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INVITED REVIEW

Reactive oxygen species inhibitory diagrams and their usability for the evaluation of antioxidant ability

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ABSTRACT

The reactive oxygen species (ROS) inhibitory diagrams on the abilities of biosubstances to eliminate hydroxyl, superoxide and alkoxyl radicals were introduced and the role of biosubstances against the oxidative stress was discussed using these diagrams.
Hydroxyl radical causes the hydrogen abstraction with alkyl compounds and the one-electron oxidation reaction with phenol compounds, and reacts with substances encountered first after the radical is born in the system without any peculiarity. In superoxide radical elimination, the disproportionation reaction becomes important than ordinal radical reaction with stable molecules. The reaction characteristics of alkoxyl radical is slightly different from both hydroxyl and superoxide radicals, which indicates the reaction peculiarity caused by its bulky structure (RO•, R = $C(CH_3)_2$-$C(^+NH_2Cl^-)NH_2$).
The ROS inhibitory diagrams, especially the oxygen radical absorbance capacity (ORAC)-1/id^s_{50} diagram, apparently indicate that each biosubstance has favorable free radical to eliminate. These excellent biosubstances in foods and beverages work as exogenous antioxidants in the biosystems for superoxide, hydroxyl and lipid peroxide radicals, and also supplement the role of endogenous antioxidants, L-ascorbic acid and reduced glutathione.
As the ROS inhibitory diagrams are based on the eliminating abilities of free radicals produced during oxygen stress, they should respond to the need to search excellent endogenous antioxidants, and be excellent presentations for healthy life.

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INTRODUCTION

Excess formation of reactive oxygen species (ROS), especially oxygen-containing free radicals such as hydroxyl and superoxide radicals, under oxygen stress react with stable molecules, such as proteins, amino acids, saccharides and lipids, and oxidize these molecules, which causes various life-style diseases [1, 2]. Biosubstances in foods and beverages having high antioxidant abilities are essential to remove these free radicals and maintain a healthy life. Various methods, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) [3-5], the trolox equivalent antioxidant capacity (TEAC) [6, 7], and the oxygen radical absorbance capacity (ORAC) [8-10] assays, are used to determine the antioxidant abilities of foods. Fundamentally, the first two assays are based on the eliminating abilities of free radicals, such as DPPH and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{\bullet+}$), none of which is in the human body. In addition, the chemical reactivity of these radicals was obviously less than that of hydroxyl and superoxide radicals. Because of the varied reaction characteristics of the different free radicals, their eliminating abilities are not always related to the antioxidant abilities of biosubstances. Only the eliminating abilities of ROS found in the human body, such as hydroxyl, superoxide, peroxy and alkoxy radicals, reflect the real antioxidant abilities of biosubstances.

The estimation of the abilities to eliminate hydroxyl and superoxide radicals was examined using the spin-trapping ESR method. The method, being already established [11-14], is based on the competitive reaction among each free radical, substrates and spin traps. As peroxy and alkoxyl radicals derived from 2,2'-azobis(2,4-amidinopropane) dihydrochloride (AAPH) in the ORAC assay should correspond to lipid peroxide and lipid oxide radicals in biosystem, the assay can be adopted as the evaluation method for the peroxy and/or alkoxy radical-elimination. The assay is based on the delay of the fluorescein extinction with peroxy (ROO•, R = $C(CH_3)_2$-$C(^+NH_2Cl^-)NH_2$) or alkoxy radical (RO•) derived from AAPH by substrates (the ORAC-FL assay), and presented as the ORAC values relative to that of Trolox [8]. As the ORAC-FL assay includes complicated reactions including fluorescein, we should introduce extra parameter on the fluorescein radical for phenol compounds [15]. Furthermore, we could obtain the data for only restricted antioxidants [8-10, 16-18]. To overcome these problems, the ORAC-ESR (electron spin resonance) assay was developed [19, 20]. The assay is based on the competitive reaction between alkoxyl radical derived from AAPH, substrates and spin-traps, which is the same technique as those of hydroxyl and superoxide radicals. The ORAC values of various biosubstances were obtained using the ORAC-ESR assay [21].

