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Phytochemical Antioxidants: Past, Present and Future

Yasuko Sakihama and Hideo Yamasaki

Abstract

Most diseases that are difficult to prevent and cure are “syndromes” that are governed by multiple components with complicated interactions. Whatever the cause of such diseases, overproduction of harmful reactive oxygen species (ROS) can often be observed in progression of the disease. Under such conditions, the cells may be challenged by “oxidative stress” due to excessively generated oxidants. Antioxidants can be defined as chemical compounds that scavenge ROS or free radicals over-produced in the cells under oxidative stress conditions. The plant pigments flavonoids and betalains, rich in fruits and vegetables, are reactive not only with ROS but also with reactive nitrogen species (RNS) and possibly with reactive sulfur species (RSS). Here, we provide an overview of updates on the antioxidative functions of the plant pigments along with some prospects for future research on phytochemical antioxidants.

Keywords: flavonoid, betalain, reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS)

1. Introduction

Fruits and vegetables are appreciated as “healthy foods” compared with beef or pork meat. Many epidemiological studies as well as clinical investigations have suggested that a vegetable-based diet is beneficial in preventing chronic diseases including cancer, coronary heart disease, stroke and hypertension [1, 2]. Meanwhile, traditional herbal medicines have used specific plant species that contain phytochemicals exhibiting pharmacological activities [3]. Novel compounds have been isolated from such plants and they have been chemically synthesized for pharmaceutical production [4]. Nobody doubts that edible plants are beneficial in human health.

In “western” medicine, a disease can be defined as dysfunction of a physiological mechanism. Based on this concept, a drug in general is presumed to act on a specific component of a physiological mechanism. In many cases, these are inhibitors of enzymes or transporters, showing the “one-to-one” relationship between drug and target molecule. While recent drug designs have drastically changed due to a rapid development of computer technology [5] as well as gene therapy [6], the hunt for novel bioactive compounds contained in plants is still active for new drug discovery.

The “one-to-one” philosophy in medicine and pharmacology works well, if the cause of a disease is ascribed to a single component such as a protein or an enzyme. However, most diseases that are difficult to prevent and cure are “syndromes” that

are governed by multiple components with complicated interactions. Whatever the cause of such diseases, overproduction of harmful reactive oxygen species (ROS) can often be observed in progression of the disease. Under such conditions, the cells may be challenged by “oxidative stress” due to excessively generated oxidants. The oxidative stress potentially impairs cellular functions eventually leading to death [7, 8]. This is a common biological feature that can be seen in all living organisms including bacteria, fungi, plants and animals. Living organisms have evolved to cope with the oxidative stress induced by biotic (pathogen attack or biological interactions) and abiotic (or environmental) stresses. Thus, under stress conditions, living organisms need to control cellular ROS levels for their survival. In this context, antioxidant systems are essential in any living organisms. This is a biological rationale for the importance of antioxidants in prevention and cure of diseases in humans.

Plant antioxidant research shows a history of twists and turns. Some early studies had suggested concepts opposite to the present recognition. Plant antioxidants had sometimes even been considered to be toxic or carcinogenic to animals. Contradictory reports in the old literatures may lead non-specialists to a state of confusion. Thus, to follow the current state of research advances in phytochemical antioxidants, understanding its historical background is of help for non-specialists and new researchers. Highlighting the research progress of plant pigments flavonoids and betalains, here, we provide an overview of phytochemical antioxidants with some prospects for future research.

2. Historical perspective of plant antioxidants

2.1 The vitamin that prevents the disease of age of discovery

A retrospective of the history of research on plant antioxidants needs to go back to the age of discovery. When voyagers such as Magellan, Columbus, Vasco da Gam and Cook were sailing over the world's oceans, more than three times as many sailors died due to the mysterious disease “scurvy” as soldiers died in the American Civil War [9]. For hundreds of years, the cause of the disease had not been clarified and there had been no cure for this disease of sailors [10]. In 1747, James Lind working as a naval surgeon at sea on the HMS *Salisbury* conducted “clinical trials” of potential cures for the disorder. In *Treatise of the Scurvy* published in 1753, he reported that there was no effect with the potential remedies vinegar, mustard, garlic purges, elixir of vitriol, but citrus fruits (orange and lemon) showed a significant cure effect [11]. It is now known that scurvy is caused by a vitamin C (L-ascorbate) deficiency due to a lack of fresh fruits and vegetables.

Historically, antioxidant and vitamin studies have developed independently in chemistry and health science, respectively. In chemistry, antioxidants were defined as chemical compounds that can suppress oxidation reactions. In early studies, oxidation was observed as absorption of molecular oxygen in a reaction such as polymerization reaction of natural rubber. On the other hand, a vitamin (the name “vitamine, vital + amine” was the original proposal and it was later renamed to “vitamin”) was defined as an organic nutrient that is essential for human health care. The major recognized vitamins are vitamin A, B1, B2, B3, B5, B6, B7, B9, B12, C, D, E, and K. The biochemical requirements of these vitamins were revealed after their chemical identifications. Among these vitamins, vitamin A, C and E have been highlighted again in the late 20th century due to their antioxidant activities that potentially reduce the oxygen toxicity.

