



HOKKAIDO UNIVERSITY

Title	Beetroot betalain inhibits peroxynitrite-mediated tyrosine nitration and DNA strand cleavage
Author(s)	Sakihama, Yasuko; Maeda, Makiko; Hashimoto, Makoto et al.
Citation	Free Radical Research, 46(1), 93-99 https://doi.org/10.3109/10715762.2011.641157
Issue Date	2012-01
Doc URL	https://hdl.handle.net/2115/64661
Type	journal article
File Information	FreeRadcRes(proof)-1.pdf



Beetroot betalain inhibits peroxynitrite-mediated tyrosine nitration and DNA strand cleavage

YASUKO SAKIHAMA¹, MAKIKO MAEDA², MAKOTO HASHIMOTO¹, SATOSHI
TAHARA¹ & YASUYUKI HASHIDOKO¹

¹Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, and ²Graduate
School of Agriculture, Hokkaido University, Sapporo, Japan

Abstract

Two major betalains, red-purple betacyanins and yellow betaxanthins, were isolated from red beetroots (*Beta vulgaris* L.), and their peroxynitrite (ONOO⁻) scavenging capacity was investigated. Apparent colors of the betalains were bleached by the addition of ONOO⁻, and the absorbance decreases were suppressed in the presence of glutathione, a ONOO⁻ scavenger. After bleaching, a new absorption maximum was observed at 350 nm in the spectrum of the resulting reaction mixture. From HPLC analysis of the reaction products of betanin, a representative constituent of red beetroot betacyanins, treated with ONOO⁻ monitoring at 350 nm, new peaks were detected, and the intensity of the major peak was positively correlated with ONOO⁻ concentration. Betanin inhibited the ONOO⁻ (0.5 mM)-dependent nitration of tyrosine (0.1 mM). Additionally, the IC₅₀ value of betanin (19.2 μM) was lower than that of ascorbate (79.6 μM). The presence of betanin (0.05–1.0 mM) also inhibited ONOO⁻

(0.5 mM)-dependent DNA strand cleavage in a concentration-dependent manner. These results suggest that betalains can protect cells from nitrosative stress in addition to protecting them from oxidative stresses.

Key words: betanin, nitrosative stress, plant pigment, reactive nitrogen species, reactive oxygen species

Correspondence: Yasuko Sakihama, Research Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo, 060-8589 Japan, FAX: +81 11 706 8142. E-mail: sakihama@abs.agr.hokudai.ac.jp

Introduction

Peroxynitrite (ONOO^-) is a powerful oxidant and a nitrating agent formed from the reaction of nitric oxide (NO) and a superoxide radical ($\text{O}_2\bullet^-$) ($\sim 1 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$) [1]. ONOO^- is the most toxic of the reactive nitrogen species (RNS) and can potentially oxidize and nitrate many biological molecules, such as the proteins of tyrosine residues, thiols, unsaturated fatty acid-containing phospholipids, and DNA [2-4]. The modification of these biomolecules leads to the inhibition, inactivation and, sometimes, activation of enzymes and channels, as well as disturbances in cellular energetic and signaling processes [5]. Thus, ONOO^- has been implicated as a key pathophysiological intermediate in various diseases, including inflammatory processes, neurodegenerative disorders, and atheroma [1,6]. Thus, the scavenging of ONOO^- is an important

intracellular response to avoid oxidative and nitrosative damage of both plant and animal cells.

Betalains are nitrogen-containing water-soluble plant pigments that are found only in plants from 10 families of the order Caryophyllales [7-9]. Betalains can be divided into two major structural groups: betacyanins and betaxanthins (Figure 1). Betacyanins contain a *cyclo*-dopa residue and are red-violet. Betaxanthins contain various amino acid or amine side chains and are yellow. Betalains have been extensively used as natural food colorants in processed meat, ice cream and candies [8,10]. Recently, various studies have reported the antioxidant activity of betacyanins and betaxanthins against reactive oxygen species (ROS), such as the $O_2^{\bullet-}$ and H_2O_2 [10-12]. This antioxidant activity is similar to ascorbic acid and anthocyanins, which are well known antioxidants *in vitro* and *in vivo* [13,14]. As a result of these data, the effects of betalains have received increased attention. However, there have been no reports of the reactivities of betalains toward RNS. In this study, we investigated the anti-nitrosative activity of beetroot betalain using multiple *in vitro* systems: direct reaction, tyrosine nitration, and DNA strand breaks.

Materials and methods

Preparation of red beet betalains and blueberry anthocyanins

Red beetroots (*Beta vulgaris* L.) were cultivated and harvested in Eniwa-shi, Hokkaido, Japan, and stored at -80 °C until use. Betacyanin and betaxanthin were isolated according to the method reported by Chiji [15]. Defrosted beetroots (400 g)