In the present work, we give the outline of the two-dimensional - ROS inhibitory - diagrams for the eliminating abilities of biosubstances for hydroxyl and superoxide radicals in combination with the ORAC-ESR assay, and discuss the role of biosubstances to protect from the injury caused by oxidative stress [21, 22].

EVALUATION OF FREE RADICAL-ELIMINATION BY BIOSUBSTANCES

The free radical-eliminating abilities were evaluated using the spin-trapping ESR method on the basis of the competitive reaction between the spin-trapping of the free radical, R•, and its elimination by substrates [21, 22]. A spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used for the hydroxyl radical (•OH)-trapping, and 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) for superoxide (O_2•$^-$) and alkoxy radical (RO•)-trapping:

$$R\bullet + ST \rightarrow ST\text{-}R \quad (1)$$

$$R\bullet + SB \rightarrow \text{Reaction Product} \quad (2)$$

$$d[ST\text{-}R]/dt = k_{ST}\,[ST][R\bullet \quad (3)$$

$$d[\text{Reaction Product}]/dt = k_{SB}\,[SB][R\bullet] \quad (4)$$

$$d[ST\text{-}R]/dt : d[\text{Reaction Product}]/dt = I : I_0 - I \quad (5)$$

$$I_0/I - 1 = (k_{SB}/k_{ST})\ ([SB]/[ST]) \quad (6)$$

-[ST] and [SB] are the concentrations of spin trap (ST) and substrate (SB);

-k_{ST} and k_{SB} are the second-order rate constants for reactions (1) and (2);

-I and I_0 are the spin concentrations of spin adduct, ST-R, in the presence and absence of a substrate, respectively.

The ESR spectra of spin adducts, DMPO-OH, CYPMPO-O_2•$^-$ and CYPMPO-OR, R = $C(CH_3)_2$-$C(^+NH_2Cl^-)NH_2$), are shown in Figure 1. The spin concentration of each spin adduct was estimated using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) as a standard after double integration of the observed ESR signals.

In the early stage;

$$[SB] = [SB]_0,\ [ST] = [ST]_0 \quad (7)$$

$$I_0/I - 1 = (k_{SB}/k_{ST})\ ([SB]_0/[ST]_0) \quad (8)$$

When $I = 0.5$;

$$k_{SB} = k_{ST}\,[ST]_0/[SB]_0 \quad (9)$$

As $[SB]_0 = ID_{50}$ (50 % inhibitory dose)

$$k_{SB} = k_{ST}\,[ST]_0/[ID_{50}] \propto 1/[ID_{50}] \quad (10)$$

To avoid confusion, ID_{50} in mole concentration unit should be described as ID^h_{50} (mM) for the hydroxyl radical and ID^s_{50} (mM) for the superoxide radical, and that in the mass concentration as id^h_{50} (mg/ml) for the hydroxyl radical and id^s_{50} (mg/ml) for the superoxide radical. The former is important to discuss in relation to the second-order rate constant, and the latter, to evaluate the antioxidant abilities of components in natural substances. Equation 10 indicates that we can adopt $1/[ID_{50}]$ as the indicator of the free radical-eliminating ability of substrate. As the values $1/[ID^h_{50}$ (mM)] and $1/[ID^s_{50}$ (mM)] of various substrates are linearly related to k_{SB} obtained by the pulse radiolysis and the flash photolysis in a logarithmic presentation, $1/[ID_{50}]$ is a very good parameter for the free radical-eliminating abilities. The ORAC values (μmol TE/g) of substrates for alkoxyl radical were, therefore, calculated from the ratio of k_{SB}/k_{ST} (substrates) to k_{SB}/k_{ST} (Trolox) [20]. The hydroxyl, superoxide radical-eliminating abilities ($1/[id^h_{50}$ (mg/ml)] and $1/[id^s_{50}$ (mg/ml)]), and the ORAC values of selected biosubstances are listed in Table 1.

