Mechanism for the peroxynitrite scavenging activity by anthocyanins

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Abstract We show that anthocyanins can function as potent inhibitors of the formation of nitrated tyrosine in vitro, and clarified how pelargonidin (Pel), which has a mono-hydroxyl group on the B-ring, can scavenge peroxynitrite ($ONOO^-$) by detection of the reaction products. Pel was reacted with $ONOO^-$, then the reaction mixture was analyzed using high-performance liquid chromatography (HPLC). The HPLC analyses showed two novel peaks assumed to be the reaction products. Based on the instrumental analyses, the reaction products were identified as *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid. Pel can protect tyrosine from undergoing nitration through the formation of *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid.

Key words: Anthocyanin; Nitric oxide; Peroxynitrite; 3-Nitrotyrosine; Antioxidant

1. Introduction

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, vegetables and red wine [1], suggesting that we ingest a significant amount of anthocyanins in our plant-based daily diets. There is now an increasing interest in the in vivo protective function of natural antioxidants contained in dietary plants against oxidative damage caused by free radical species [2,3]. The antioxidant activity of phenolic phytochemicals has been widely investigated [4,5]. Anthocyanins, which are included in the phenolic phytochemicals, are stable under acidic conditions but not stable and rapidly decomposed under neutral conditions [6]. Based on this background, anthocyanins have not been recognized as a physiological antioxidant [6]. However, we demonstrated in an in vitro and in vivo study that anthocyanins have antioxidative activity under physiological conditions [7–10]. We also recently reported how cyanidin 3-*O*-β-D-glucoside (C3G), which is a typical anthocyanin, had significant antioxidant activity, is absorbed and metabolized in vivo using rats [11].

Nitric oxide (NO) is one of the free radicals produced in vivo. It plays an important role in physiological function, but it is also associated with the pathogenesis of inflammatory diseases. NO can react with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$), and the formed $ONOO^-$ is a powerful oxidant and induces various oxidative damages. 3-Nitrotyrosine (NT) is generated when $ONOO^-$ is added to tyrosine or proteins containing tyrosine residues under physiological conditions. Recently, Eiserich et al. reported that nitrite (NO_2^-) promotes tyrosine nitration through formation of nitryl chloride (NO_2Cl) by the reaction of hypochlorous acid (HOCl) or myeloperoxidase [12–14].

NT has been detected in various diseases using specific monoclonal and polyclonal antibodies that recognize NT in proteins [15–17]. NT was also detected in atherosclerotic lesions of human coronary arteries using immunohistochemical detection, indicating that oxidants derived from NO are generated in human atherosclerosis and are involved in its pathogenesis [15,18]. Therefore, it is important to inhibit this protein nitration. Some phenolic antioxidants have been effective in reducing the formation of NT [19–22]. Recent reports showed that the phenolic compounds contained in red wine may play an important role as inhibitors of low-density lipoprotein (LDL) oxidation [23], and result in anti-atherosclerotic activity. Red wine contains a large amount of anthocyanins [24], indicating that anthocyanins may contribute to the inhibitory effect of the oxidation of LDL. However, there have been no prior studies how anthocyanins react with $ONOO^-$ and showed scavenging activity.

In the present study, we demonstrated the mechanism for the scavenging activity of $ONOO^-$ by pelargonidin (Pel), which is one of the typical anthocyanidins, due to the formation of *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid by reacting with $ONOO^-$.

2. Materials and methods

2.1. Materials and chemicals

Pel was obtained from Extrasynthèse (Genay, France), and its purity was greater than 99%. The chemical structure of Pel is shown in Fig. 1. $ONOO^-$ was synthesized and quantified prior to use as previously described [25]. Briefly, an acidic solution (0.6 M HCl containing 0.7 M H_2O_2) was mixed with 0.6 M $NaNO_2$ in a cold bath, then 1.5 M NaOH was immediately added to the solution. The solution was frozen at −20°C and the yellowish top layer was collected. The concentration was determined by ultraviolet (UV) absorbance spectroscopy at 302 nm in 1.2 M NaOH ($\varepsilon_{302\,nm} = 1670\ M^{-1}\ cm^{-1}$) [26]. Antiserum against anti-3-NT was obtained from a rabbit as previously described [19].

