Journal of Berry Research 1 (2011) 169–176
DOI:10.3233/BR-2011-018
IOS Press

Invited Review

Antioxidant capacity: Which capacity and how to assess it?

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Received 29 December 2010; accepted 2 March 2011

Abstract. The role of antioxidants in the maintenance of health and prevention of disorders and diseases has received much attention. Above all, the action and effects of natural antioxidants contained in foods, fruits, spices, beverages and dietary supplements have been the subjects of extensive studies. The capacity of antioxidants has been assessed by various methods, but they often give inconsistent and conflicting results. Animal and human studies do not always support the beneficial effects of antioxidants and the methods for assessment of antioxidant effect are critically needed. In the present article, the methods of assessment of antioxidant capacity are critically reviewed.

Keywords: Antioxidant capacity assay, free radical, lipid peroxidation, natural antioxidant

1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) are essential for humans to maintain homeostasis and health, but uncontrolled and excess ROS/RNS have been implicated in the pathogenesis of various diseases. The aerobic organisms are protected from oxidative stress induced by ROS/RNS by a fine defense network, in which various antioxidants with multiple functions play their respective roles such as reduction of hydrogen peroxide and hydroperoxides, sequestration of metal ions, scavenging of reactive species, and repairing damage [1]. Some antioxidants are small molecular weight compounds, while others are high molecular weight proteins and enzymes. Free radical scavenging is one of the important functions of antioxidants, since free radicals induce oxidation of lipids, proteins, and DNA, which results in disturbance and functional loss of biological membranes and enzymes and production of toxic compounds. Thus, the role of free radical scavenging antioxidants has attracted much attention of not only scientists but also general public. Above all, the natural antioxidants contained in fruits, vegetables, spices, beverages, and dietary supplements have received much attention [2]. For example, many studies have shown that berries are rich in many kinds of antioxidants [3, 4].

The assessment of the capacity of pure antioxidant compounds and products containing complex mixture of antioxidants have been the subjects of extensive studies and arguments [1, 5, 6, and references cited therein]. A number of methods and variations have been developed and applied for the assessment of antioxidant capacity but very often

different assays give inconsistent results. There is lack of correlation between activities determined by the same antioxidant by different assays and between activities determined by the same assay in different laboratories. The antioxidant capacity means different things at different occasions for different people and it has been expressed by the terms such as ability, activity, capacity, efficacy, parameter, potential, power, and reactivity. It is important to specify what capacity is measured by which method. In this brief article, the methods for the assessment of antioxidant capacity of free radical scavenging will be critically reviewed.

2. Assessment of free radical scavenging capacity *in vitro*

The capacity of free radical scavenging *in vitro* is assessed by kinetic and stoichiometric factors, that is, how much does the antioxidant reduce the rate of oxidation and how long does it suppress the oxidation. These are determined by the rate of scavenging free radicals by antioxidant and the number of free radicals it can scavenge, respectively. Many methods have been developed and applied to measure these parameters [1, 7, 8]. Each method has its merits and demerits. Recently, the capacity of free radical scavenging has been often and widely assessed by either the reaction with stable free radical or by competition method using conventional UV/visible absorption spectrophotometer. The details of these methods have been described in many review articles [1, 9, and references cited therein].

2.1. Assessment by the reaction with reference free radical

This assay using a reference free radical is simple, rapid, and needs only a UV/visible absorption spectrophotometer. Above all, galvinoxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) are commercially available, stable, and easy to handle. Galvinoxyl is an oxygen-centered radical, while DPPH nitrogen-centered radical. Both galvinoxyl and DPPH have strong absorption in visible region, which is bleached by the reaction with antioxidants. Therefore, the reaction with antioxidant can be followed from the decrease in their characteristic absorption, 428 and 520 nm in ethanol respectively. The 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) cation radical is used similarly [10]. ABTS cation radical has to be generated from ABTS before measurement.