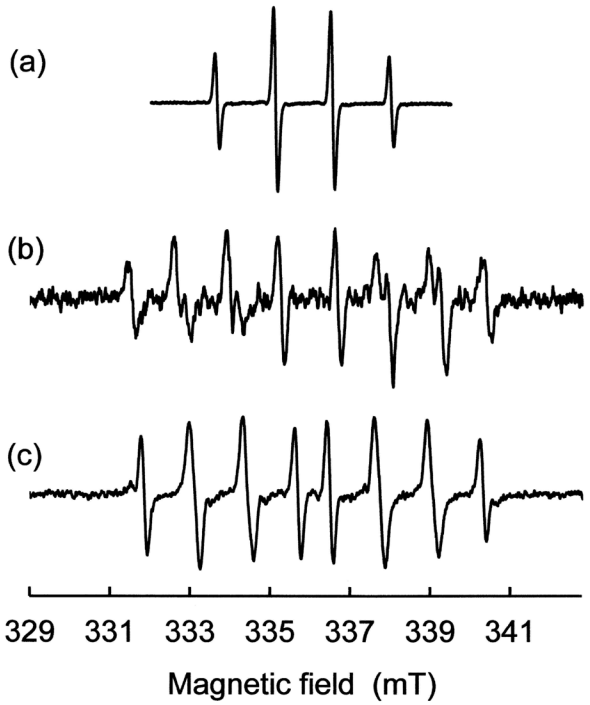

Figure 1. ESR spectra of spin adducts using for the evaluation of the free radical elimination. **(a)** spin adduct for hydroxyl radical (DMPO-OH); **(b)** spin adduct for superoxide radical (CYPMPO-O_2•$^-$); and **(c)** spin adduct for alkoxy radical (CYPMPO-OR, R = $C(CH_3)_2$-$C(^+NH_2Cl^-)NH_2$).

HYDROXYL RADICAL-ELIMINATING ABILITIES OF BIOSUBSTANCES

Hydroxyl radical causes the hydrogen abstraction with alkyl compounds and the one-electron oxidation reaction with phenol compounds. In general, the second-order rate constants of biosubstances, such as proteins, amino acids, saccharides, carboxylic acids, and polyphenols, are so large ($10^7 \sim 10^{10}$/M/s) [22, 23], hydroxyl radical react with substances encountered first after the radical is born in the system without any peculiarity. Targets of the hydrogen abstraction by hydroxyl radical are methyl, methylene, methine, hydroxyl, carboxyl and amino groups. Alkyl amino acids having larger number of these functional groups have larger hydroxyl radical-eliminating abilities, *cf.* L-leucine ~ L-isoleucine > L-valine > L-α-alanine > glycine, and acetic acid < propionic acid < butyric acid < hexanoic acid. As sulfhydryl group is easily oxidized to disulfide or sulfoxide, L-cysteine has a high radical-eliminating ability. Peptides composed of the same amino acid residue with larger polymerization grade have higher radical-eliminating abilities, *cf.* glycylglycylglycine > glycylglycine > glycine and L-alanylalanylalanine ~

L-alanylalanine > L-α-alanine. The radical-eliminating abilities of peptides with different kind of amino acids, such as L-glutathione (reduced, GSH), indicate the similar values as that of amino acid residue (L-cysteine) with highest abilities. The value of cytchrome *c* (cyt-*c*) is larger than those of other proteins, which should be caused by its oxidation reaction of DMPO to DMPOX through DMPO-OH [24]. However, the values of proteins are in the intermediate range of those of amino acids. Saccharides having a large number of methine and hydroxyl groups indicate the large radical-eliminating abilities in comparison with alkyl amino acids. Increase of pyranose and furanose rings increases the radical-eliminating abilities, *cf*. trisaccharide (D-raffinose) > disaccharide (D-cellobiose, D-sucrose) > monosaccharides (D-galactose, D-glucose). The delocalization of unpaired electron in aromatic ring and the formation of stable semiquinone radical in catechol, galloyl, and chroman groups increase the radical-eliminating abilities of these compounds. One- and two-electron oxidation of L-ascorbic acid (vitamin C), being strong antioxidant in human body, forms stable ascorbyl radical and dehydroascorbic acid, which indicates high radical-eliminating abilities.