was squeezed with a juicer and filtered through two layers of Kimwipes (Crecia, Japan). The filtrate (200 mL) was centrifuged at 10 000 rpm for 10 min at 4°C, and 120 mL of supernatant was applied to a Dowex 50W-X2 (H⁺-type, 90 × 80 cm, Wako, Japan) column. After washing with a 2-fold volume of 0.1% HCl, betalain pigments were eluted with an 8-fold volume of RO water. This betalain extract contained both betacyanins and betaxanthins. Betacyanins and betaxanthins were purified using a HITACHI-HPLC system (Japan) with an L-6320 Intelligent pump, an L-2450 UV/VIS Detector, a D-2500 Chromato Integrator and an Inertsil Prep-ODA column (250 × 20 mm, GL Sciences Inc., Japan). The elution was performed with 5% (v/v) formic acid and 5% (v/v) acetonitrile in H₂O as a solvent at a flow rate of 3 mL min⁻¹ and was monitored at 500 nm. The separated betacyanins and betaxanthins were lyophilized and redissolved in a small volume of H₂O. The betacyanin fraction included betanin and a less than 10% isobetanin, which estimated from the peak intensities of HPLC analysis. The betaxanthin fraction was deduced to the mixture of vulgaxanthins which have been reported as the main betaxanthin pigments of beetroot [16]. These fractions were used as betanin and betaxanthins for subsequent assays. The concentrations of betanin and betaxanthins were estimated using the molar extinction coefficients $\epsilon_{538} = 60\,000\text{ M}^{-1}\text{cm}^{-1}$ and $\epsilon_{480} = 48\,000\text{ M}^{-1}\text{cm}^{-1}$, respectively [17].

Fresh blueberries were obtained from a local market in Sapporo and immersed in a 5-fold volume of methanol containing 0.1% HCl for 3 h. The extract was filtered through filter paper and concentrated *in vacuo* at 30°C. To precipitate anthocyanins, a 3-fold volume of diethyl ether was added to the concentrated extract. The precipitated

blueberry anthocyanins were collected and the concentration of anthocyanin was determined using the molar extinction coefficient $\epsilon_{538} = 15\,600\text{ M}^{-1}\text{cm}^{-1}$ for malvidin glycoside, a major anthocyanin in blueberry [18].

Peroxynitrite (ONOO^-) synthesis

ONOO^- was synthesized by the method described by Patel and DarleyUsmar [19]. Ice-cold 2 M sodium nitrite (10 mL) and acidified 1.85 M hydrogen peroxide (in 4.0 M HCl, 10 mL) were mixed by pumping both liquids into a Y-shaped connector. The resulting acidic ONOO^- was immediately quenched with 4.2 M NaOH (10 mL). Excess hydrogen peroxide was removed by adding 1.3 g manganese dioxide (MnO_2). MnO_2 was removed by centrifugation (4 000 rpm for 5 min at 4°C). The solution was frozen at -80°C; ONOO^- tended to form a yellow top layer due to freeze fractionation, which was scraped for further studies (typical concentrations were 200 –300 mM). The concentration of ONOO^- was determined using the molar extinction coefficient $\epsilon_{302} = 1\,670\text{ M}^{-1}\text{cm}^{-1}$.

UV-visible spectral analysis of ONOO^- scavenging activity of betalains

The reactivity of betalains against ONOO^- was measured with a spectrophotometer. The reaction mixture (1 mL total) contained 0.25 mM sodium phosphate buffer (pH 7.4) and 20 μM betanin or betaxanthin, and various concentrations of ONOO^- were added. The absorbance spectra of betanin before and after adding ONOO^- (0.3 mM) were measured. A high-concentration reaction mixture,

1 containing 0.6 M sodium phosphate (pH 7.4), 0.47 mM betanin and 9.3 mM ONOO⁻,
2 was used for the analysis of the reaction products. The same experiment was performed
3 under acidic conditions (50 mM citric acid, pH 5.0).

5 *HPLC analysis of the reaction products*

6 The reaction products of betanin and ONOO⁻ were analyzed with a Waters
7 HPLC system (Massachusetts, USA), a 600 controller, and a 996 Photodiode Array
8 Detector with Empower software and an L-column2 ODS column (250×10 mm, Kanto
9 Chemicals, Japan). The following solvent system was used: 5% (v/v) HCOOH and 5%
10 (v/v) acetonitrile in H₂O at a flow rate of 1 mL min⁻¹ for 0–30 min and 3 mL min⁻¹ for
11 30–60 min.

13 *Inhibition of ONOO⁻-dependent L-tyrosine nitration*

14 The inhibitory effect of betanin on the ONOO⁻ nitration of L-tyrosine was
15 measured using the method described by Pannala *et al.* [20]. The reaction mixture (1
16 mL total) contained 0.5 M sodium phosphate buffer (pH 7.4) and 0.1 mM L-tyrosine in
17 the presence of varying concentrations (0.001–0.1 mM) of the antioxidants betanin,
18 anthocyanin or ascorbic acid. The reaction was started by the addition of 0.5 mM
19 ONOO⁻ to the reaction mixture. The reaction mixtures were analyzed by HPLC with
20 the same condition described above, and the level of tyrosine was monitored at 275 nm
21 during the formation of nitrotyrosine. The exposure of 0.1 mM of tyrosine to 0.5 mM
22 of ONOO⁻ without any antioxidant resulted in the 60% nitration of tyrosine and a

subsequent decrease in tyrosine levels. The ONOO^- scavenging ability was expressed as the percentage inhibition of the decrease in tyrosine compared to control samples.

