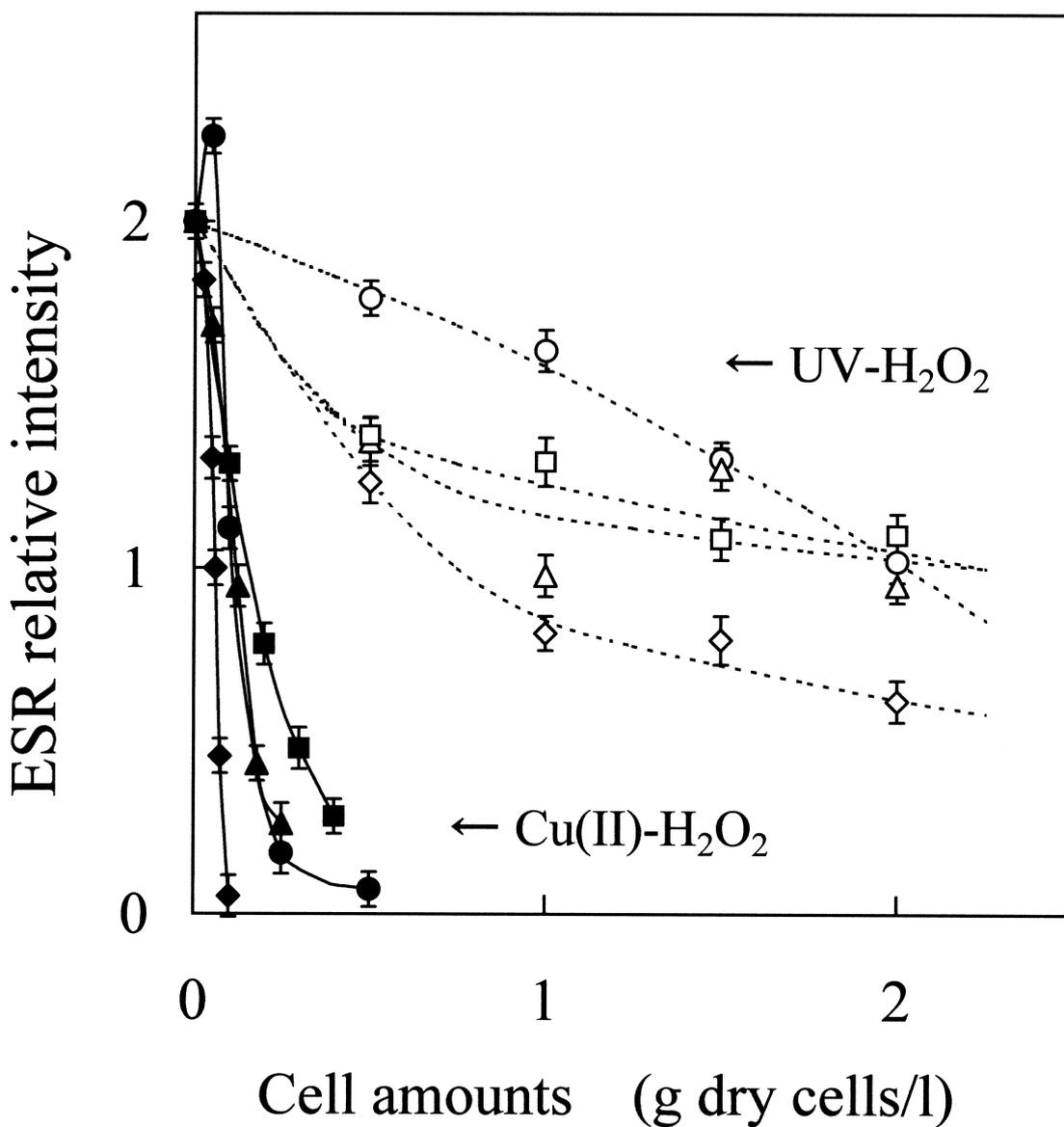


Fig. 2. The effect of microbial cells on DMPO-OH formation in the Cu(II)-H₂O₂-DMPO and UV-H₂O₂-DMPO systems. *Arthrobacter nicotianae* : Cu (closed circle), UV (open circle); *Bacillus subtilis* : Cu (closed triangle), UV (open triangle); *Micrococcus luteus* : Cu (closed square), UV (open square); Egg albumin : Cu (closed diamond), UV (open diamond).