Table 1. ORAC values, hydroxyl-radical-eliminating capacities and superoxide-radical-eliminating capacities of functional biosubstances

Biosubstances	ORAC value (μmol TE/g[d])	Hydroxyl radical (1/{id^h_{50}[mg/ml]}[f])	Superoxide radical (1/{id^h_{50}[mg/ml]}[f])
Glycine	0.57	0.017	---[g]
L-α-Alanine	0.61	0.067	---[g]
L-Phenylalanine	1.66	3.3	---[g]
L-Glutamine	1.31	0.2	---[g]
L-Tryptophane	3390	4.3	0.4
L-Proline	0.48	0.5	0.0031
L-Cysteine	11200	2.6	77
L-Methionine	471	2.2	0.61
L-Lysine	0.39	0.5	0.012
L-Arginine	0.87	0.28	0.01
L-Histidine	78.3	2.8	0.036
L-Homoserine	4.86	1.3	0.02
L-Anserine	38.4	1.4	0.024
L-Carnosine	68.2	1.4	0.013
L-Glutathione (reduced) (GSH)	3810	0.77	11
Gelatin	3.33	0.18	0.53
Casein	13.6	0.33	2.6
Bovine serum albumin (BSA)[a]	10.8	0.4	0.23
Cytochrome c[b]	12.7	3	2.3
Superoxide dismutase (SOD)[c]	0.92	0.4	13000
Acetic acid	0.52	0.14	0.014
2-Deoxy-D-ribose	9.37	0.83	0.017
D-Glucose	2.28	0.71	---[g]
D-Mannitol	3.46	0.5	---[g]
Chondroitin sulfate	12.4	0.5	0.083
L-Ascorbic acid (Vitamin C)	26000	83	910
Trolox	3995[e]	56	67
(+)-Catechin	2660	1.3	2600
(–)-Epicatechin	3250	1.2	1400
(–)-Epigallocatechin (EGC)	10300	1.3	5300
(–)-Epigallocatechin gallate (EGCG)	12100	3.2	6300
Dopamine	3510	1.2	3100
Gallic acid (GA)	15500	7.7	12000
Caffeic acid	11600	15	3700
L-DOPA	3100	24	4200

[a]Molecular weight 66 kDa; [b]molecular weight 12.384 kDa (from horse heart); [c]molecular weight 32.5kDa (from bovine erythrocyte); [d]TE means trolox equivalent (Cao and Prior [9]); [e]μmol of 1 g Trolox (molecular weight 250.2); [f]calculated from tables of Nakajima et al [22]; [g]the id^s_{50} (mg/ml) values of these substances were greater than their solubilities.

As a whole, the hydroxyl radical-eliminating abilities are in the order of Trolox, L-ascorbic acid > 3,4-dimethyl-L-phenylalanine (L-DOPA) ~ caffeic acid > (–)-epigallocatechin gallate (EGCG), gallic acid (GA) > aromatic amino acids > sulfur-containing amino acids ~ proteins.

SUPEROXIDE RADICAL-ELIMINATING ABILITIES OF BIOSUBSTANCES

In superoxide radical elimination, the disproportionation reaction becomes important than ordinal radical reaction with stable molecules. The rate constant of spontaneous disproportionation reaction in neutral range is $8.5 \times 10^4 \sim 8.5 \times 10^5$/M/s, and the reaction proceeds more slowly in basic range and more rapidly in acidic range [25]. In biosystems, there exist the superoxide-regulation enzyme, superoxide dismutase (SOD), and superoxide is disproportionated to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). As superoxide radical has not so strong oxidation potential, most of alkyl amino acids, saccharides and carboxylic acids indicate little or no radical-eliminating abilities. L-Cysteine and GSH have larger radical-eliminating abilities in similar manner as hydroxyl radical. Substances having catechol and galloyl groups also indicate larger radical-eliminating abilities in similar manner as hydroxyl radical. Increase of the number of galloyl group increases the ability. As a whole, the superoxide radical-eliminating abilities are in the order of SOD > EGCG > GA ≥ (–)-epigallocatechin (EGC) > catechins > L-ascorbic acid > cyt-*c* ≥ Trolox > L-cysteine ≥ GSH.