Inhibition of ONOO^- -dependent plasmid DNA cleavage

DNA cleavage was analyzed by detecting the conversion of the supercoiled form of pUC19 plasmid DNA (Invitrogen, U.S.A.) to open circular and linear forms [4]. DNA (300 ng) was incubated with 0.5 mM ONOO^- in the presence or absence of betanin in 50 mM sodium phosphate buffer (pH 7.4) for 5 min at room temperature. After the incubation, the DNA was precipitated in cold ethanol and left at -20°C overnight. The DNA was collected by centrifugation and resuspended in 20 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The forms of plasmid DNA were analyzed by electrophoresis in 1% agarose/TBE gels. After electrophoresis, the gels were stained with $0.5\ \mu\text{g mL}^{-1}$ ethidium bromide for 30 min and then photographed under UV illumination. Signal intensity was calculated with an NIH-image software.

Results

Bleaching of beetroot betalains by ONOO^-

Betanin isolated from red beet (inc. <10% isobetanin) showed red-purple in neutral pH buffer (pH 7.4); this color was bleached by the addition of ONOO^- in 0.5 M NaOH, with a rapid decrease in absorbance at 538 nm (Figure 2A). Subsequently, a new absorbance increase was observed at 350 nm (Figure 2A dashed line). Although betalains are unstable at neutral to alkaline pH, the absorbance changes of betanin and

betaxanthin were negligible with the addition of 0.5M NaOH instead of ONOO^- during measurement (3 min). Similar bleaching was observed under acidic pH conditions (pH 5.0). This bleaching of betanin strongly depended on the ONOO^- concentration (Figure 2B). Like betanin, the yellow betaxanthins isolated from beetroot exhibited a similar bleaching reaction to ONOO^- (Figure 2). These bleaching of betanin and betaxanthins were approximately 90% suppressed in the presence of 10 mM glutathione, which is known to be an ONOO^- scavenger.

Analysis of betanin and ONOO^- reaction products

The reaction mixture was analyzed with reverse phase HPLC by monitoring the photo diode array detector. When monitoring the reaction mixture without ONOO^- at 500 nm, two peaks were detected at 32.3 and 37.5 min and were identified as betanin and isobetanin, respectively, in accordance with the retention times of each authentic compound (Figure 3A). In the presence of ONOO^- , the peak of betanin completely disappeared (Figure 3A). Conversely, in the absence of ONOO^- treatment, the solution showed no peak at 350 nm; however, there were three peaks (peaks a-c) detected in the ONOO^- treatment mixture (Figure 3A). UV/vis absorption spectra of the peaks a, b and c showed absorption maxima at 349 and 504 nm for peak a, 355 nm for peak b, and 345, 357, 371 and 395 nm for peak c. The intensities of peaks b and c showed positive correlations with concentrations of ONOO^- . Several peaks were detected from the peak c fraction as a result of performing HPLC analysis using a different mobile solvent. The observation of several absorption maxima of the peak c fraction supported the

assumption that the reaction was a mixture of several compounds. Figure 3B shows the negative correlation between peak intensities of betanin and reaction product. Betanin decreased as the ONOO^- concentration increased and completely disappeared in the presence of 20 molar equivalents of ONOO^- . Whereas betanin decreased, the area of peak b increased with a sigmoidal curve toward the ONOO^- concentration. The peak of betanin decreased to less than 30% of the control value when 5 molar equivalents of ONOO^- were added to the reaction mixture. However, product B corresponding compound to the peak b was not detected under these conditions, suggesting that betanin was converted to product B via undetectable intermediates in this study. The molecular weight of product B was estimated to be 358 from LC-TOFMS analysis, which corresponded to decarboxylated and nitrated *cyclo*-dopa cleaved from betanin at the Schiff base.

Inhibition activity against L-tyrosine nitration and DNA breakage mediated by ONOO^-

Exposure of tyrosine to ONOO^- resulted in production of 3-nitrotyrosine, and subsequent decrease in the levels of tyrosine was observed. The half maximal inhibitory concentration (IC_{50}) values of betanin, blueberry anthocyanins and ascorbic acid were 19.2, 13.8 and 79.6 μM , respectively (Figure 4). The betanin IC_{50} was lower than those reported for plant polyphenols, such as chlorogenic acid and caffeic acid (Table 1) [20,21].

Many reports have indicated that ONOO^- induces plasmid DNA breakage [4,22]. Figure 5 shows the results of incubating pUC19 plasmid DNA with 0.5 mM

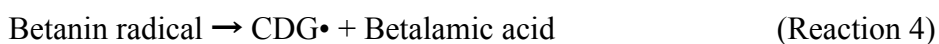
1 ONOO⁻. Most of the supercoiled (SC) form of pUC19 DNA was converted to the open
2 circular (OC) form by ONOO⁻ incubation, which resulted in single strand breaks. In the
3 presence of betanin in the reaction mixture, however, the OC form of DNA decreased
4 and the remaining SC form increased in a dose-dependent manner (Figure 5, lanes 3–7).
5 In the presence of 1 mM betanin (lane 7), approximately 60% of the DNA retained the
6 SC form after ONOO⁻ exposure; the same concentration of betanin (1 mM) alone did
7 not affect the SC form of DNA (data not shown). These results indicate that betanin can
8 protect tyrosine and plasmid DNA from ONOO⁻ attack.