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Abbreviations: C3G, cyanidin 3-*O*-β-D-glucoside; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MS, mass spectrometry; NO, nitric oxide; NT, 3-nitrotyrosine; $ONOO^-$, peroxynitrite; Pel, pelargonidin; UV, ultraviolet

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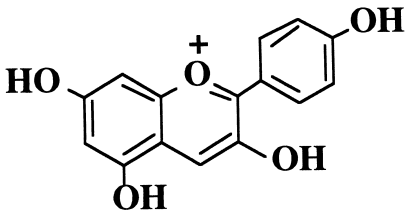
2.2. Reaction conditions and identification of reaction products of Pel with ONOO⁻

$ONOO^-$ (0–3 mM) was added to Pel (0.5 mM) in 0.1 M phosphate buffer (pH 7.4) and then immediately mixed. Decomposed $ONOO^-$ was prepared by adding the $ONOO^-$ to the buffer and kept at room temperature for 10 min. Pel was then added to the decomposed reaction mixture with continuous mixing. After that, phosphoric acid (20 μl) was added to the mixture to avoid pH dependent degradation of Pel. The identification of the reaction products was performed by UV spectrum and retention time using the photodiode array detector, and high-performance liquid chromatography-mass spectrometry (HPLC-MS). To confirm the UV spectrum, HPLC connected to a photodiode array detector (MD-910, Jasco, Tokyo, Japan) was performed on a Develosil ODS-HG-5 column (Nomura Chemical Co., Ltd., Aichi, Japan, 4.6 mm×250 mm) using 25% acetonitrile in water containing 0.01% acetic acid as the solvent at a flow rate of 1.0 ml/min. For HPLC-MS, an aliquot of the reaction mixture was applied to the HPLC system, which was connected to a mass spectrometer (PLATFORM II, Fisons Instruments, UK) on a Develosil ODS-HG-5 column (4.6 mm×250 mm) using 25% acetonitrile in water containing 0.01% acetic acid as the solvent. The electrospray ionization (ESP) mode (negative) was used for the detection.

3. Results and discussion

Although the data were not shown, anthocyanins significantly inhibited the nitration of LDL-determined enzyme-linked immunosorbent assay using anti-NT antibody. To clarify the inhibitory mechanism of the anthocyanins, anthocyanin was reacted with $ONOO^-$, and the reaction products were then analyzed. Previous studies showed that mono-phenolic groups such as *p*-coumaric acid, genistein and daidzein were nitrated by $ONOO^-$ [19,20,27]. On the other hand, nitration is not seen with the catecholates, caffeic acid and chlorogenic acid [21,28]. They are converted into *o*-quinone derivatives. However, there have been no prior studies on the reactions of anthocyanins with $ONOO^-$. Among the anthocyanins tested, Pel has the simplest chemical structure in the tested pigments, and it was easy to analyze the reaction products based on a preliminary experiment. Therefore, we focused on Pel, which has a mono-hydroxyl moiety on the B-ring, and the reaction products of Pel with $ONOO^-$ were then analyzed.

Fig. 2A shows the HPLC chromatogram of the reaction mixture of Pel (0.5 mM) with $ONOO^-$ (2 mM). The reaction of Pel with $ONOO^-$ resulted in the formation of two novel products (P-1: retention time = 5.8 min, and P-2: retention time = 12.3 min) which were assumed to be the reaction products. Some minor peaks were detected in the chromatograms of the reaction mixture of Pel with decomposed $ONOO^-$ (data not shown). However, these peaks were quite different from that of the reaction products with $ONOO^-$. Therefore, P-1 and P-2 are derived from the reaction of Pel with



Pelargonidin (Pel)

Fig. 1. Chemical structure of Pel.