2.2 The oxygen toxicity and ROS

Although molecular oxygen (O_2) is required for respiration in animals, a high concentration or high partial pressure of oxygen often damages the central nervous and pulmonary systems, which leads to disease or death. Oxygen toxicity in the central nervous system and that in pulmonary system had been referred to as the Paul Bert effect and the Lorrain Smith effect, respectively [12]. Although the toxicity of oxygen itself was implied by Joseph Priestley in 1774 (dephlogisticated air at that time) [13], the modern style of experimental science has been opened up by Bert (1833–1886), the Father of Aviation of Medicine [14, 15]. In his *La Pression Barometrique* (1878), Bert described that a high partial pressure of breathing oxygen (hyperoxia) can lead to death of animals, the first experimental demonstration for the toxicity of pure oxygen [14]. Since his pioneering discovery had not been appreciated for a long time, unfortunately, eye damage (retinopathy of prematurity) to premature infants frequently occurred due to the use of pure oxygen [16].

The biochemical basis of the oxygen toxicity is ascribed to overproduction of reactive oxygen species (ROS) in cells. The ROS firstly produced in cells is mostly superoxide radical (O_2^-), which is the reaction product of the one electron reduction of molecular oxygen (O_2) [17]. Whereas chemists have known the inorganic reaction that produces O_2^- from O_2 , the biological relevance of the reaction had not been considered in biochemistry. At that time most biochemists were fascinated by the oxidative phosphorylation that is the final step of ATP synthesis in aerobic respiration. For mitochondrial ATP synthesis, the presence of O_2 is prerequisite to drive the respiratory electron transport. Therefore, the toxicity of O_2 had been overlooked. The discovery of the enzyme superoxide dismutase (SOD) that destroys O_2^- is a landmark in the research history of oxygen toxicity [18]. The discovery of the antioxidant enzyme SOD has drastically changed our recognition: O_2 might be toxic for living organisms.

To prevent oxygen toxicity, it has been revealed that antioxidant enzyme systems are essential for the survival of all living organisms, including humans. The ROS O_2^- and H_2O_2 can be removed by the enzymatic reactions of SOD and peroxidases, but other unstable ROS molecules, hydroxyl radicals ($\bullet OH$) for example, cannot be destroyed by those enzymatic reactions. These molecules are scavenged by antioxidants. Vitamin A or carotenoid can scavenge singlet oxygen (1O_2) that could be produced in the eyes or skin under ultraviolet (UV) light [19]. Vitamin E, or α -tocopherol, can react with the ROS radicals produced in lipophilic environments such as in lipid membranes. Vitamin C (ascorbate) serves as a universal reducing power to the antioxidant enzyme systems while the ascorbate molecule itself scavenges various types of ROS (except H_2O_2) by its spontaneous reactions [20]. It is important to note that humans need to acquire these essential antioxidant vitamins (A, C, E) from dietary foods, largely from fruits and vegetables.

2.3 Vitamin P concept and plant pigments

Historically, there was a short-lived Vitamin P concept. Albert Szent-Györgyi, a Nobel prize winner who isolated ascorbate, demonstrated that flavonoid glycosides rich in citrus fruits can behave similar to ascorbate in maintaining capillary permeability [21]. Based on his observations, Szent-Györgyi proposed that the plant flavonoids, as a group of plant pigments, are also essential nutrients and referred to them as vitamin P (permeability) [22]. However, this vitamin P concept did not gain broad acceptance due to the chemical diversity of flavonoids. More recently, his idea that flavonoids can complement the function of ascorbate has been renewed with the development of the antioxidant hypothesis.

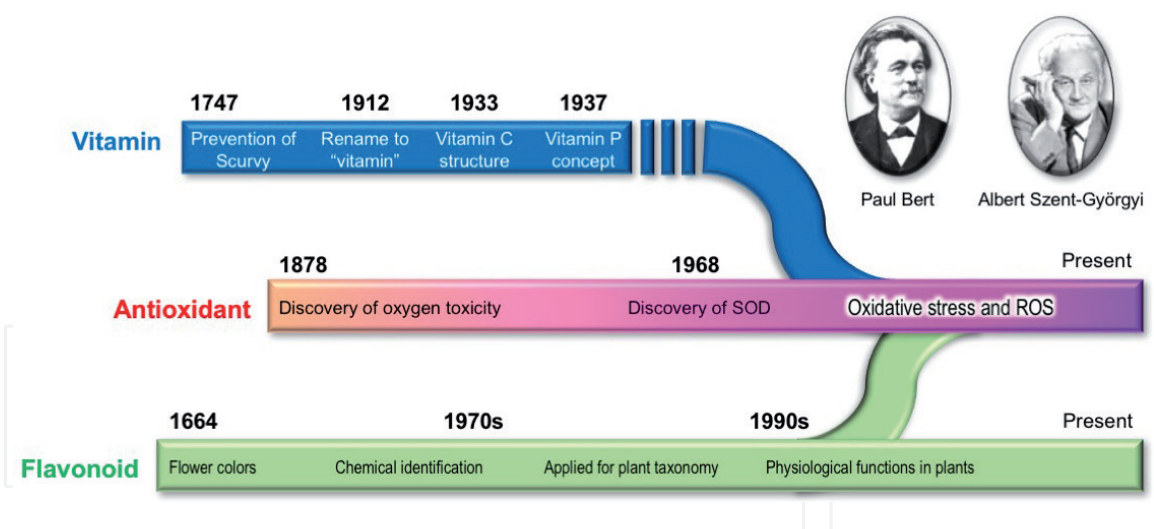


Figure 1.