ORAC VALUES OF BIOSUBSTANCES

Largest radical-eliminating ability is found in L-ascorbic acid, and L-cysteine, EGC, EGCG, GA and caffeic acid have also very large abilities. Polyphenols containing

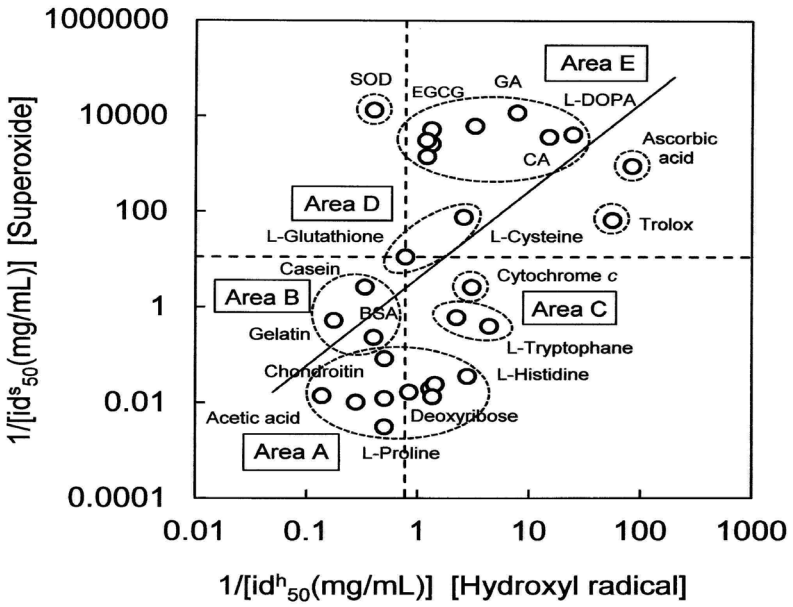

Figure 2. ROS inhibitory diagram for the hydroxyl radical-eliminating abilities $1/[id^h_{50}$ (mg/ml)] (abscissa) and the superoxide radical-eliminating abilities $1/[id^s_{50}$ (mg/ml)] (ordinate).

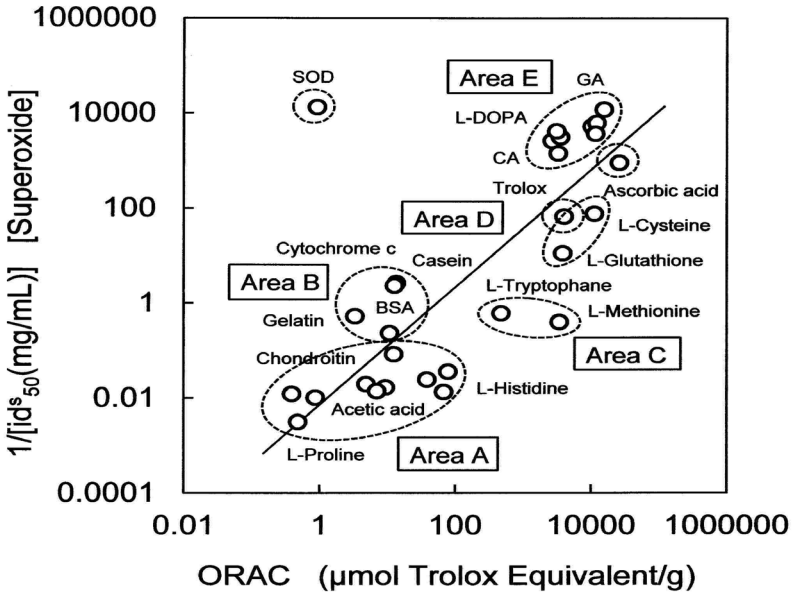

Figure 3. ROS inhibitory diagram for the ORAC values (abscissa) and the superoxide radical-eliminating abilities $1/[id^s_{50}$ (mg/ml)] (ordinate).

catechol group, such as caffeic acid, dopamine, L-DOPA and catechin, present also large abilities. L-Tryptophane and GSH have also similar radical-eliminating ability as polyphenols. The ability of GSH is smaller than L-cysteine because of its higher molecular weight. Proteins, other amino acids, saccharides, and carboxylic acid have very small radical-eliminating abilities. In these biosubstances, L-histidine, L-carnosine and L-anserine have relatively larger abilities. Though SOD has the highest superoxide radical-eliminating ability, it has smaller alkoxyl radical-eliminating abilities than other proteins, which indicates the peculiarity of SOD for the disproportionation reaction. The ORAC value of cyt-*c* is in similar range as those of other proteins. The reaction characteristics of alkoxyl radical is slightly different from both hydroxyl and superoxide radicals, which indicates the peculiarity caused by its bulky structure (RO•, R = $C(CH_3)_2$-$C(^+NH_2Cl^-)NH_2$). As a whole, the ORAC values are in the order of L-ascorbic acid > GA ≥ EGCG ~ caffeic acid ~ L-cysteine ~ EGC > Trolox ~ GSH ~ L-tryptophane ~ dopamine ~ L-DOPA ~ catechins > L-hystidine and its peptides > others.

