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Title	Beetroot betalain inhibits peroxynitrite-mediated tyrosine nitration and DNA strand cleavage
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Instructions for use

1 **Beetroot betalain inhibits peroxynitrite-mediated tyrosine nitration and DNA**

2 **strand cleavage**

3

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9

10 **Abstract**

11 Two major betalains, red-purple betacyanins and yellow betaxanthins, were

12 isolated from red beetroots (*Beta vulgaris* L.), and their peroxynitrite (ONOO⁻)

13 scavenging capacity was investigated. Apparent colors of the betalains were bleached

14 by the addition of $ONOO^-$, and the absorbance decreases were suppressed in the

15 presence of glutathione, a $ONOO^-$ scavenger. After bleaching, a new absorption

16 maximum was observed at 350 nm in the spectrum of the resulting reaction mixture.

17 From HPLC analysis of the reaction products of betanin, a representative constituent of

18 red beetroot betacyanins, treated with $ONOO^-$ monitoring at 350 nm, new peaks were

19 detected, and the intensity of the major peak was positively correlated with $ONOO^-$

20 concentration. Betanin inhibited the $ONOO^-$ (0.5 mM)-dependent nitration of tyrosine

21 (0.1 mM). Additionally, the IC_{50} value of betanin (19.2 μM) was lower than that of

22 ascorbate (79.6 μM). The presence of betanin (0.05–1.0 mM) also inhibited $ONOO^-$

1   (0.5 mM)-dependent DNA strand cleavage in a concentration-dependent manner. These

2   results suggest that betalains can protect cells from nitrosative stress in addition to

3   protecting them from oxidative stresses.

4

7

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11

Introduction

13   Peroxynitrite ($ONOO^-$) is a powerful oxidant and a nitrating agent formed from

14   the reaction of nitric oxide (NO) and a superoxide radical ($O_2 \bullet^-$) ($\sim 1 \times 10^{10}$ $M^{-1}s^{-1}$) [1].

15   $ONOO^-$ is the most toxic of the reactive nitrogen species (RNS) and can potentially

16   oxidize and nitrate many biological molecules, such as the proteins of tyrosine residues,

17   thiols, unsaturated fatty acid-containing phospholipids, and DNA [2-4]. The

18   modification of these biomolecules leads to the inhibition, inactivation and, sometimes,

19   activation of enzymes and channels, as well as disturbances in cellular energetic and

20   signaling processes [5]. Thus, $ONOO^-$ has been implicated as a key pathophysiological

21   intermediate in various diseases, including inflammatory processes, neurodegenerative

22   disorders, and atheroma [1,6]. Thus, the scavenging of $ONOO^-$ is an important

1 intracellular response to avoid oxidative and nitrosative damage of both plant and

2 animal cells.

3   Betalains are nitrogen-containing water-soluble plant pigments that are found

4 only in plants from 10 families of the order Caryophyllales [7-9]. Betalains can be

5 divided into two major structural groups: betacyanins and betaxanthins (Figure 1).

6 Betacyanins contain a *cyclo*-dopa residue and are red-violet. Betaxanthins contain

7 various amino acid or amine side chains and are yellow. Betalains have been

8 extensively used as natural food colorants in processed meat, ice cream and candies

9 [8,10]. Recently, various studies have reported the antioxidant activity of betacyanins

10 and betaxanthins against reactive oxygen species (ROS), such as the $O_2 \bullet^-$ and H_2O_2

11 [10-12]. This antioxidant activity is similar to ascorbic acid and anthocyanins, which

12 are well known antioxidants *in vitro* and *in vivo* [13,14]. As a result of these data, the

13 effects of betalains have received increased attention. However, there have been no

14 reports of the reactivities of betalains toward RNS. In this study, we investigated the

15 anti-nitrosative activity of beetroot betalain using multiple *in vitro* systems: direct

16 reaction, tyrosine nitration, and DNA strand breaks.