An example for the measurement of stoichiometric number and reactivity by using galvinoxyl is described here [11]. Antioxidant compound or material containing mixture of antioxidants is added to an ethanol solution of galvinoxyl and the decrease in the absorption at 428 nm is followed. When all the antioxidants react with galvinoxyl, the absorption becomes constant. The fall in absorbance is proportional to the concentrations of antioxidant or amount of material and the plot of absorption fall as a function of antioxidant concentration gives a straight line. The stoichiometric number n can be calculated from the following Eq. 1:

$$A = n\varepsilon lC \tag{1}$$

where A, n, ε, l, and C denote absorption fall, stoichiometric number, molar extinction coefficient, cell length, and concentration of antioxidant or material, respectively. The molar extinction coefficient of galvinoxyl at 428 nm in ethanol is $1.50 \times 10^5\ M^{-1}cm^{-1}$.

The reactivity of antioxidant can be estimated from the initial rate of decrease in the absorption at 428 nm. The relative reactivities of a series of antioxidants may be assessed from the relative rate of decrease. Furthermore, the rate constants can be estimated from detailed kinetic analysis [11].

In some studies, the amount of decrease or percentage of remaining reference radical at specific time or the concentration of antioxidant or the time needed to reach a 50% decrease of the initial reference radical concentration is measured for a basis of assessment. However, it should be pointed out that these methods do not distinguish between reactivity and stoichiometric number and furthermore these results depend on the reaction conditions employed and cannot be generalized.

2.2. Assessment from competition method

Competition method has been used widely for the assessment of antioxidant capacity for scavenging free radicals. The capacity is assessed from the extent of suppression by antioxidant of probe consumption induced by free radicals. Various probes have been applied (Table 1) and their consumption is measured by UV/visible absorption, fluorescence,

Table 1
Reference compound used as a probe for assessment of radical scavenging capacity of antioxidant

Hydrophilic compound	Lipophilic compound
ABTS	β-Carotene
Alizarin red	BODIPY
DCFH	cis-Parinaric acid
Fluorescin	Crocin
Phycoerythrin	DPPD
Propylgallate	
Pyranine	
Pyrogallol red	

ABTS: 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical, soluble also in organic solvent; BODIPY: 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DCFH: 2′,7′-dichlorodihydrofluorescin; DPPD: *N*,*N*′-diphenyl-*p*-phenylenediamine.

chemiluminescence, or electron paramagnetic/spin resonance (EPR/ESR) spectroscopy. The free radicals are often generated by the decomposition of azo initiators, which undergo thermal decomposition at constant rate to yield two radicals (Reaction 2) which react with oxygen rapidly to give peroxyl radicals (Reaction 3):

$$\text{R-N} = \text{N-R} \longrightarrow 2\text{R}\cdot + \text{N}_2 \tag{2}$$

$$\text{R}\cdot + \text{O}_2 \longrightarrow 2\text{ROO}\cdot \tag{3}$$

Peroxyl radicals are most appropriate radicals to evaluate the activity of antioxidants, since they play an important role as a chain-carrying radical in lipid peroxidation and their reactivity is such that antioxidant can compete well with the lipids. In contrast, hydroxyl radical and even alkoxyl radical are too reactive for antioxidants to scavenge efficiently *in vivo*, while superoxide is too unreactive to induce lipid peroxidation per se.

The reactivity of the antioxidant toward peroxyl radicals can be estimated from the competition with a probe such as pyrogallol red (PGR) [12, 13]. The rate of PGR consumption induced by radicals generated from azo initiator is measured from the decrease in absorption in the absence and presence of antioxidant. The ratio of the rates of PGR consumption in the absence and presence of antioxidant, R_0 and R_{IH} respectively, is given by the Eq. 4:

$$R_0/R_{\text{IH}} = 1 + (k_{\text{IH}}[\text{IH}]/k_{\text{PGR}}[\text{PGR}]) \tag{4}$$

where k_{IH} and k_{PGR} are the rate constant for the reactions of radicals with antioxidant and PGR respectively [12, 14]. A plot of R_0/R_{IH} as a function of [IH]/[PGR] should give a straight line, whose slope is $k_{\text{IH}}/k_{\text{PGR}}$. The relative reactivities of a series of antioxidants can be obtained from $k_{\text{IH}}/k_{\text{PGR}}$ measured for different antioxidants.