10 Discussion

11 In the case of phenolic compounds, such as flavonoids and chlorogenic acid, it
12 is widely accepted that polyphenols featuring catechol structures are more efficient
13 antioxidants than are monophenols [23,24]. These catecholate compounds can be
14 oxidized with two electrons as a result of scavenging ROS, resulting in the production
15 of the corresponding quinones. However, monophenolic compounds become to
16 corresponding radical by one-electron oxidation, forming dimers or other products [25].

17 Recently, a similar betanin ROS scavenging mechanism was proposed in the
18 aqueous/lipid system by Tesoriere *et al.* [26]. When betanin reacts with a peroxy
19 radical (LOO•), an electron is donated from the phenol moiety and an intermediate
20 betanin radical is generated. After splitting the aldimine bond via the nucleophilic
21 attack of water, a *cyclo*-dopa 5-*O*- β -D-glucoside radical (CDG•) is also produced as an
22 intermediate [26]. This reaction is similar to those of monophenols that scavenge ROS.

Catecholate compounds also scavenge ONOO^- , possibly leading to the formation of the corresponding quinones and nitrite [20,27]. Monophenolic compounds become corresponding radicals by one-electron oxidation, and the radicals then react with NO_2 radicals, resulting in the production of nitrated compounds [20,28]. Therefore, when betanin reacts with ONOO^- , it is assumed that the following reactions occur. ONOO^- is in equilibrium with ONOOH ($\text{pK}_a=6.8$) (Reaction 1), and ONOOH is decomposed to an $\bullet\text{OH}$ and NO_2 radical pair (Reaction 2). Betanin then reacts with $\bullet\text{OH}$, resulting in the generation of a betanin radical by one-electron oxidation (Reaction 3). Solvolytic cleavage of betanin then produces $\text{CDG}\bullet$ and betalamic acid (Reaction 4). $\text{CDG}\bullet$ then reacts with the NO_2 radical, producing nitrated CDG (Reaction 5).



In this study, we detected a decarboxylated and nitrated CDG as a reaction product of betanin with ONOO^- (product B). Product B is likely produced via the assumed reactions (Reactions 1–5), though the decarboxylation mechanism of product B remains unclear. Decarboxylated products during high temperature treatment of

betanin have been reported [29,30]. Herbach has proposed that CDG is generated after hydrolytic cleavage of betanin then CDG is decarboxylated[29]. In the reaction of betanin and ONOO⁻, the reaction order of CDG nitration and decarboxylation is not clear yet. However, nitration maybe precedes decarboxylation, because CDG radical is generated by cleavage of betanin (Reaction 4) and generally the reaction between radicals (Reaction 5) very fast. Structural analysis of the products from betalain that react with ONOO⁻ is necessary to elucidate the betalain ONOO⁻ scavenging mechanism. Further study using simple chemical models of betalains will be an effective approach to clarify this mechanism.

In conclusion, our study demonstrated the effective inhibitory activity of betanin against tyrosine nitration and DNA damage induced by ONOO⁻, one of the most toxic RNS, indicating that betanin is a strong scavenger against ONOO⁻ comparable to anthocyanin [13]. Because betaxantins was bleached by ONOO⁻ as well as betanin, it is expected that the ONOO⁻ scavenging activity would be a common ability of betalain pigments. It has been reported that dietary betalains are incorporated into human blood cells, which may protect these cells from oxidative hemolysis [31]. Zou *et al.* also reported that betalains in cactus pear extract participated in growth inhibition in an *in vivo* mouse model of ovarian cancer [32]. According to these reports, it is plausible that dietary betalains can function in human cells as scavengers of ROS and RNS. Thus, betalains have a great potential in the food and pharmaceutical industries, not only as oxidative stress protectors but also as nitrosative stress protectors.

Acknowledgements

We thank Dr. Y. Fukushi of Hokkaido University and Dr. H. Chiji of Fuji Women's University for their helpful suggestions for betalain purification and for providing authentic betanin. This research was supported by Grant-in-Aid A (20248033 to YH).

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

References

- [1] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620-1624.
- [2] Bonny MG, Auguso O. Carbon dioxide stimulates the production of thiyl, sulfinyl, and disulfide radical anion from thiol oxidation by peroxynitrite. *J Biol Chem* 2001; 276: 9749-9754.
- [3] Ohshima H, Friesen M, Brouet I, Bartsch H. Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem Toxicol* 1990; 9: 647-652.
- [4] Grace SC, Salgo MG, Pryor WA. Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA. *FEBS Lett* 1998; 426: 24-28.
- [5] Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov* 2007; 6: 662-680.
- [6] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007; 87: 315-424.
- [7] Clement JS, Mabry TJ. Pigment evolution in the Caryophyllales: A systematic overview. *Bot Acta* 1996; 109: 360-367.
- [8] Strack D, Vogt T, Schliemann W. Recent advances in betalain research. *Phytochemistry* 2003; 62: 247-269.