17

18 **Materials and methods**

19 *Preparation of red beet betalains and blueberry anthocyanins*

20   Red beetroots (*Beta vulgaris* L.) were cultivated and harvested in Eniwa-shi,

21 Hokkaido, Japan, and stored at -80 °C until use. Betacyanin and betaxanthin were

22 isolated according to the method reported by Chiji [15]. Defrosted beetroots (400 g)

1   was squeezed with a juicer and filtered through two layers of Kimwipes (Crecia, Japan).

2   The filtrate (200 mL) was centrifuged at 10 000 rpm for 10 min at 4°C, and 120 mL of

3   supernatant was applied to a Dowex 50W-X2 (H$^+$-type, 90 × 80 cm, Wako, Japan)

4   column. After washing with a 2-fold volume of 0.1% HCl, betalain pigments were

5   eluted with an 8-fold volume of RO water. This betalain extract contained both

6   betacyanins and betaxanthins. Betacyanins and betaxanthins were purified using a

7   HITACHI-HPLC system (Japan) with an L-6320 Intelligent pump, an L-2450 UV/VIS

8   Detector, a D-2500 Chromato Integrator and an Inertsil Prep-ODA column (250 × 20

9   mm, GL Sciences Inc., Japan). The elution was performed with 5% (v/v) formic acid

10   and 5% (v/v) acetonitrile in H$_2$O as a solvent at a flow rate of 3 mL min^{-1} and was

11   monitored at 500 nm. The separated betacyanins and betaxanthins were lyophilized and

12   redissolved in a small volume of H$_2$O. The betacyanin fraction included betanin and a

13   less than 10% isobetanin, which estimated from the peak intensities of HPLC analysis.

14   The betaxanthin fraction was deduced to the mixture of vulgaxanthins which have been

15   reported as the main betaxantnin pigments of beetroot [16]. These fractions were used

16   as betanin and betaxantins for subsequent assays. The concentrations of betanin and

17   betaxanthins were estimated using the molar extinction coefficients ε_{538} = 60 000

18   M^{-1}cm^{-1} and ε_{480} = 48 000 M^{-1}cm^{-1}, respectively [17].

19   Fresh blueberries were obtained from a local market in Sapporo and immersed

20   in a 5-fold volume of methanol containing 0.1% HCl for 3 h. The extract was filtered

21   through filter paper and concentrated *in vacuo* at 30°C. To precipitate anthocyanins, a

22   3-fold volume of diethyl ether was added to the concentrated extract. The precipitated

4

1 blueberry anthocyanins were collected and the concentration of anthocyanin was

2 determined using the molar extinction coefficient ε_{538} = 15 600 M^{-1}cm^{-1} for malvidin

3 glycoside, a major anthocyanin in blueberry [18].

4

5 *Peroxynitrite (ONOO$^-$) synthesis*

6   ONOO$^-$ was synthesized by the method described by Patel and DarleyUsmar

7 [19]. Ice-cold 2 M sodium nitrite (10 mL) and acidified 1.85 M hydrogen peroxide (in

8 4.0 M HCl, 10 mL) were mixed by pumping both liquids into a Y-shaped connector.

9 The resulting acidic ONOO$^-$ was immediately quenched with 4.2 M NaOH (10 mL).

10 Excess hydrogen peroxide was removed by adding 1.3 g manganese dioxide (MnO$_2$).

11 MnO$_2$ was removed by centrifugation (4 000 rpm for 5 min at 4°C). The solution was

12 frozen at -80°C; ONOO$^-$ tended to form a yellow top layer due to freeze fractionation,

13 which was scraped for further studies (typical concentrations were 200 –300 mM). The

14 concentration of ONOO$^-$ was determined using the molar extinction coefficient ε_{302} = 1

15 670 M^{-1} cm^{-1}.

16

17 *UV-visible spectral analysis of ONOO$^-$ scavenging activity of betalains*

18   The reactivity of betalains against ONOO$^-$ was measured with a

19 spectrophotometer. The reaction mixture (1 mL total) contained 0.25 mM sodium

20 phosphate buffer (pH 7.4) and 20 μM betanin or betaxanthin, and various

21 concentrations of ONOO$^-$ were added. The absorbance spectra of betanin before and

22 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).