Various competition methods have been developed and applied and of those oxygen radical absorbance capacity (ORAC) has been most widely used [1, 9, 15]. In the typical ORAC assay, the loss of probe such as fluorescein and phycoerythrin induced by free radicals generated from azo initiator such as 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) is followed over time in the absence and presence of antioxidant. The area under the decay curve (AUC) is measured and the net increase in the AUC produced by antioxidants is used for assessment of antioxidant capacity. ORAC values, expressed usually as Trolox equivalent, have been measured for many antioxidant compounds and extracts of fruits, spices, and vegetables.

ORAC values are determined by both reactivity and stoichiometry of antioxidant. The shape and AUC depends on antioxidant and also on the probe. When a probe with low reactivity toward free radical is used, a clear lag phase is observed and the AUC is determined primarily by the content of antioxidants, and the capacity of antioxidants with low reactivity is overestimated. On the other hand, a probe with high reactivity yields obscure lag phase and reflects reactivity rather than the content of antioxidants. It should be pointed out that ORAC value does not distinguish stoichiometry and reactivity.

2.3. Assessment by reduction of metal ion

Antioxidant can stabilize free radicals by donating electron and the antioxidant capacity by this mechanism is measured by the capacity of reduction of metal ions such as ferric ion (Fe^{3+}) or cupric ion (Cu^{2+}): FRAP (Ferric Reducing Antioxidant Power) [16, 17] and CUPRAC (Cupric Reducing Antioxidant Power) [17], respectively. Recently, a new method for measuring reducing capacity by chemiluminescence was reported [18]. A method of determination by on-line HPLC-CUPRAC assay with post-column detection [19] or by copper (II)-neocuproine complex immobilized onto a cation-exchanger film [20] has been proposed. The capacity measured by FRAP or CUPRAC method does not always correlate well with that for radical scavenging.

3. Antioxidant capacity against lipid peroxidation

The capacity of free radical scavenging of antioxidant measured by the method described above does not always correlate with the capacity of antioxidation such as lipid peroxidation inhibition. The capacity for inhibition of lipid peroxidation has been measured in many different systems such as in homogeneous solution, aqueous dispersions of micelles, liposomal membranes, isolated low density lipoprotein (LDL), red blood cells and plasma. The antioxidation capacity can be assessed from the extent of suppression of lipid peroxidation by antioxidant compared with that in its absence. It is determined not only by the capacity for scavenging free radicals but also by the localization of antioxidant, fate of antioxidant-derived radical, interaction with other antioxidants, and mobility of antioxidant at the microenvironment. These factors should be considered to understand the action and capacity of antioxidants [1].

There are both hydrophilic and lipophilic antioxidants and they act in aqueous and lipophilic compartments respectively, and under certain circumstances they interact to inhibit lipid peroxidation synergistically [21]. Lipophilic as well as hydrophilic azo initiators are used to elucidate the action of antioxidants in heterogeneous systems [22].

The absolute or relative rate constants for scavenging lipid peroxyl radical by antioxidants have been measured by the kinetic analysis of lipid peroxidaiton [23]. This is a direct measure of the capacity of antioxidant for radical scavenging and enables us to understand the dynamics of antioxidant action. The rate and extent of lipid peroxidation can be measured from oxygen uptake, substrate consumption, or product formation. The conjugated diene formed by the oxidation of polyunsaturated lipids such as linoleic acids have strong absorption at 234 nm, which is a convenient marker of lipid peroxidation to measure.