- [9] Stafford HA. Anthocyanins and betalains - Evolution of the mutually exclusive pathways. *Plant Sci* 1994; 101: 91-98.
- [10] Azeredo HMC. Betalains: properties, sources, applications, and stability – a review. *Int J Food Sci Technol* 2009; 44: 2365–2376.
- [11] Kanner J, Harel S, Granit R. Betalains - A new class of dietary cationized antioxidants. *J Agric Food Chem* 2001; 49: 5178-5185.
- [12] Cia Y-Z, Sunb M, Corke H. Characterization and application of betalain pigments from plants of the Amaranthaceae. *Trends Food Sci Technol* 2005; 16: 370-376.
- [13] Stintzing FC, Carle R. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends Food Sci Technol* 2004; 15: 19-38.
- [14] Yamasaki H, Uefuji H, Sakihama Y. Bleaching of the red anthocyanin induced by superoxide radical. *Arch Biochem Biophys* 1996; 332: 183-186.
- [15] Chiji H. Studies on betalain pigments of centrospermae plants with special reference to a violet red pigment in *Salicornia europaea* L. *Memories of the Faculty of Agriculture, Hokkaido University* 1976; 9: 303-372.
- [16] Vincent KR, Scholz R. Separation and Quantification of Red Beet Betacyanins and Betaxanthins by High-Performance Liquid Chromatography. *J Agric Food Chem* 1977; 26: 812-816.
- [17] Kugler F, Stintzing FC, Carle R. Identification of betalains from petioles of differently colored Swiss chard (*Beta vulgaris* L. ssp *cicla* [L.] Alef. Cv. Bright lights) by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J Agr Food Chem* 2004; 52: 2975-2981.

- 1 [18] Mateus N, de Freitas V. Evolution and stability of anthocyanin-derived pigments
2 during port wine aging. J Agric Food Chem 2001; 49: 5217-5222.
- 3 [19] Patel RP, DarleyUsmar VM. Using peroxynitrite as oxidant with low-density
4 lipoprotein. Method Enzymol 1996; 269: 375-384.
- 5 [20] Pannala A, Razaq R, Halliwell B, Singh S, Rice-Evans CA. Inhibition of
6 peroxynitrite dependent tyrosine nitration by hydroxycinnamates: Nitration or
7 electron donation? Free Radic Biol Med 1998; 24: 594-606.
- 8 [21] Kerry N, Rice-Evans C. Inhibition of peroxynitrite-mediated oxidation of
9 dopamine by flavonoid and phenolic antioxidants and their structural
10 relationships. J Neurochem 1999; 73: 247-253.
- 11 [22] Kennedy LJ, Moore K, Caulfield JL, Tannenbaum SR, Dedon PC. Quantitation of
12 8-oxoguanine and strand breaks produced by four oxidizing agents. Chem Res
13 Toxicol 1997; 10: 386-392.
- 14 [23] RiceEvans CA, Miller J, Paganga G. Antioxidant properties of phenolic
15 compounds. Trends Plant Sci 1997; 2: 152-159.
- 16 [24] Villano D, Fernandez-Pachon MS, Troncoso AM, Garcia-Parrilla MC. Comparison
17 of antioxidant activity of wine phenolic compounds and metabolites *in vitro*.
18 Anal Chim Acta 2005; 538: 391-398.
- 19 [25] Barclay LRC, Baskin KA, Dakin KA, Locke SJ, Vinqvist MR. The antioxidant
20 activities of phenolic antioxidants in free-radical peroxidation of phospholipid-
21 membrans. Can J Chem-Rev Can Chim 1990; 68: 2258-2269.
- 22 [26] Tesoriere L, Allegra M, Gentile C, Livrea MA. Betacyanins as phenol antioxidants.

- Chemistry and mechanistic aspects of the lipoperoxyl radical-scavenging activity in solution and liposomes. Free Radic Res 2009; 43: 706 -717.
- [27] Hogg N, Joseph J, Kalyanaraman B. The oxidation of alpha-tocopherol and trolox by peroxyxynitrite. Arch Biochem Biophys 1994; 314: 153-158.
- [28] Sakihama Y, Tamaki R, Shimoji H, Ichiba T, Fukushi Y, Tahara S, Yamasaki H. Enzymatic nitration of phytophenolics: evidence for peroxyxynitrite-independent nitration of plant secondary metabolites. FEBS Lett 2003; 553: 377-380.
- [29] Herbach KM, Stintzing FC, Carle R. Identification of heat-induced degradation products from purified betanin, phyllocactin and hydrocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 2005; 19: 2603-2616.
- [30] Wybraniec S. Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC-MS/MS. J Agric Food Chem 2005; 53: 3483-3487.
- [31] Tesoriere L, Butera D, Allegra M, Fazzari M, Livrea MA. Distribution of betalain pigments in red blood cells after consumption of cactus pear fruits and increased resistance of the cells to *ex vivo* induced oxidative hemolysis in humans. J Agr Food Chem 2005; 53: 1266-1270.
- [32] Zou D-M, Brewer M, Garcia F, Feugang JM, Wang J, Zang R, Liu H, Zou C. Cactus pear: a natural product in cancer chemoprevention. Nutr J 2005; 4: 25

Figure legends

Figure 1. Chemical structures of beetroot betacyanins and betaxanthins.

Betanin: R_1 = glucose, Isobetanin: C_{15} epimer of betanin, vulgaxanthin I: R_2 = NH_2 ,
vulgaxanthin II: R_2 = OH.