ROS INHIBITORY DIAGRAMS OF BIOSUBSTANCES

The hydroxyl and superoxide radical-eliminating abilities were depicted two-dimensionally, taking $1/[id^h_{50}$ (mg/ml)] as the abscissa and $1/[id^s_{50}$ (mg/ml)] as the ordinate (Figure 2, $1/id^h_{50}$–$1/id^s_{50}$ diagram). As shown in Figure 2, each group of biosubstances are placed into separated five major areas as A to E on a two-dimensional map; area A, amino acid analogs and carbohydrates (saccharides and carboxylic acids) without redox-active moiety; area B, proteins without SOD and cyt-*c*; area C, L-tryptophane and methionine; area D, L-cysteine and GSH; and area E, polyphenols. L-Ascorbic acid, Trolox, SOD and cyt-*c* are depicted separately. Hydroxyl radical-eliminating abilities, $1/[id^h_{50}$ (mg/ml)], are ranged in narrower range from 0.017 to 84, and the ratio between the largest $1/[id^h_{50}$ (mg/ml)] value and the smallest one is about 5000. As described in section 3, hydroxyl radical react with substances encountered first after the radical is born in the system without any peculiarity. On the other hand, superoxide radical-eliminating abilities, $1/[id^s_{50}$ (mg/ml)], are ranged in very broad range from 0.0003 to

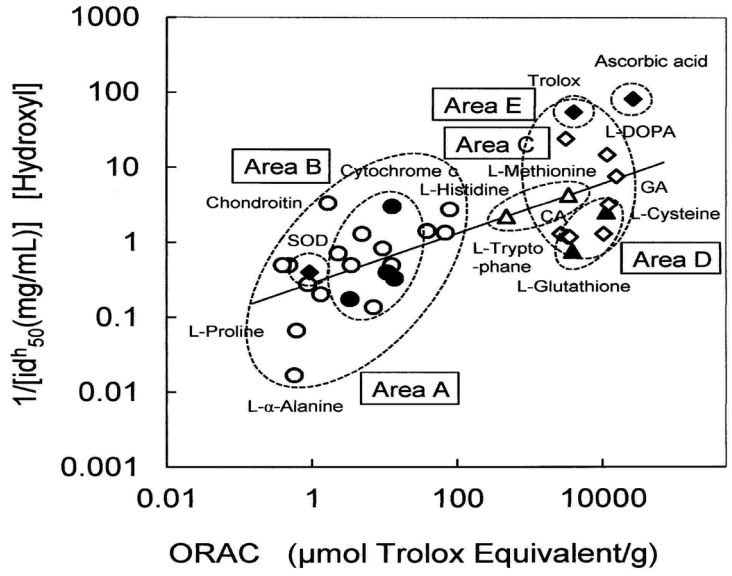

Figure 4. ROS inhibitory diagram for the ORAC values (abscissa) and the hydroxyl radical-eliminating abilities $1/[id^h_{50}$ (mg/ml)] (ordinate). The open circles indicate biosubstances included in **area A**, the closed circle, those in **area B**, the open triangle, those in **area C**, the closed triangle, those in **area D**, the open diamond, those in **area E**, and the closed diamond, isolated biosubstances (L-ascorbic acid, Trolox and SOD).