As described above, the capacity of inhibition of lipid peroxidation may be assessed from the extent of inhibition of lipid peroxidation in the oxidation of lipids in aqueous dispersions such as micelles, liposomal membranes, isolated LDL, and plasma. Above all, the plasma oxidation is recommended because it is biologically relevant and the capacity of both water-soluble and lipid-soluble antioxidants can be assessed including their synergistic interaction. In some cases, plasma is diluted to 1% to follow the increase in absorption of 234 nm due to conjugated diene, but it one should be bear in mind that the lipid concentration under such condition is very low, about 20 μM, which makes it difficult to measure the capacity of lipophilic antioxidants under physiological conditions.

Cultured cells have been used also as substrate for assessment of antioxidant capacity. In general, antioxidants are added to the cell culture medium, but the concentration of antioxidants should be carefully chosen considering the amount of lipids in the medium and also the rate of intake into the cells. For example, tocotrienols appear to be more potent than the corresponding tocopherols, primarily because of faster intake of tocotrienols than tocopherols [1].

4. Assessment of antioxidant capacity *in vivo*

The capacity of antioxidants *in vivo* against oxidative stress is determined by several factors in addition to those described above which are important *in vitro*. Above all, the bioavailability, that is, absorption, transportation, distribution, retainment, metabolism, and excretion of antioxidants, is one of the major factors in determining the capacity *in vivo*. The effects of dosage and duration of antioxidant administration on time course change in the concentrations of metabolites as well as the intact antioxidant in biological fluids and tissues have been measured. Some antioxidants undergo biotansformation through enzymatic conjugation with sulfate, methyl, or glucuronide groups [24].

Table 2
Biomarkers of oxidative stress

Lipid	Protein	DNA
Ethane and pentane	Protein carbonyl	Comet assay
in exhaled gas	Hydroperoxide	Thymine glycol
TBARS	Nitro-, chloro-, bromo-amino acid	5-Hydroxyuracil
Conjugated diene	Disulfide -SS-	2-, 8-Hydroxyadenine
Hydroperoxide	-SOH, -SOOH, -SOOOH	8-Hydroxyguanine
Aldehyde	Aldehyde-modified protein	8-Nitro-, chloro-, bromo-guanine.
Ketone	Hydroperoxide-modified protein	
Isoprostane	Crosslinked protein	
Neuroprostane	Dityrosine	
Isofuran	Albumin dimer	
Neurofuran	Advanced oxidation products	
HODE	Creatol	
HETE	Myeloperoxidase	
Lyso PC	Lipofuscin	
Oxidized LDL‘	Cleavage products	
Oxysterols	Antibody	

TBARS: thiobarbituric acid reactive substances; HODE: hydroxyoctadecadienoic aicd; HETE: hedroxyeicosatetraenoic acid; Lyso PC: lysophosphatidylcholine; LDL: low density lipoprotein.

It has been pointed out that the prooxidants contained in diet such as metal ions, lipid oxidation products and nitrite may be involved in the oxidative stress in gastrointestinal tract to induce stomach ulcer and develop stomach, colon, and rectal cancer [25, 26]. The antioxidants contained in diet may exert their antioxidant function in gastrointestinal tract before they are absorbed or excreted.

The capacity of antioxidants has been assessed also from the change in “total antioxidant capacity” (TAC) value after intake of antioxidant or antioxidant-containing products [27, 28]. The TAC database for vegetables, fruits, beverages, spices and other food stuff has been measured by ORAC or other methods [29–31]. It should be noted, however, that TAC values measured by competition method are semi-quantitative at best and do not always show the capacity of antioxidation nor inhibition of oxidative damage as pointed out above.