4

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 H_2O at a flow rate of 1 mL min^{-1} for 0–30 min and 3 mL min^{-1} for

11 30–60 min.

12

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

1 subsequent decrease in tyrosine levels. The ONOO⁻ scavenging ability was expressed

2 as the percentage inhibition of the decrease in tyrosine compared to control samples.

3

4 *Inhibition of ONOO⁻-dependent plasmid DNA cleavage*

5   DNA cleavage was analyzed by detecting the conversion of the supercoiled

6 form of pUC19 plasmid DNA (Invitrogen, U.S.A.) to open circular and linear forms [4].

7 DNA (300 ng) was incubated with 0.5 mM ONOO⁻ in the presence or absence of

8 betanin in 50 mM sodium phosphate buffer (pH 7.4) for 5 min at room temperature.

9 After the incubation, the DNA was precipitated in cold ethanol and left at -20°C

10 overnight. The DNA was collected by centrifugation and resuspended in 20 mL of TE

11 buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The forms of plasmid DNA were

12 analyzed by electrophoresis in 1% agarose/TBE gels. After electrophoresis, the gels

13 were stained with 0.5 μg mL^{-1} ethidium bromide for 30 min and then photographed

14 under UV illumination. Signal intensity was calculated with an NIH-image software.

15

16 **Results**

17 *Bleaching of beetroot betalains by* ONOO⁻

18   Betanin isolated from red beet (inc. <10% isobetanin) showed red-purple in

19 neutral pH buffer (pH 7.4); this color was bleached by the addition of ONOO⁻ in 0.5 M

20 NaOH, with a rapid decrease in absorbance at 538 nm (Figure 2A). Subsequently, a

21 new absorbance increase was observed at 350 nm (Figure 2A dashed line). Although

22 betalains are unstable at neutral to alkaline pH, the absorbance changes of betanin and

1   betaxanthin were negligible with the addition of 0.5M NaOH instead of ONOO⁻ during

2   measurement (3 min). Similar bleaching was observed under acidic pH conditions (pH

3   5.0). This bleaching of betanin strongly depended on the ONOO⁻ concentration (Figure

4   2B). Like betanin, the yellow betaxanthins isolated from beetroot exhibited a similar

5   bleaching reaction to ONOO⁻ (Figure 2). These bleaching of betanin and betaxanthins

6   were approximately 90% suppressed in the presence of 10 mM glutathione, which is

7   known to be an ONOO⁻ scavenger.

8

9   *Analysis of betanin and ONOO⁻ reaction products*

10   The reaction mixture was analyzed with reverse phase HPLC by monitoring the

11   photo diode array detector. When monitoring the reaction mixture without ONOO⁻ at

12   500 nm, two peaks were detected at 32.3 and 37.5 min and were identified as betanin

13   and isobetanin, respectively, in accordance with the retention times of each authentic

14   compound (Figure 3A). In the presence of ONOO⁻, the peak of betanin completely

15   disappeared (Figure 3A). Conversely, in the absence of ONOO⁻ treatment, the solution

16   showed no peak at 350 nm; however, there were three peaks (peaks a-c) detected in the

17   ONOO⁻ treatment mixture (Figure 3A). UV/vis absorption spectra of the peaks a, b and

18   c showed absorption maxima at 349 and 504 nm for peak a, 355 nm for peak b, and 345,

19   357, 371 and 395 nm for peak c. The intensities of peaks b and c showed positive

20   correlations with concentrations of ONOO⁻. Several peaks were detected from the peak

21   c fraction as a result of performing HPLC analysis using a different mobile solvent.