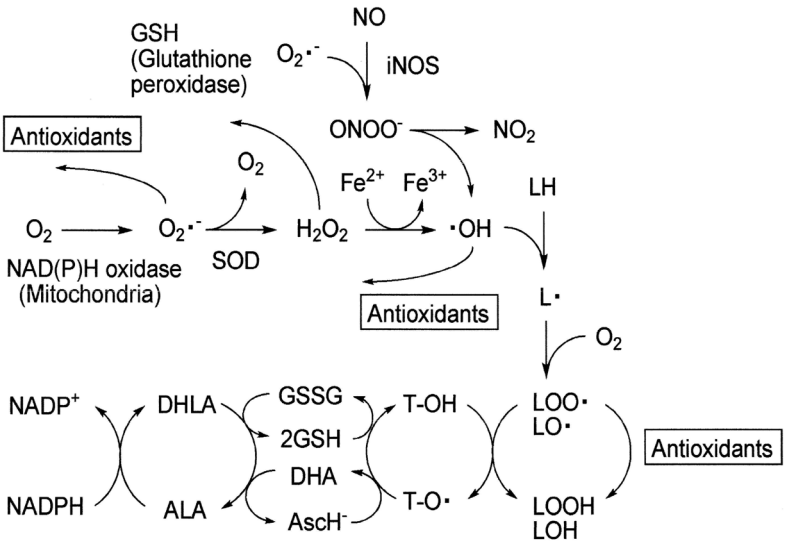

Figure 5. Pathway of ROS formation, the lipid peroxidation, and the role of antioxidants in the management of oxidative stress. **ALA**, α-lipoic acid; **AscH**, L-ascorbic acid; **DHA**, dehydroascorbic acid; **DHLA**, dihydrolipoic acid; **GSH**, glutathione; **GSSH**, glutathione disulfide; **iNOS**, inducible nitric oxide synthase; **LH**, membrane lipid; **NADP+**, nicotinamide adenine dinucleotide phosphate (oxidised); **NADPH**, nicotinamide adenine dinucleotide phosphate (reduced); **ONNO–**, peroxynitrate; **T-O•**, tocopheroxyl radical; **T-OH**, α-tocopherol; **SOD**, superoxide dismutase.

14000, and the ratio between the largest $1/[id^h_{50}$ (mg/ml)] value and the smallest one is about 47×10^6. Superoxide radical has not so strong oxidation potential, and decomposes to molecular oxygen and hydrogen peroxide (the disproportionation reaction), which reveal the heavy peculiarity for the radical-eliminating abilities of biosubstances. Little correlation was observed in both radical-eliminating abilities (the correlation factor = 0.24 in a logarithmic presentation).

The ORAC values and superoxide radical-eliminating abilities were depicted two-dimensionally, taking the ORAC values (μmol Trolox equivalent/g) as the abscissa and $1/[id^s_{50}$ (mg/ml)] as the ordinate (Figure 3, the ORAC–$1/id^s_{50}$ diagram). The ORAC values are ranged in fairly broad range from 0.39 to 26000, and the ratio between the largest $1/[id^h_{50}$ (mg/ml)] value and the smallest one is about 67000. As shown in Figure 3, each group of biosubstances is placed into separated five major areas A to E on a two-dimensional map in similar manner as the $1/id^h_{50}$–$1/id^s_{50}$ diagram. The areas are more clearly separated each other than in Figure 2, and the correlation factor is 0.73 in a logarithmic presentation. Remarkable difference between Figures 2 and 3 is observed in cyt-*c*. In Figure 2, it locates around area C near L-tryptophane and L-methionine. While in Figure 3, it included in area B, the protein group, which indicates that metal-enzymes, such as SOD and cyt-*c*, simply act as ordinal proteins in the alkoxy radical elimination. The diagram can use for the identification of components in foods and beverages.

The ORAC values and hydroxyl radical-eliminating abilities were depicted two-dimensionally, taking the ORAC values (μmol Trolox equivalent/g) as the abscissa and $1/[id^h_{50}$ (mg/ml)] as the ordinate (Figure 4, the ORAC–$1/id^h_{50}$ diagram). Though the correlation factor in a logarithmic presentation is intermediate of Figures 2 and 3 (0.51), five areas are overlapped each other; *cf.* area A and B, area C, D, and E, and cannot draw area separately among the kind of biosubstances. The overlap of area A (mainly including amino acids) and area B (proteins) indicates that both hydroxyl and alkoxyl radical-eliminations are mainly based on the radical reaction with stable molecules, which is quite different from superoxide radical mainly on the disproportionation to oxygen molecule and hydrogen peroxide. The diagram cannot use for the identification of components in foods and beverages.