A timeline of antioxidant research of phytochemicals. Flavonoids are major plant pigments that are widely appreciated as natural antioxidants. Historically, antioxidant studies, vitamin studies and flavonoid studies have independently progressed in health science, biochemistry and botany, respectively. These different lines of studies have been integrated into the present plant antioxidant studies.

Plant fruits and flowers display beautiful colorations ranging from blue to red. These plant colorations are produced with three major pigments i.e., chlorophylls, carotenoids and flavonoids. In plants, biological functions of chlorophylls and carotenoids have been known as the photosynthetic pigments that absorb light energy to drive photosynthesis. In contrast, only the visual attraction for flower pollinators such as bees or butterflies had been proposed as a biological function of colored flavonoids for a long time [23]. The chemical diversity of flavonoids found across plant species had made it difficult to consider common physiological or biochemical functions. Conversely, the huge chemical diversity of flavonoids was useful for plant taxonomy until amino acid or DNA sequence information available.

In 1990s, red anthocyanin, a flavonoid subgroup, was highlighted to account for the paradoxical epidemiological observation termed the “French paradox”. French people have a relatively low incidence of coronary heart disease even though they consume a diet relatively rich in saturated fats [24]. Researchers were interested in anthocyanins and polyphenols contained in red wine that may suppress heart disease through their antioxidant activities [24]. Similarly, the longevity of Japanese people was explained by their daily consumption of green tea rich in catechin, another subgroup of flavonoid [25, 26]. These epidemiological reports have stimulated biochemical screening of natural antioxidants contained in plants.

To date, health science, biochemistry, botany and other different field of studies have been integrated into antioxidant research. A timeline for antioxidant research of phytochemicals is summarized in **Figure 1**.

3. Plant pigment flavonoid

3.1 Flavonoids in plants

Flavonoids are representative secondary metabolites of land plants. The pigments commonly accumulate in epidermal cells of the organs such as in flowers, leaves, stems, roots, seeds and fruits [27, 28]. Flavonoids are found as glycosidic forms (glycosides) and non-glycosidic forms (aglycones). Subcellular localization of the glycosides is largely confined to hydrophilic regions such as vacuoles and apoplasts. The aglycones are localized in lipophilic regions e.g., oil glands and waxy layers.

The term “flavonoid” originated from its yellow color (the *Latin* word *flavus* means yellow). Bioactive flavonoids such as flavones and flavonols are sometimes referred as to “bioflavonoids”. **Figure 2** shows the basic structures of flavonoids. The general structure of flavonoids includes a C6-C3-C6 carbon skeleton with two phenyl rings (A- and B-rings) and a heterocyclic ring (C-ring). Based on the structure of the aglycones, flavonoids can be classified into subgroups: chalcone, flavanone, flavone, isoflavone, flavonol, and anthocyanidin (**Figure 3**). According to the IUPAC nomenclature, flavonoids are recommended to be subcategorized into flavonoids (bioflavonoids), isoflavonoids and neoflavonoids [29]. Since this classification has yet not been widely adopted, in this chapter, traditional phytochemical names and classifications are used to avoid confusions. Most of these subgroups show yellowish coloration while anthocyanins exhibit multiple colorations depending on the aglycone structure, the presence of metal, pH and conjugation with other molecules (**Figure 3**).

Common glycosylation positions are the 7-hydroxyl in flavones, isoflavones and dihydroflavones; the 3- and 7- hydroxyl in flavonols and dihydroflavones; the 3- and 5-hydroxyl in anthocyanidins [30]. The typical sugars involved in glycoside formation are glucose, galactose, rhamnose, xylose and arabinose. In addition to the glycosylation, methylation, isoprenylation and dimerization occur at those positions [30].

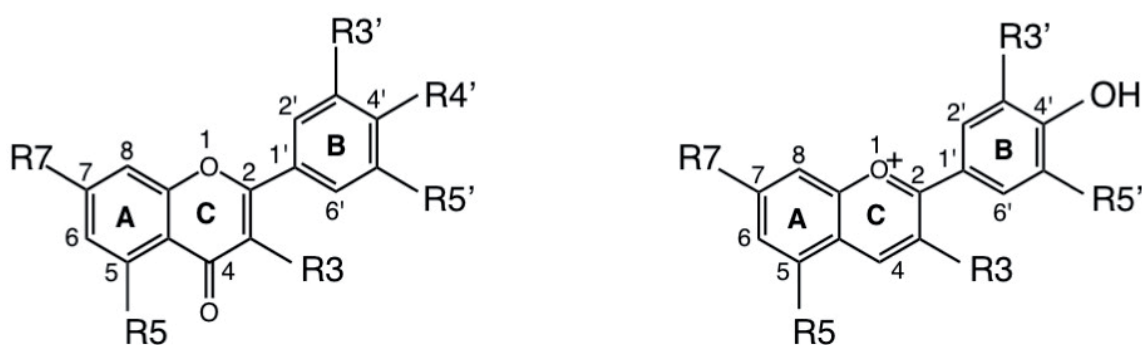


Figure 2.
 Chemical structures of flavonoids. Chemical structures of flavonoids include a C6-C3-C6 carbon skeleton with two phenyl rings (A- and B-rings) and a heterocyclic ring (C-ring). Left, the basic structures of a flavone, isoflavone and flavonol. Right, the basic structures of anthocyanin. The –R on the rings can be replaced by other molecules including sugars to make a huge variety of chemical structures of flavonoids.