22   The observation of several absorption maxima of the peak c fraction supported the

1   assumption that the reaction was a mixture of several compounds. Figure 3B shows the

2   negative correlation between peak intensities of betanin and reaction product. Betanin

3   decreased as the $ONOO^-$ concentration increased and completely disappeared in the

4   presence of 20 molar equivalents of $ONOO^-$. Whereas betanin decreased, the area of

5   peak b increased with a sigmoidal curve toward the $ONOO^-$ concentration. The peak of

6   betanin decreased to less than 30% of the control value when 5 molar equivalents of

7    $ONOO^-$ were added to the reaction mixture. However, product B corresponding

8   compound to the peak b was not detected under these conditions, suggesting that

9   betanin was converted to product B via undetectable intermediates in this study. The

10   molecular weight of product B was estimated to be 358 from LC-TOFMS analysis,

11   which corresponded to decarboxylated and nitrated *cyclo*-dopa cleaved from betanin at

12   the Schiff base.

13

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

15   Exposure of tyrosine to $ONOO^-$ resulted in production of 3-nitrotyrosine, and

16   subsequent decrease in the levels of tyrosine was observed. The half maximal

17   inhibitory concentration (IC_{50}) values of betanin, blueberry anthocyanins and ascorbic

18   acid were 19.2, 13.8 and 79.6 μM, respectively (Figure 4). The betanin IC_{50} was lower

19   than those reported for plant polyphenols, such as chlorogenic acid and caffeic acid

20   (Table 1) [20,21].

21   Many reports have indicated that $ONOO^-$ induces plasmid DNA breakage

22   [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.

9

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 peroxyl

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.

1   Catecholate compounds also scavenge $ONOO^-$, possibly leading to the

2   formation of the corresponding quinones and nitrite [20,27]. Monophenolic compounds

3   become corresponding radicals by one-electron oxidation, and the radicals then react

4   with NO_2 radicals, resulting in the production of nitrated compounds [20,28]. Therefore,

5   when betanin reacts with $ONOO^-$, it is assumed that the following reactions occur.

6    $ONOO^-$ is in equilibrium with ONOOH (pK_a=6.8) (Reaction 1), and ONOOH is

7   decomposed to an •OH and NO_2 radical pair (Reaction 2). Betanin then reacts with

8   •OH, resulting in the generation of a betanin radical by one-electron oxidation (Reaction

9   3). Solvolytic cleavage of betanin then produces CDG• and betalamic acid (Reaction 4).

10   CDG• then reacts with the NO_2 radical, producing nitrated CDG (Reaction 5).

11

12   $ONOO^- + H^+ \rightleftharpoons ONOOH$ (Reaction 1)

13   $ONOOH \rightarrow •OH + NO_2$ radical   (Reaction 2)

14   Betanin + •OH $\rightarrow$ Betanin radical   (Reaction 3)

15   Betanin radical $\rightarrow$ CDG• + Betalamic acid   (Reaction 4)

16   CDG• + NO_2 radical $\rightarrow$ nitrated CDG   (Reaction 5)

17

18   In this study, we detected a decarboxylated and nitrated CDG as a reaction

19   product of betanin with $ONOO^-$ (product B). Product B is likely produced via the

20   assumed reactions (Reactions 1–5), though the decarboxylation mechanism of product

21   B remains unclear. Decarboxylated products during high temperature treatment of

1   betanin have been reported [29,30]. Herbach has proposed that CDG is generated after

2   hydrolytic cleavage of betanin then CDG is decarboxylated[29].   In the reaction of

3   betanin and ONOO,the reaction order of CDG nitration and decarboxylation is not clear

4   yet, However, nitration maybe precedes decarboxylation, because CDG radical is

5   generated by cleavage of betanin (Reaction 4) and generally the reaction between

6   radicals (Reaction 5) very fast. Structural analysis of the products from betalain that

7   react with $ONOO^-$ is necessary to elucidate the betalain $ONOO^-$ scavenging mechanism.

8   Further study using simple chemical models of betalains will be an effective approach

9   to clarify this mechanism.

10   In conclusion, our study demonstrated the effective inhibitory activity of

11 betanin against tyrosine nitration and DNA damage induced by $ONOO^-$, one of the

12 most toxic RNS, indicating that betanin is a strong scavenger against $ONOO^-$

13 comparable to anthocyanin [13]. Because betaxantins was bleached by $ONOO^-$ as well

14 as betanin, it is expected that the $ONOO^-$ scavenging activity would be a common

15 ability of betalain pigments. It has been reported that dietary betalains are incorporated

16 into human blood cells, which may protect these cells from oxidative hemolysis [31].