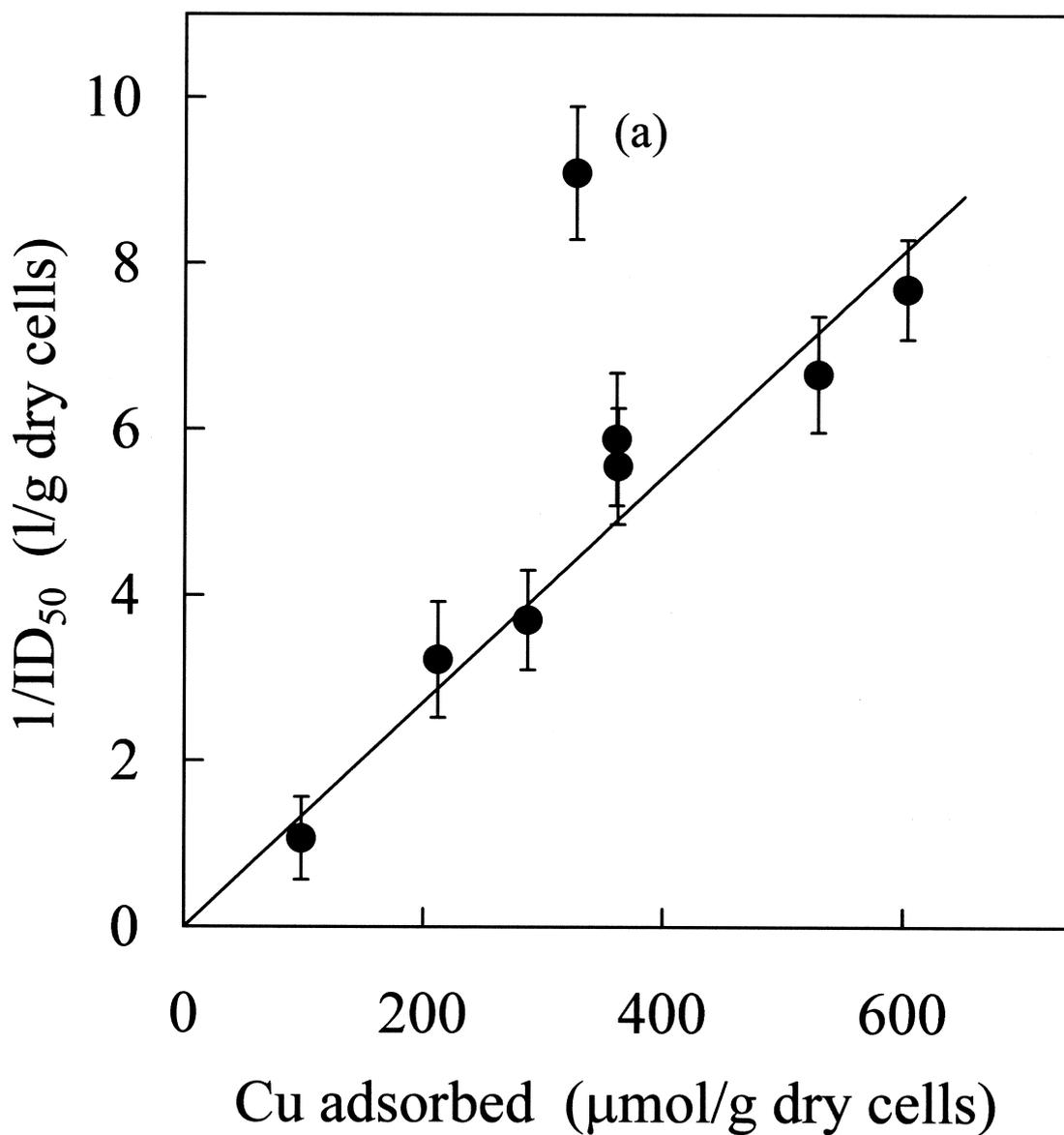


Fig. 3. Relationship between the copper biosorption amounts, Q_0 , and the inverse of the median inhibitory doses for DMPO-OH formation, ID_{50} . The solid line was calculated using the obtained results listed in Table 1 except for *Bacillus subtilis* IAM 1026 (a) by the least square method