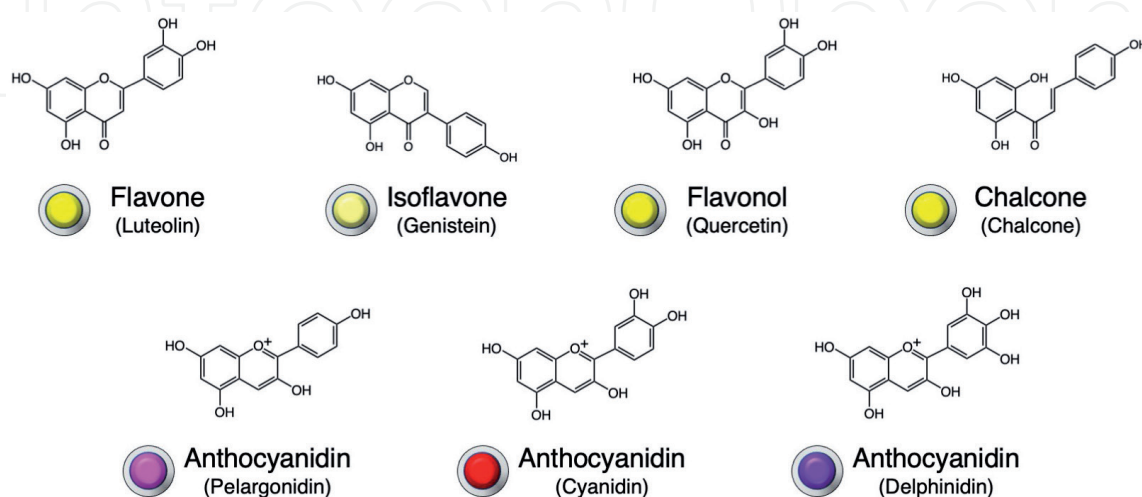


Figure 3.
 Representative flavonoid subgroups. Based on the aglycone structures, flavonoids can be classified into flavone, isoflavone, flavonol, chalcone and anthocyanidin. Representative flavonoids with parenthesis along with apparent visual colorations are shown.

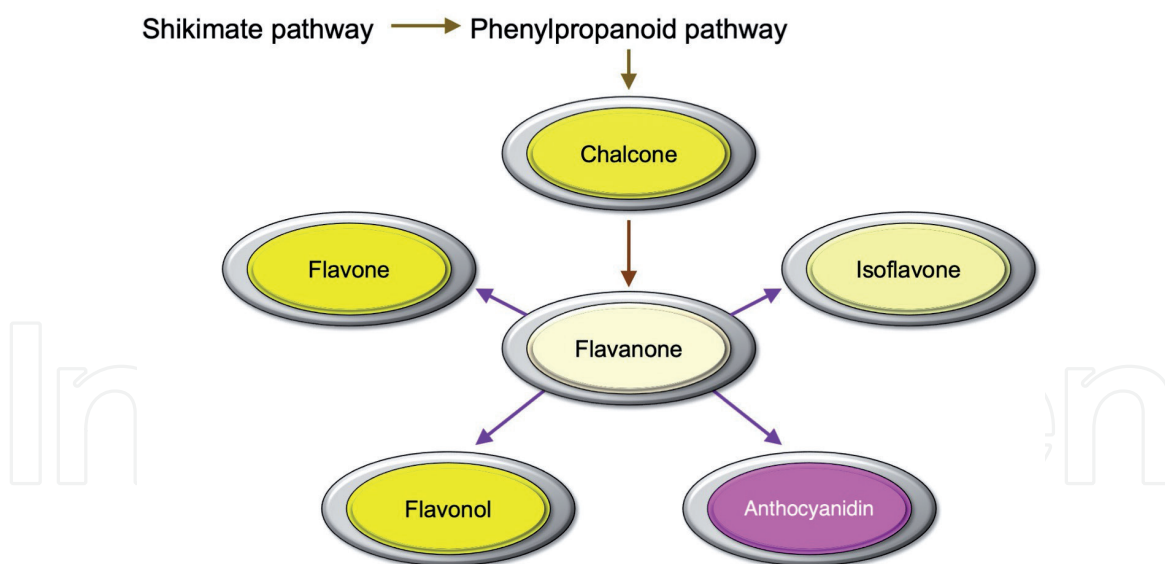


Figure 4.

An outline of flavonoid biosynthesis pathways in plants. The synthesis of the flower pigment anthocyanins requires multiple steps including the shikimate pathway, phenylpropanoid pathway, via chalone and flavanone. The number of required enzymatic steps reflects the evolutionary order of the pigments.

These modifications produce a huge structural diversity of flavonoids. More than 9,000 chemical structures of flavonoids have been reported to date [31].

Enzymes and genes involved in flavonoid biosynthesis have been identified [27, 32–35]. **Figure 4** shows an outline of biosynthetic pathways of the major sub-classes of flavonoids. Flavonoids are synthesized from phenylalanine, an aromatic amino acid produced in the shikimate pathway. Phenylalanine is sequentially metabolized by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase, and 4-coumarate CoA ligase to 4-coumaroyl CoA. This 4-coumaroyl CoA and 3 molecules of malonyl CoA are condensed by chalcone synthase to form the flavonoid chalcone (yellow). Chalcone is isomerized to the flavanone naringenin (colorless) by chalcone isomerase. Naringenin is further converted to flavones (pale yellow) and isoflavone (pale yellow) catalyzed by flavone synthase and isoflavone synthase, respectively. Naringenin is hydroxylated to dihydroflavonol by flavanone 3-hydroxylase and further metabolized to flavonol (yellow) by flavonoid synthetase. Dihydroflavonol is converted to anthocyanidin (red, red-violet or blue-violet), an aglycone of anthocyanin, by dihydroflavonol 4-reductase and anthocyanidin synthase. Anthocyanidin is glycosylated by UDP-glucose-dependent glycosyltransferase. Manipulation of those genes has been challenged to change of flower or fruits coloration [28].