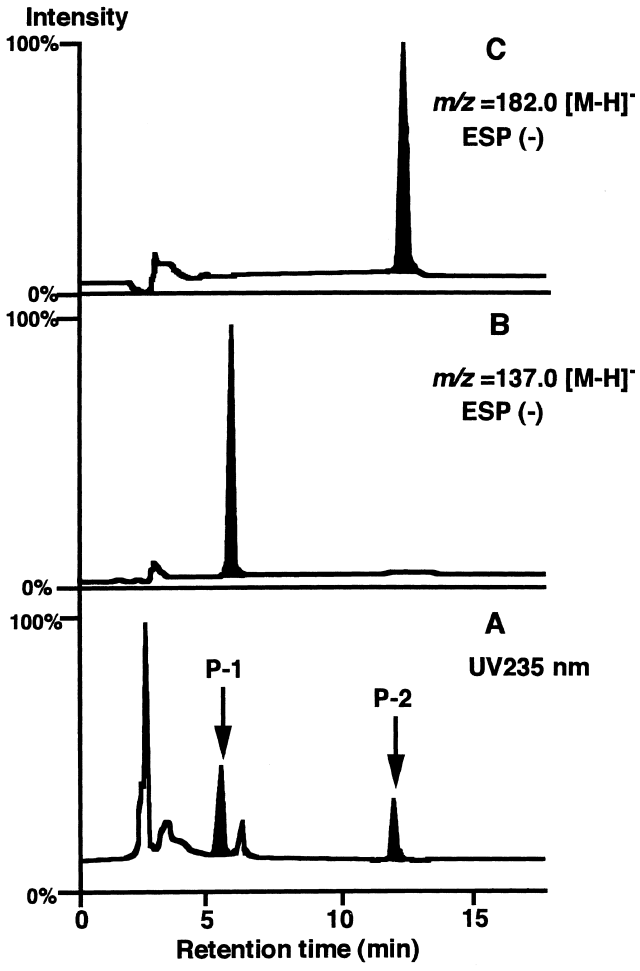


Fig. 2. HPLC-MS analysis for the reaction products of Pel with $ONOO^-$. The aliquot of the reaction mixture was applied to the HPLC system connected to an MS using 25% acetonitrile in water containing 0.01% acetic acid as the solvent. (A) The elution was monitored at UV 235 nm. (B) The elution was scanned in the ESP mode by *m/z* 137.0 as the $[M\text{-}H]^-$ of *p*-hydroxybenzoic acid. (C) The elution was scanned in the ESP mode by *m/z* 182.0 as the $[M\text{-}H]^-$ of 4-hydroxy-3-nitrobenzoic acid.

$ONOO^-$. P-1 and P-2 showed UV spectral data λ_{max} values at 255, and at 235 and 343 nm, respectively (data not shown). P-1 and P-2 had corresponding signals at $m/z = 137.0$ and 182.0 based on HPLC-MS (Fig. 2B,C). Considering these instrumental results, P-1 and P-2 could be tentatively postulated as *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid, respectively. To confirm these chemicals, instrumental analyses of authentic *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid were performed and compared with the P-1 and P-2 results. The data of the authentic compounds (retention time in the HPLC analysis, UV spectrum, and HPLC-MS) were in complete agreement with those of P-1 and P-2. Therefore, P-1 was identified as *p*-hydroxybenzoic acid and P-2 was identified as 4-hydroxy-3-nitrobenzoic acid.

Fig. 3 shows the amount of the reaction products and unreacted Pel as the concentration of $ONOO^-$ increased. Pel was rapidly decreased and completely broken down when the concentration of $ONOO^-$ was over 2 mM. The formation of *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid was increased as the concentration of $ONOO^-$ was increased.

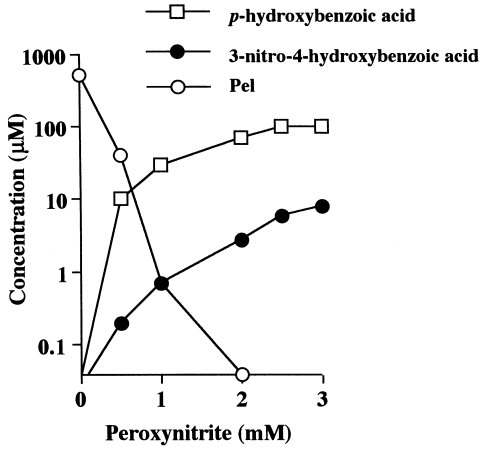


Fig. 3. Degradation of Pel and formation of *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acid when the concentration of $ONOO^-$ was changed from 0 to 3 mM. Values are means ± S.D. of three determinations.

p-Hydroxybenzoic acid was increased up to 2 mM of $ONOO^-$, then it was not significantly increased over 2 mM of $ONOO^-$. However, the formation of 4-hydroxy-3-nitrobenzoic acid was linear over the range of 0–3 mM of $ONOO^-$. These results suggest that 4-hydroxy-3-nitrobenzoic acid was formed after cleavage of Pel and formation of *p*-hydroxybenzoic acid by $ONOO^-$.