Figure 2. Bleaching of betanin and betaxanthin by the addition of $ONOO^-$. The reaction mixture (1 mL total) contained 0.25 M Na-phosphate (pH 7.4) and 20 μ M betanin or betaxanthin. The reaction was initiated by addition of various concentrations of $ONOO^-$, and the absorbances were measured after a 3-min incubation. A: absorbance spectrum of betanin and betaxanthin before (solid line) and after (dashed line) the addition of 0.3 mM $ONOO^-$. B: the dependence of betanin and betaxanthin bleaching on $ONOO^-$ concentrations. Data are presented as mean values $\pm$ SD (n=3).

Figure 3. HPLC analysis of the reaction products of betanin and $ONOO^-$. The reaction mixture (1 mL total) contained 0.6 M Na-phosphate (pH 7.4) and 0.47 mM betanin. A: HPLC chromatograms of the reaction products. Upper trace, with $ONOO^-$ (9.3 mM); lower trace, without $ONOO^-$. The arrow indicates isobetanin peak. B: change of the betanin and reaction product (peak b) amounts according to the $ONOO^-$ concentration. The inset shows the UV/Vis absorption spectrum of the reaction product (peak b). Data are presented as mean values $\pm$ SD (n=3).

1 Figure 4. Inhibition of ONOO^- -dependent tyrosine nitration by betanin. The reaction
2 mixture (1 mL) contained 0.5 M Na-phosphate buffer (pH 7.4), L-tyrosine (0.1 mM) and
3 varying concentrations (0.001–0.1 mM) of betanin, blueberry anthocyanin and ascorbic
4 acid. The reaction was initiated by the addition of ONOO^- (0.5 mM). Nitrotyrosine
5 formation was estimated from remaining tyrosine analyzed by HPLC.

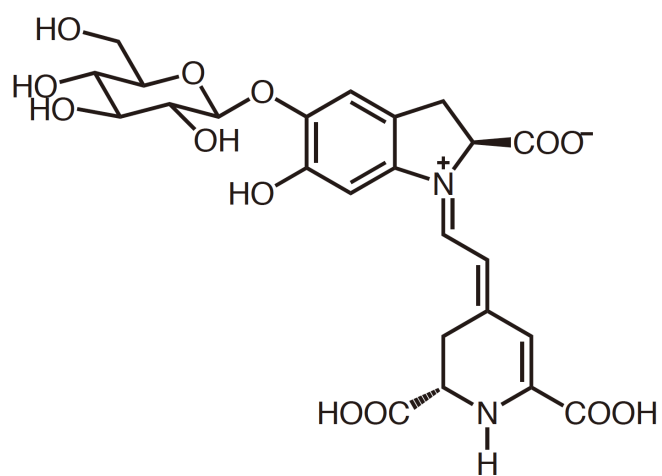
6
7 Figure 5. Inhibition of ONOO^- -induced DNA strand breaks by betanin. Supercoiled
8 (SC) pUC19 DNA (300 ng) was incubated with 0.5 mM ONOO^- in 50 mM sodium
9 phosphate (pH 7.4) for 5-min and was analyzed by agarose gel electrophoresis. Betanin
10 was added to the reaction mixture at the concentrations indicated above each lane. OC,
11 open circular DNA.

Table 1 Concentrations inhibiting 50% (IC₅₀) of ONOO⁻-dependent L-tyrosine

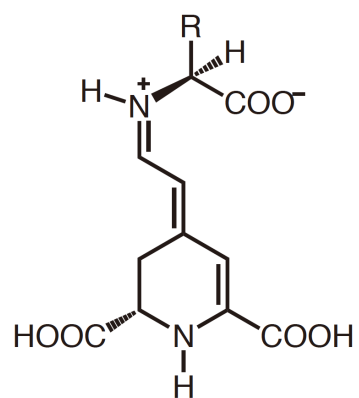
nitration

Antioxidants	IC ₅₀ (μM)
Betanin*	19.2
Blueberry anthocyanins*	13.8
Dopamine ^[21]	37.5
Chlorogenic acid ^[20]	31.4
Caffeic acid ^[20]	28.6
Ascorbic acid*	79.6

*present study, [21] Kerry *et al.*, J Neurochem 1999,
[20] Pannala *et al.*, Free Radic Biol Med 1998



Betanin (betacyanin)