3.2 Antioxidant activity of flavonoids

Antioxidant activity or antioxidant capacity of flavonoids has been experimentally evaluated with either assays based on hydrogen atom transfer (HAT) reaction or assays based on electron transfer [36]. There are several protocols or assays that have been proposed. The ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant parameter) and crocin bleaching assays are based on HAT. TEAC (Trolox equivalent antioxidant capacity), ABTS (2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid)) and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays are based on the electron transfer activity. Among these protocols, the DPPH assay has been widely used for plant materials because it is an easy and accurate method suitable for measuring antioxidant activity of fruits, vegetable juices or plant extracts [36]. Inhibition of the lipid peroxidation reaction is also a measure to assess the antioxidant activity of plant polyphenols [37].

In addition to the reactions with model radical substrates, it has been demonstrated that flavonoids can directly react with a various type of ROS. The flavonol quercetin was demonstrated to show quenching activity for the singlet oxygen ($^1\text{O}_2$), a non-radical ROS molecule [38]. The flavonol kaempferol [39] and the anthocyanidin cyanidin [40] *in vitro* were shown to scavenge superoxide radical (O_2^-). The flavonol quercetin was reported to scavenge hydroxyl radicals ($\bullet\text{OH}$) produced by radiolysis of water [41, 42]. The flavonols rutin and quercetin were demonstrated to scavenge the hydroperoxide of linoleic acid ($\text{LOO}\bullet$) to inhibit lipid peroxidation [43]. It is now evident that flavonoids are natural plant antioxidants contained in fruits and vegetables.

In principle, the OH groups on the aromatic rings of flavonoids are responsible for the antioxidant or free radical scavenging activity. Most antioxidant flavonoids share the catechol structure with two hydroxy groups ($-\text{OH}$) and/or the double bond between C2-C3 and carbonyl structure [44, 45]. Antioxidant flavonoids satisfying such criteria bear multiple hydroxy groups in a molecule, thereby the name of “polyphenols” being synonymously used for plant antioxidants by the public. It should be noted that polyphenol structure can be found not only in flavonoids but also in other plant phenolic compounds such as hydroxycinnamic acid [35].

When polyphenols scavenge ROS, either through a direct chemical reaction or as an electron donor for an enzymatic reaction, polyphenolic compounds are oxidized and phenoxyl radicals are generated [46]. The phenoxyl radicals are unstable, forming dimers or polymers as a result of spontaneous reaction. Tannin and lignin are the polymerization products of such phenoxyl radical reactions. In the presence of reductant such as ascorbate, the phenoxyl radicals produced are rapidly regenerated into their parent compounds [46]. The enzyme monodehydroascorbate reductase (MDAR) was demonstrated to regenerate flavonoids from their phenoxyl radicals, a possible recycling system of antioxidants [47]. In plants, it has been proposed that flavonoids complement the ascorbate antioxidant system [35].

4. Betalain in red beets and cactus

4.1 Structures and biosynthesis of betalains in plants

Plant coloration can be mostly attributed to spectral property of the colored flavonoids, i.e., anthocyanidins. The plant pigment betalains are exceptional. The term “betalain” comes from the *Latin* name of the common beet (*Beta vulgaris*) from which betalains were first extracted. Betalains are a class of tyrosine-derived pigments that are distributed in only 13 families of Caryophyllales order such as red beet (Amaranthaceae) and cactus (Cactaceae), and in some fungi [48], where they replace anthocyanin pigments [32]. To date, anthocyanins and betalains have never been detected jointly in plant tissues [48]. The biological meaning of the mutually exclusive relationship between betalains and anthocyanidins is still unknown [49, 50].

Betalains are immonium derivatives of betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid] [48]. Betalains are classified into two groups: betacyanin (red-violet) and betaxanthin (yellow) as shown in **Figure 5**. Betacyanin is a conjugate with *cyclo*-dopa and its glycoside, while betaxanthin is a conjugate with amino acid or amine (**Figure 5**).

In contrast with flavonoids, biosynthetic pathway of betalains in plants has not been fully clarified [32, 50, 51]. Hydroxylation of tyrosine by tyrosinase or polyphenol oxidase produces L-dopa, which is catalyzed by 4,5-dopa dioxygenase