The capacity of antioxidants *in vivo* may be assessed by the effects of antioxidants on the level of oxidative stress in biological fluids and tissues such as plasma, erythrocytes, urine, and cerebrospinal fluids from humans and experimental animals. Saliva and tear may also be used as non-invasive samples. Reliable biomarkers are required for this purpose and many biomarkers have been applied to measure the level of oxidative stress *in vivo* [1, 7, 32]. Various oxidation products of lipids [33–35], proteins [36, 37], and DNA [38] have been used as oxidative stress biomarkers as summarized in Table 2. Isoprostanes formed by the free radical-mediated oxidation of arachidonates are accepted to be most reliable biomarker of oxidative stress *in vivo*. Furthermore, the levels, ratio of oxidized/reduced forms, and oxidized products of antioxidants have been used as biomarker of oxidative stress *in vivo* (Table 3). It has been observed often that the ratio of oxidized glutathione [GSSG] to reduced glutathione [GSH] increases with increasing oxidative stress. Recently, the imaging technique is receiving much attention for detecting oxidative damage in vivo, for example a technique using radio isotope and antibody against oxidation products by single-photon emission computed tomography (SPECT) positron emission tomography (PET) [39]. The imaging technique may be used as a novel tool for assessment of antioxidant capacity *in vivo*.

The effects of various antioxidants on the levels of biomarkers described above have been evaluated in many human studies and experimental animal studies under normal conditions and oxidative stress. The effects of antioxidants have been assessed also in some human intervention studies [40].

Table 3
Antioxidants as oxidative stress biomarker

1. Antioxidant compound level
Antioxidant vitamins, Total antioxidant capacity (TAC)
2. Antioxidant enzyme level
SOD, catalase, GPx, Trx, Prx, Glutathione reductase, GST
3. Oxidation product of antioxidant
Tocopheryl quinone, 5-nitro-γ-tocopherol, allantoin, biopyrrin
4. Ratio of oxidized to reduced form
GSSG/GSH, TQ/TQH_2, UQ/UQH_2

SOD: superoxide dismutase; GPx: glutathione peroxidase; Trx: thioredoxin; Prx: peroxyredoxin; GST: glutathione-S-transferase; GSSG: oxidized glutathione; GSH: reduced glutathione; TQ: tocopheryl quinone; TQH_2: tocopheryl hydroquinone; UQ: ubiquinone; UQH_2: ubiquinol.

5. Concluding remarks

The term "antioxidant capacity" means different things to different people and occasions. The "antioxidant capacity" may mean capacity of scavenging free radicals, capacity of inhibition of oxidation, or capacity to prevent a disease. When assessing the capacity, it should be clearly defined which capacity is being measured. It is also important to distinguish between stoichiometry and reactivity for scavenging radicals, that is, between reactivity and amount of antioxidants. The capacity may be assessed for pure antioxidant compound, mixtures of antioxidant compounds, extracts, or commercial products. The capacity may be evaluated in absolute number or relative value.

The capacity of scavenging free radicals can be assessed most easily from the reaction of the test material with a stable free radical such as galvinoxyl and DPPH. These radicals are soluble in organic solvents such as hydrocarbons and ethanol and therefore this method is suitable for lipophilic antioxidants, but not for water-soluble antioxidants. A stoichiometric and kinetic numbers, that is, content and reactivity respectively, can be measured separately as described above.

The competition method may be applied for almost all kinds of test compounds and materials. The ORAC method is most frequently used, but this value does not distinguish reactivity and content. The stoichiometric number or the content of antioxidants in the test materials may be assessed from the lag phase produced for the decay of a probe with low reactivity such as pyranine, while the reactivity from the effect on the decay rate of a probe with high reactivity such as pyrogallol red.

It is important to note that the capacity of free radical scavenging does not always correlate well with the capacity to inhibit oxidation. The capacity of antioxidants for inhibition of oxidation including interactions between antioxidants may be assessed from the effects on the oxidation of plasma or lipid peroxidation in aqueous dispersions.

The antioxidant capacity *in vivo* including bioavailability, which can not be assessed by a simple extrapolation of the *in vitro* results, may be assessed from the effects of antioxidant supplementation on appropriate biomarkers in biological fluids and tissues.

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