Betaxanthin

Fig. 1

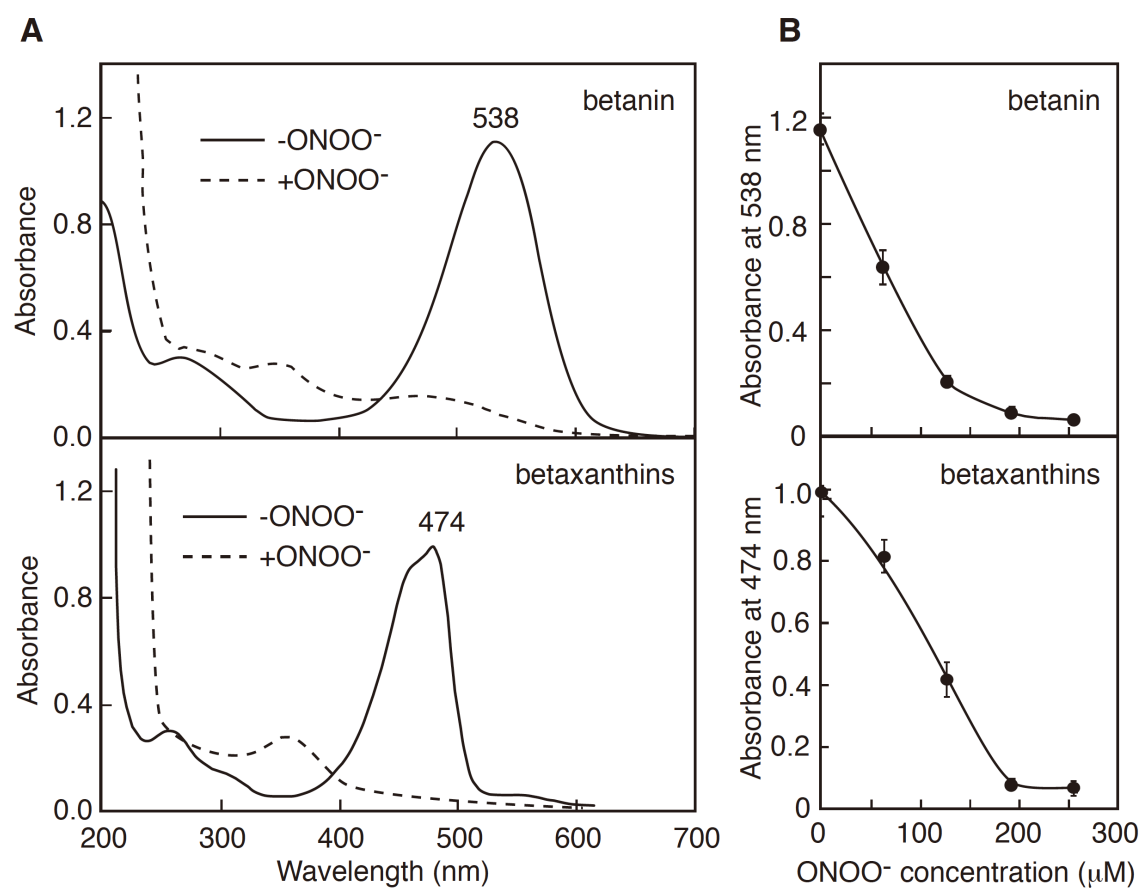


Fig. 2

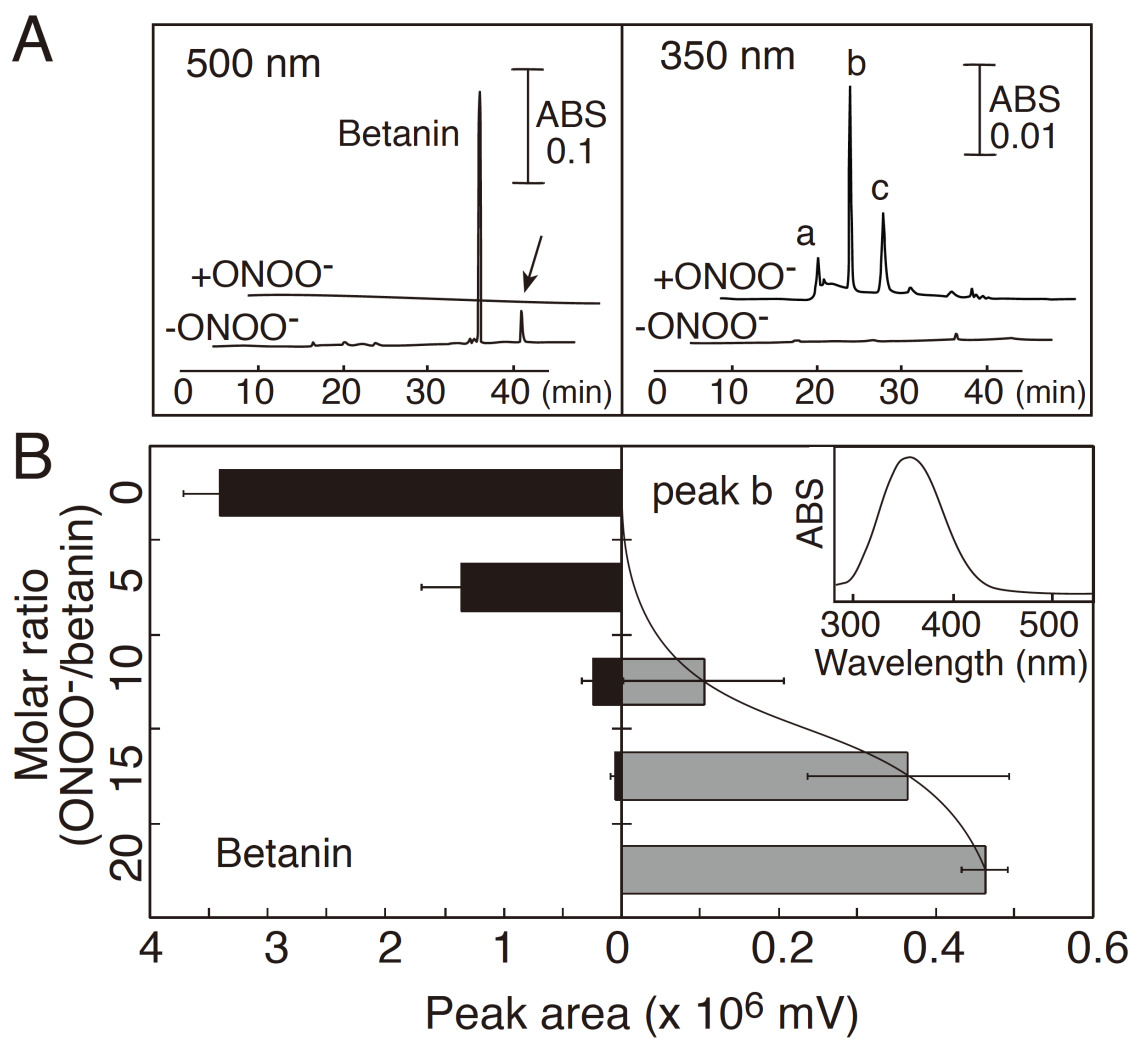