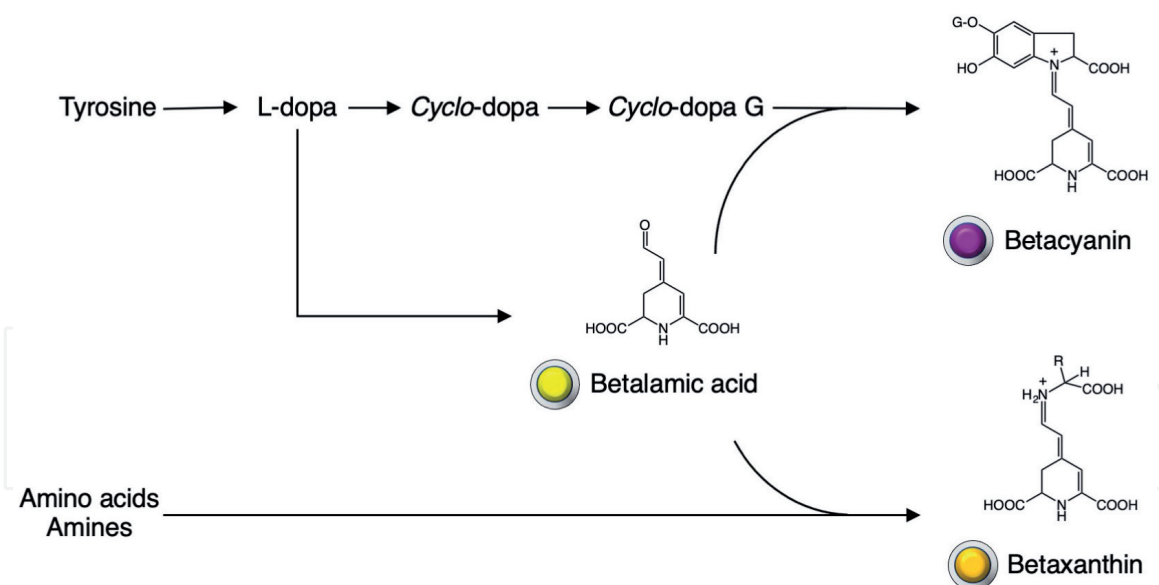


Figure 5.

Structures and biosynthesis pathways of betalains. Betalains are synthesized from L-tyrosine via L-dopa. The intermediate betalamic acid is condensed with cyclo-dopa glycoside or amino acid/amine to betacyanin and betaxanthin, respectively.

to form betalamic acid, the basic common skeleton of betalains. *Cyclo-dopa*, a component of betacyanin, had been considered to be formed by spontaneous chemical reaction after L-dopa is oxidized to dopaquinone by tyrosinase. Recently, the cytochrome P450 CYP76AD1 has been identified as the enzyme which catalyzes the conversion of L-dopa to *cyclo-dopa*, a novel biosynthesis route [52]. CYP76AD1 is a bifunctional enzyme that catalyzes tyrosine hydroxylation as well as *cyclo-dopa* synthesis. This P450 enzyme appears to play important roles not only in betacyanin synthesis but also in betalain synthesis. Furthermore, CYP76AD6 that catalyzes only tyrosine hydroxylation has also been reported [53]. No enzyme for condensing the obtained betalamic acid with a *cyclo-dopa* or an amino acid/amine has been found to date; instead, these condensations likely occur by a spontaneous reaction to form betacyanin or betaxanthin, respectively. Betacyanin usually accumulates as a glycoside, and two routes are estimated for glycosylation: *cyclo-dopa* being condensed with betalamic acid after it is glycosylated and *cyclo-dopa* and betalamic acid being condensed to be betacyanin and then glycosylated. Both are catalyzed by glucosyltransferases [54].

4.2 Antioxidant functions of betalains

Similar to flavonoids, betalains exhibit antioxidant or radical scavenging activity [55, 56]. In contrast with flavonoids, however, the chemistry of the antioxidant mechanism of betalains is less understood. It has been suggested that the common skeleton betalamic acid may contribute to their antioxidant activities [57–59]. Phenolic hydroxy group in *cyclo-dopa* moiety of betacyanin and the amino acid/amine portion of betaxanthin may increase the radical scavenging activities of betalamic acids [58]. Betalains can act as an electron donor for the enzyme peroxidases to detoxify hydrogen peroxide (H_2O_2) [60]. In food chemistry it has been suggested that the degradation of betalains during storage is suppressed in the presence of ascorbate, suggesting that betalain radicals formed by the oxidation might be reduced by ascorbate back to the parent molecules, similar to flavonoids.

5. Reactions of the phytochemicals with RNS and RSS

5.1 RNS and RSS

It is now evident that plant antioxidants remove ROS and free radicals that increase under oxidative stress conditions within cells. In addition to ROS, new players behaving similar to ROS have recently been identified, namely, reactive nitrogen species (RNS) and reactive sulfur species (RSS) [61]. As ROS refers to a group of reactive molecular species originating from molecular oxygen (O_2), RNS and RSS are named for the groups of reactive molecular species derived from nitric oxide (NO) and hydrogen sulfide (H_2S), respectively. Both NO and H_2S are simple gaseous molecules that had initially been appreciated within the life sciences only for their toxicity [62]. Recent investigations have confirmed that NO and H_2S are essential biomolecules synthesized in plants and animals. RNS and RSS are involved in the regulation of a variety of physiological processes. Along with carbon monoxide (CO), NO and H_2S are categorized as “gasotransmitters” [62]. Until recently, many enzymes that produce NO and H_2S have been identified in plants, animals and bacteria.

It is important to note that NO and H_2S are involved not only in physiological regulations (positive effect) but also in dysfunctions or disorders (negative effect). Similar to ROS, unregulated RNS and/or RSS production potentially causes dysfunction of metabolism under biotic as well as abiotic stress conditions, leading to sickness or death in humans [17]. Although a limited number of studies are available on anti-RNS and anti-RSS functions of phytochemicals, it has been reported that flavonoids and betalains could remove RNS and possibly RSS too.