ROLE OF BIOSUBSTANCES FOR OXIDATIVE STRESS

A ROS, such as hydroxyl radical, is formed in the mitochondria from molecular oxygen via a superoxide anion radical and hydrogen peroxide [26]. Hydroxyl radical is also produced by the *N*-methyl-D-aspartate (NMDA) receptor activation in PC12 cells through the peroxynitrite formation [27]. Ikeno *et al* [28] showed that inducible nitric oxide synthase (iNOS) appeared in the brain cortex after hypoxic-ischemic (HI) insult, and peroxynitrite was produced. A brief scheme of the ROS formation and its elimination process is summarized in Figure 5. Endogenous antioxidants, such as α-tocopherol, L-ascorbic acid and GSH, eliminate ROS synergistically in biosystems. The ROS inhibitory diagrams, especially the ORAC–$1/id^s_{50}$ diagram, clearly indicate that each biosubstance has favorable free radicals to eliminate; *cf.* SOD, gallic acid, EGCG, EGC, L-DOPA, caffeic acid, dopamin, catechin and epicatechin to superoxide radical, L-ascorbic acid, Trolox, L-DOPA and caffeic acid to hydroxyl radical, L-ascorbic acid, gallic acid, EGCG, L-cysteine, caffeic acid and EGC to alkoxyl radical. Trolox, GSH, L-tryptophane, dopamine, epicatechin, L-DOPA and catechin are also fairly good eliminator for alkoxyl radical. These excellent biosubstances work as exogeneous antioxidants in the biosystems for superoxide, hydroxyl and lipid peroxide radicals in Figure 5, and also supplement the role of L-ascorbic acid and GSH.

Various methods, such as the DPPH [3-5], the TEAC [6, 7] and the ORAC [8, 10] assays, are used to determine the antioxidant abilities of foods. Especially, the ORAC assay was adopted for foods by many authors [16-18, 29-32], and the ORAC databases for selected foods (2007 and 2010) were listed up to the website of the United States Department of Agriculture (USDA). However, the USDA's Nutrient Data Laboratory (NDL) removed the database from the NDL website due to mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health. Though the antioxidant ability is thought to be a good marker for health, it needs some medical grounds [33]. As the ROS inhibitory diagrams are based on the eliminating abilities of free radicals produced in the oxygen stress, they should respond to the need, and be usable presentations for healthy life.

In the present review, we discussed the antioxidant abilities of water-soluble biosubstances only. Lipophilic antioxidants, such as α-tocopherol and carotenoids, are also important to protect from the injury caused by oxidative stress. They mainly play an important role in the protection for the lipid peroxidation in cell membrane, in similar manner as α-tocopherol in Figure 5. The antioxidant abilities of lipophilic biosubstances should be measured for the lipophilic free radical, such as lipophilic peroxy and alkoxyl radicals. Tsuchiya *et al* [34] examined the radical-eliminating effects of α-tocopherol and Trolox, a hydrophilic homolog of α-tocopherol, by the AAPH-phycoerythrin assay in aqueous system and by the 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN)-*cis*-parinaric acid assay in hexane, respectively. AMVN is a lipophilic azo-initiator and *cis*-parinaric acid, a lipophilic fluorescent. They observed that the hydrophilic system could be inapplicable to quantitate the efficiency of free radical-elimination by lipophilic antioxidants, and *vice versa*. Using the competitive reaction of lipophilic antioxidants with *cis*-parinaric acid for peroxy radical, the second-order rate constants of α-tocopherol, β-carotene, and ubiquinol 10 with peroxy radical in dioleoylphosphatidylcholine-liposome were estimated to be 8.76×10^5, 1.97×10^5 and 1.84×10^5/M/s, respectively [35]. The value of α-tocopherol, being the highest antioxidant ability among three antioxidants tested, was smaller than that obtained in solution or

suspension. Furthermore, these results were obtained using the fluorescent decay of the *cis*-parinaric acid in a special lipophilic environment, and we could not compare with biosubstances in Table 1 directly. Only the data of Trolox was referred to α-tocopherol. Further information on the free radical-eliminating abilities of lipophilic biosubstances should be needed, especially using the spin-trapping ESR method with some lipophilic azo-initiator, such as AMVN in an appropriate lipophilic environment.

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