17 Zou *et al*. also reported that betalains in cactus pear extract participated in growth

18 inhibition in an *in vivo* mouse model of ovarian cancer [32]. According to these reports,

19 it is plausible that dietary betalains can function in human cells as scavengers of ROS

20 and RNS.   Thus, betalains have a great potential in the food and pharmaceutical

21 industries, not only as oxidative stress protectors but also as nitrosative stress protectors.

22

1　**Acknowledgements**

6

7　**Declaration of Interest**

8　　　The authors report no conflicts of interest. The authors alone are responsible

9　for the content of the paper.

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21

1   **Figure legends**

2   Figure 1. Chemical structures of beetroot betacyanins and betaxanthins.

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

4   vulgaxanthin II: R_2 = OH.

5

6   Figure 2. Bleaching of betanin and betaxanthin by the addition of ONOO⁻. The

7   reaction mixture (1 mL total) contained 0.25 M Na-phosphate (pH 7.4) and 20 μM

8   betanin or betaxanthin. The reaction was initiated by addition of various concentrations

9   of ONOO⁻, and the absorbances were measured after a 3-min incubation. A:

10   absorbance spectrum of betanin and betaxanthin before (solid line) and after (dashed

11   line) the addition of 0.3 mM ONOO⁻. B: the dependence of betanin and betaxanthin

12   bleaching on ONOO⁻ concentrations. Data are presented as mean values ± SD (n=3).

13

14   Figure 3. HPLC analysis of the reaction products of betanin and ONOO⁻. The reaction

15   mixture (1 mL total) contained 0.6 M Na-phosphate (pH 7.4) and 0.47 mM betanin. A:

16   HPLC chromatograms of the reaction products. Upper trace, with ONOO⁻ (9.3 mM);

17   lower trace, without ONOO⁻. The arrow indicates isobetanin peak. B: change of the

18   betanin and reaction product (peak b) amounts according to the ONOO⁻ concentration.

19   The inset shows the UV/Vis absorption spectrum of the reaction product (peak b). Data

20   are presented as mean values ± SD (n=3).

21

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.