NO reacts rapidly with O_2^- to produce the RNS peroxynitrite ($ONOO^-$) following the reaction:



$ONOO^-$ at physiological pH is unstable and is in rapid equilibrium with its conjugate acid, peroxynitrous acid ($ONOOH$, pK_a 6.8) [63]. In early studies, NO was considered to act as an antioxidant because NO removes O_2^- from a solution as the consequence of the spontaneous reaction. However, this is half-side of a coin since the reaction product $ONOO^-$ attacks proteins and nucleic acids. The nitrated amino acid 3-nitrotyrosine (3- NO_2 -Tyr) is produced when $ONOO^-$ reacts with tyrosine residues of proteins, which potentially disturbs enzyme activities that may lead dysfunction of metabolism, a situation referred as to “nitrosative stress” [64]. It is now widely accepted that $ONOO^-$ is a major cytotoxic agent of RNS.

H_2S is synthesized in plants and animals by multiple enzyme systems [62]. Biogenic H_2S production is involved in various physiological mechanisms as a signaling molecule [62]. Analogous to ROS and RNS, H_2S (or HS^-) produces many reactive molecular species such as persulfide, polysulfide, polysulfane and others [65]. These RSS modify thiol ($-SH$) groups of the cysteine residue of proteins and change enzymatic activities, resulting in both positive regulation and negative inhibition. Uncontrolled overproduction of RSS is a potential risk to damage the cells. Although there is yet little evidence to confirm that flavonoids and betalains scavenge RSS, results of epidemiological studies imply that dietary phytoantioxidants also contribute to reduce the cytotoxicity of RSS in humans [66].

5.2 Chemical reactions with RNS and RSS

Plant phenolic compounds, such as anthocyanin [67, 68] and *p*-hydroxybenzoic acid [69], have been reported to scavenge ONOO^- [70]. Betalains also react with ONOO^- [71, 72]. As the consequence of these reactions, the phytochemical antioxidants inhibit the ONOO^- -induced L-tyrosine nitration and DNA damage [35, 71]. In flavonoids, -OH group at the C3 position of the C-ring has been proposed to be involved in the ONOO^- scavenging activity [69, 73]. As the result of the reaction with ONOO^- , the phytochemical antioxidants are nitrated [74]. These *in vitro* studies have suggested that flavonoids and betalains potentially protect the cells from the nitrosative stress that may induce disorders or mutations [75, 76].

Reactions of the phytochemicals that contribute to reduce the toxicity of RSS are largely unknown. The plant phenolic hydroxycinnamic acids are known to be sulfated by sulfotransferases highly expressed in the human liver and intestine [66]. Flavonoids act as inhibitors of the human sulfotransferases (SULTs) [66]. In plants, sulfate esters of flavonoids are rare compounds [77, 78] that are found in species occurring coastal and swampy areas as well as arid habitats [78]. Functions of sulfated flavonoids in plants and animals are not clear [79]. Sulfated flavonoids, such as quercetin 3-sulfate or quercetin persulfate, have been demonstrated with animals to show antioxidant activity, anti-inflammatory activity, antitumor activity and anticoagulant activity [80–83]. These different lines of studies may imply that sulfated phytochemicals might be associated with physiological regulations in stress tolerance or disease in plants and animals. Although, at present, it must be a speculation to consider specific reactions of flavonoids and betalains with RSS, it is promising that the future investigations of S-containing phytochemicals including sulfated flavonoids or sulfoflavonoids will open up a new research field in life sciences.

6. Antioxidant phytochemicals in human health

In modern science, a great number of studies have suggested health benefits of vegetable-based diets for humans. Many compounds identified from plants have been tested to evaluate their biochemical or pharmacological actions in prevention, mitigation and cure of diseases. According to the “one-to-one” principle, researchers have searched for novel bioactive phytochemicals that interact with specific target enzymes or molecules associated with disorders or diseases. The pharmacokinetic action of antioxidants, however, does not follow the “one-to-one” principle. The actual target is not a specific enzyme or protein but ROS. Since production of ROS is exclusively involved in any types of diseases including cancer, antioxidant activity of phytochemicals has attracted attention not only from researchers but also from the public due to their perceived “cure-all” actions. Nowadays, the antioxidant hypothesis described above has been accepted as the most probable explanation for the health benefits of vegetable-based diets.

Recent progress in medical science has clarified that unregulated RNS and RSS production are observed in many disorders or diseases, echoing findings from ROS research. Although a little is known how plants and animals might regulate RNS and RSS in the cells to achieve a fine balance, there is accumulating evidence to support the hypothesis that phytochemical antioxidants, such as flavonoids and betalains, also reduce the toxicity of RNS and RSS. The occurrence of nitrated flavonoids as well as sulfated flavonoids may imply the possible associations of the phytochemical antioxidants with RNS and/or RSS metabolisms in plants and animals. In this context, the term “antioxidant” for phytochemicals may need to be given a new name to reflect the latest research findings.