Fig. 4 shows the proposed mechanism for the inhibition of protein nitration by Pel. There are two possible steps by which Pel can inhibit the $ONOO^-$-mediated nitration of tyrosine. At first, Pel is broken by $ONOO^-$, and *p*-hydroxybenzoic acid is then formed. In the second step, *p*-hydroxybenzoic acid is reacted with $ONOO^-$, which results in the formation of 4-hydroxy-3-nitrobenzoic acid. Namely, not only Pel itself but the decomposed Pel, *p*-hydroxybenzoic acid, can protect tyrosine from undergoing nitration through the formation of 4-hydroxy-3-nitrobenzoic acid.

In conclusion, we have shown that anthocyanins can function as a potent inhibitor of the formation of nitrated tyrosine in vitro, and clarified how Pel, which has one hydroxyl group on the B-ring, scavenges $ONOO^-$ by detection of the nitrated reaction products (4-hydroxy-3-nitrobenzoic acid). We recently demonstrated that C3G, which is one of the typical anthocyanins, has antioxidant activity and protective effects against hepatic ischemia-reperfusion injury in vivo [9,10]. C3G has *o*-dihydroxyl moieties on the B-ring, suggesting that it shows a different mechanism from Pel to protect against the $ONOO^-$-mediated nitration of tyrosine. The inhibitory effects of anthocyanins on the formation of tyrosine nitration and the detection of the reaction products in an in vivo system are now in progress.

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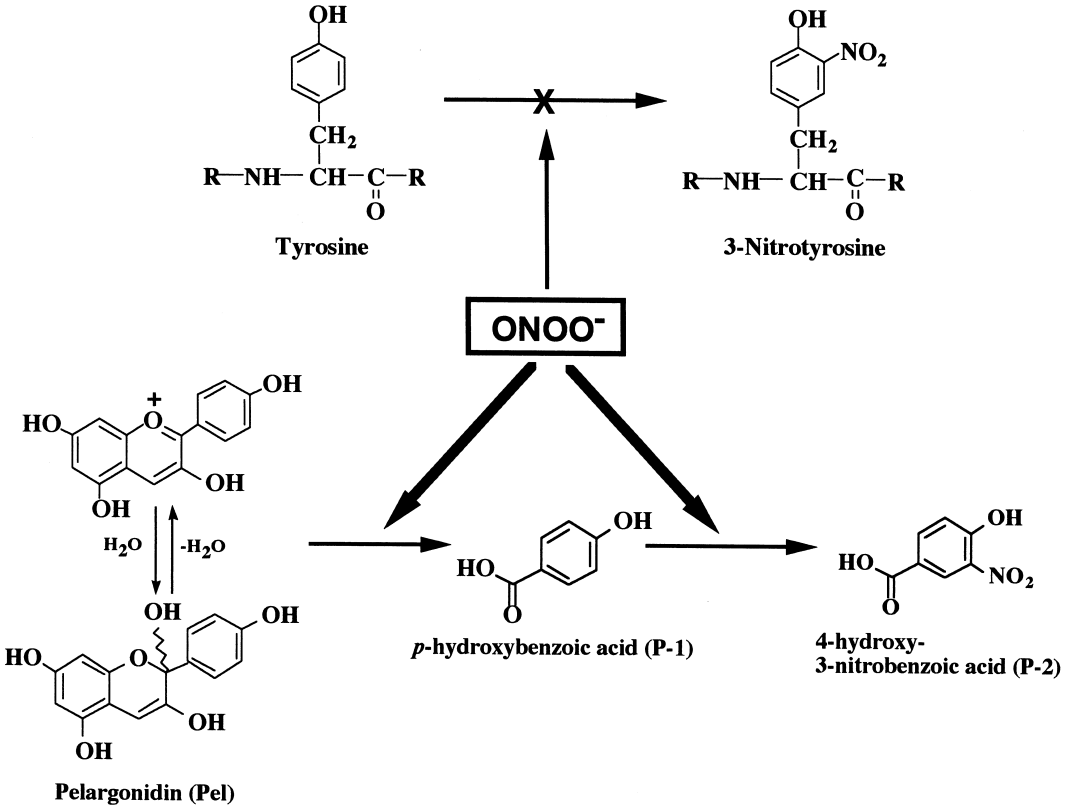

Fig. 4. Proposed mechanism for the inhibition of $ONOO^-$-induced protein nitration by Pel.

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