12

1   Table 1 Concentrations inhibiting 50% (IC_{50}) of $ONOO^-$-dependent L-tyrosine

2   nitration

3

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

4   *present study, [21] Kerry *et al.*, J Neurochem 1999,

5   [20] Pannala *et al.,* Free Radic Biol Med 1998

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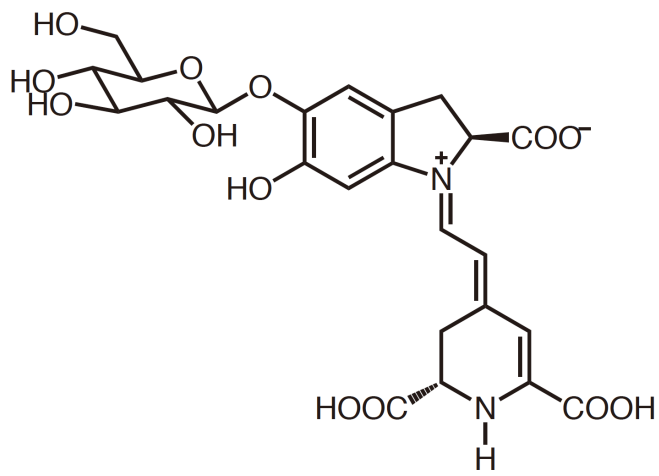
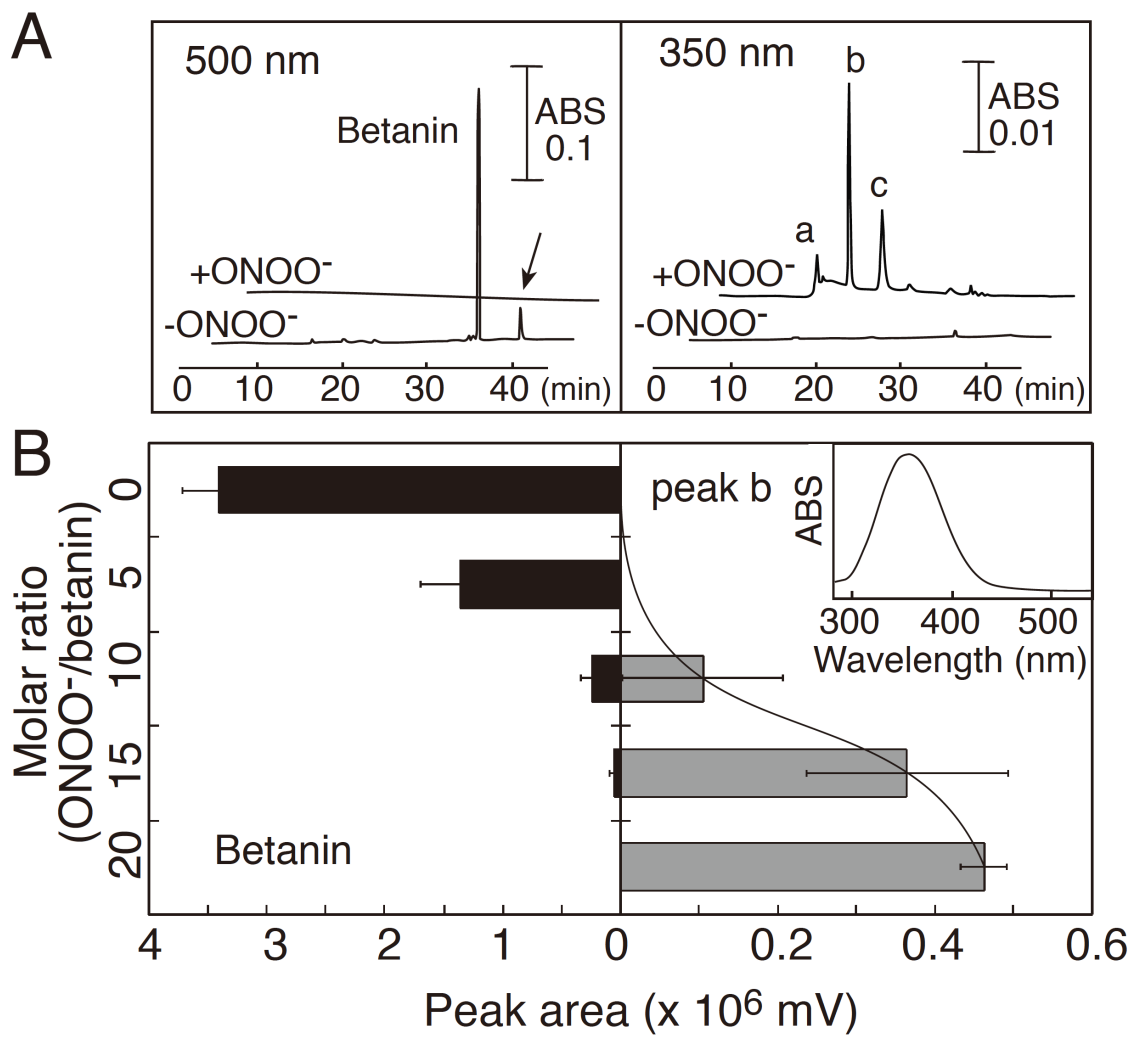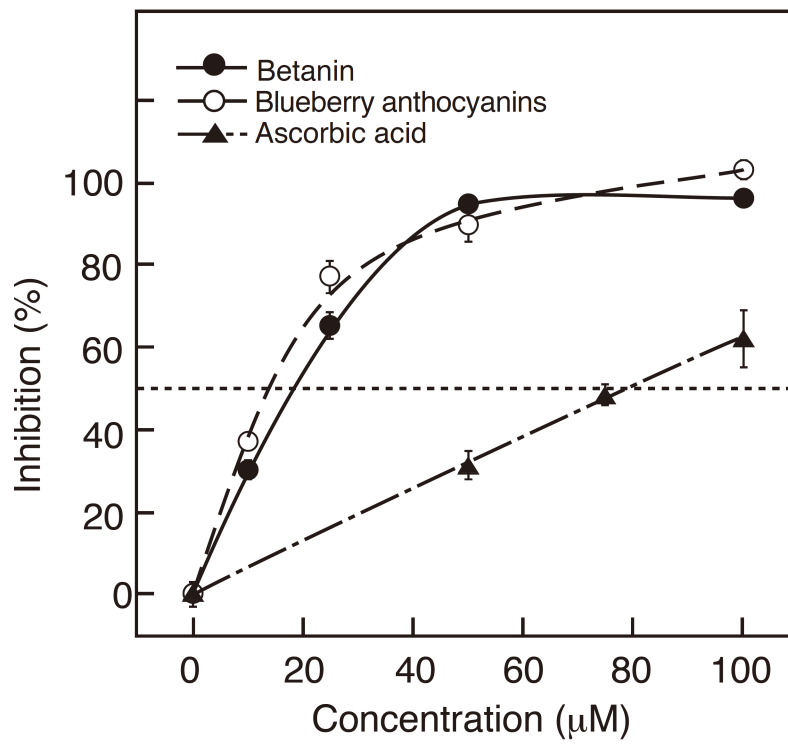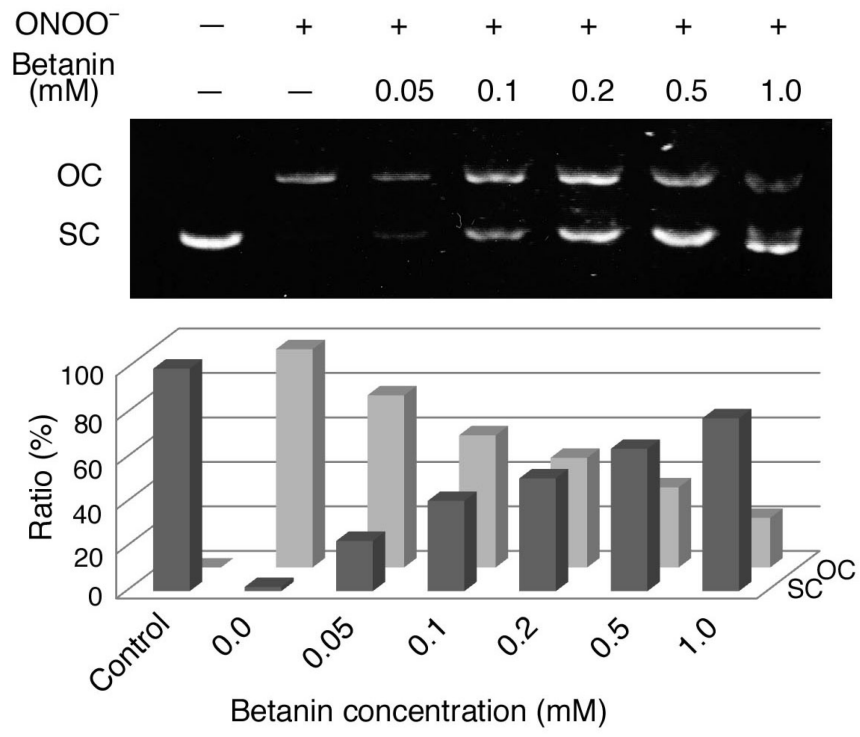Betanin (betacyanin)   Betaxanthin
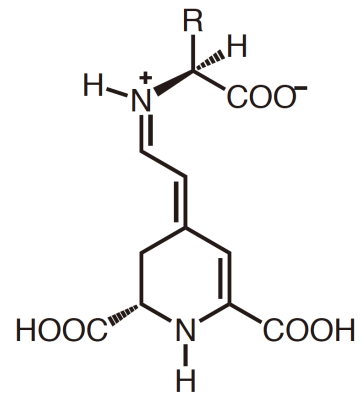
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5