Fig. 3

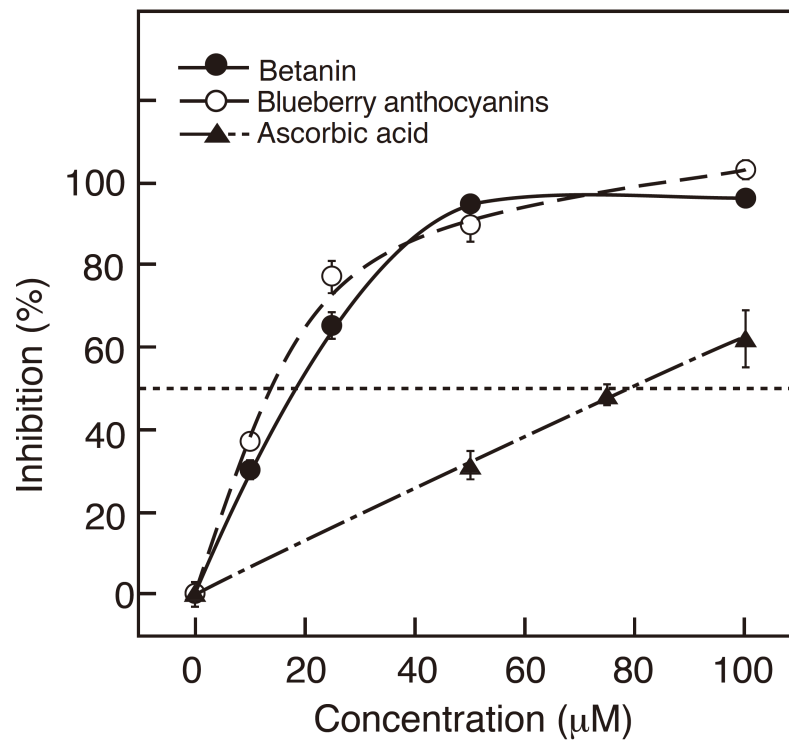


Fig. 4

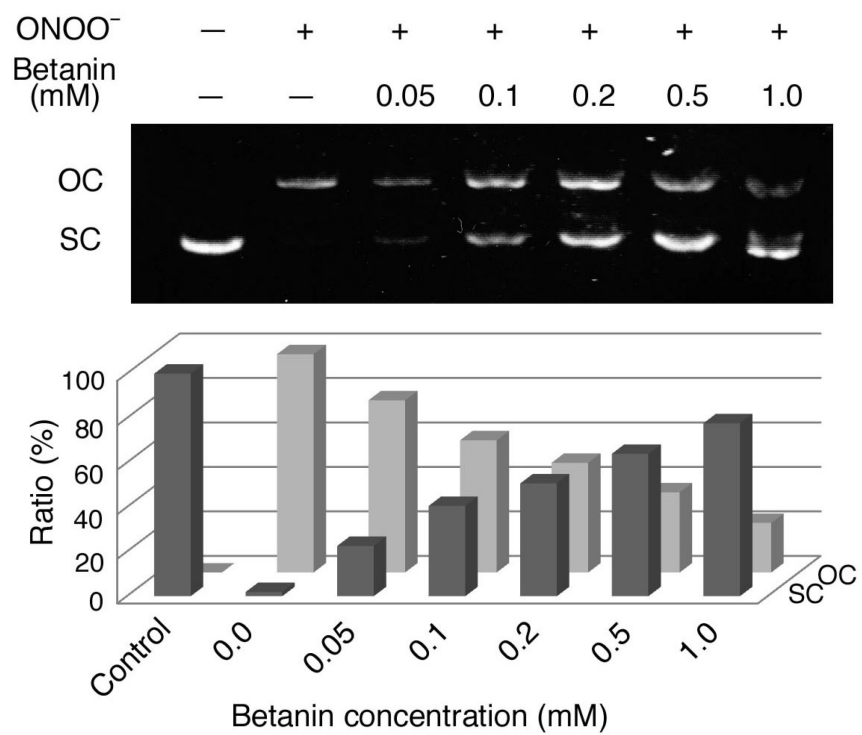


Fig. 5