In 2020, more than million people died due to the coronavirus disease 2019 (COVID-19) pandemic. There is no promising specific drug or treatment (as of December 2020) for the severe hospitalized patients. A “cytokine storm” occurs in severe cases of COVID-19 and the anti-inflammatory steroid dexamethasone has been applied to lower mortality [84]. COVID-19 and the common “cold” both present a syndrome of disease states. It seems unrealistic to rely on a single drug or chemical to cure the disease. In prevention of the infection, ascorbate and vegetables appears to be effective. The antioxidant flavonoids can reduce inorganic nitrite (NO_2^-) to generate NO in an acidic solution [85]. The vegetable diets and beverages such as the beet juice have been reported to prevent hypertension probably because of increase in NO bioavailability due to nitrite-dependent NO production [2, 86]. It is likely that vegetable-based foods and beverages could prevent or mitigate COVID-19 through their phytochemical antioxidant activities along with their provision of nitrate/nitrite supplementation [84, 87].

7. Prospects for future research

Oxygen toxicity can be attributed ultimately to the biological evolution of oxygenic photosynthesis. In the ancient earth, H_2S and NO concentrations are considered to have been much higher than the present day due to active volcanism [62]. The concentration of these “old” gasses fell down following the evolutionary development of oxygenic photosynthesis in cyanobacteria [62]. It is presumed that most living organisms that were dominant at that time went extinct but some of them successfully developed antioxidant systems to cope with new oxic environments. The survivors from the lethal environments are the ancestors of the present animals. Even for plants, a high partial pressure of O_2 made by photosynthesis is yet a great risk. To protect the photosynthetic apparatus, green plants have developed their unique antioxidant systems along with creation of many types of antioxidant molecules [88]. The left panel of **Figure 6** represents a conceptual illustration for ROS, RNS and RSS in biological evolution in the earth history from past to the

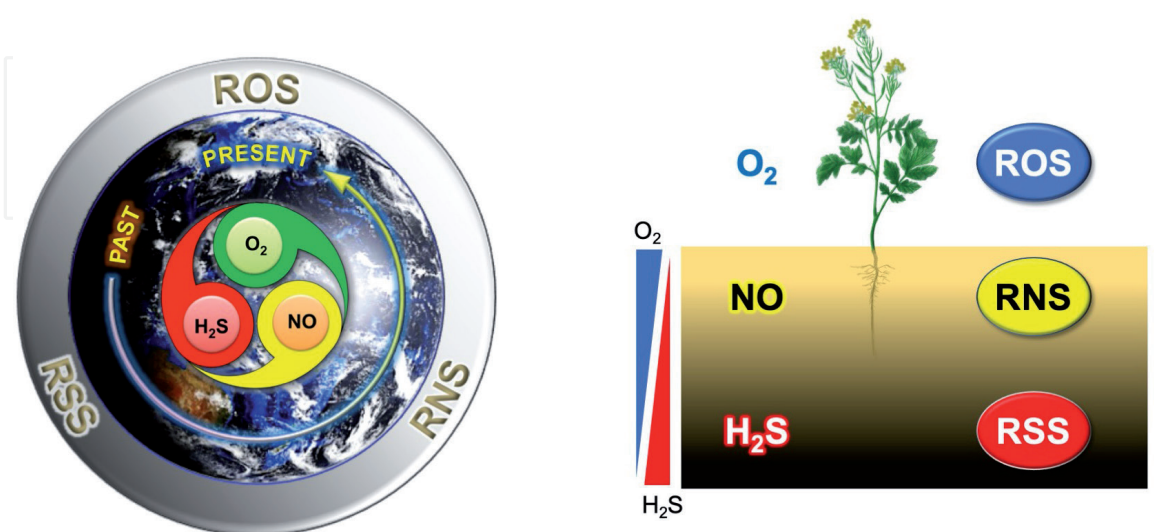


Figure 6.
 The ONS gradient in evolution and habitats. In plants, antioxidants can be found abundantly in leaves where oxygenic photosynthesis occurs, with a risk of overproduction of ROS. If oxidative stress is defined as a condition of disturbance of the fine-tuned redox balance, knowing the interplays among ROS, RNS and RSS is important for understanding cellular homeostasis. Oxygen tension would alter the best balance for each living organism in the field where there is the ONS (O_2 -NO- H_2S) gradient from surface to the deep in soils, which also reflects the order of their evolutionary development (from ancient to the present) [54].

present. The order (ROS→RNS→RSS) can be found in ecological niches from surface to deep such as in soils (**Figure 6**, right). In the case of plants grown in the field, leaves are in oxic environments and roots are in hypoxic environments where there exists a gradient of O₂, NO and H₂S. Taking into account that sulfated plant phenolic compounds are found in plants inhabiting harsh environments, we consider it plausible that novel bioactive phytochemicals associated with RNS and RSS metabolisms might be found in the roots grown in such hypoxic environments [89].

8. Conclusions

Flavonoids and betalains are natural antioxidants that mitigate oxidative stress in plants and animals. In life sciences, oxidative stress can be defined as an imbalance of pro-oxidants and antioxidants in cells. Oxidative stress can be also defined as a disruption of redox signaling and control, emphasizing the importance of a dynamic but fine-tuned redox balance in cellular homeostasis [90]. According to this new definition, the ROS scavenging activity may be just a part of the pleiotropic functions of phytochemicals. Flavonoids and betalains could tune a fine redox balance through modulating the interplays among ROS, RNS and RSS. We are now entering into the next stage of plant “antioxidant” research.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Yasuko Sakihama¹ and Hideo Yamasaki^{2*}

¹ Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

² Faculty of Science, University of the Ryukyus, Okinawa, Japan

*Address all correspondence to: yamasaki@sci.u-ryukyu.ac.jp

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