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Graphical Abstract

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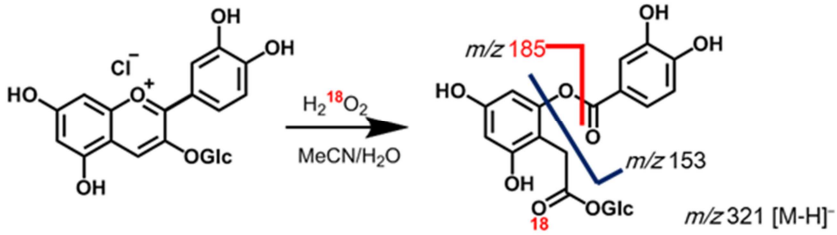

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ABSTRACT

The oxidation of cyanidin-3-*O*-glucoside by hydrogen peroxide was investigated in a range of solvents. The reaction products had chemical structures identical to those formed by the reaction of this compound with the alkylperoxyl radical 2,2′-azobis(2,4-dimethyl)valeronitrile. A plausible oxidation mechanism was proposed based on the obtained reaction products, and this mechanism was confirmed by HPLC–MS experiments using ^{18}O-labeled reagents. Further, the reaction conditions were found to influence both the reaction rate and the products formed during the transformation, which validated the proposed mechanism.

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1. Introduction

Anthocyanins, which are widely distributed in the plant kingdom, are responsible for the red, blue, and violet colors of the flowers and fruits of various plants. Structurally, anthocyanins are the sugar-containing analogs of anthocyanidin aglycones, which contain a characteristic flavylium (2-phenylchromenium) cation. In addition, as a type of polyphenol, anthocyanins are known to exhibit antioxidant properties.[1–3]

Reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and superoxide anion radicals are widely present in the human body. The excessive generation of reactive oxygen species is believed to cause damage to proteins, lipids, carbohydrates, and DNA. One of the key roles of anthocyanins in plants is the protection against oxidative damage. For example, it has been reported that the concentration of anthocyanins in plant tissues is enhanced under oxidative conditions to protect the plants from oxidation.[4,5] In the case of the human body, the generation of reactive oxygen species is believed to be a factor in the development of various diseases, including cancer and heart disease. It has been reported that the presence of dietary polyphenols such as anthocyanins can defend cells from oxidative damage.[6,7]

Although various reports have discussed the oxidation reactions of anthocyanins,[8–13] the details of their radical scavenging mechanism have not yet been revealed. In our previous study, we investigated the reaction of cyanidin-3-*O*-glucoside (**1**) with the alkylperoxyl radical 2,2′-azobis(2,4-dimethyl)valeronitrile (AMVN) and proposed an oxidation mechanism based on the chemical structures of the obtained products.[14] In this study, we investigated the reaction of **1** with H_2O_2 and hydroxyl radicals in detail and identified the oxidation products (Fig. 1). In addition, the reaction of **1** with H_2O_2 was examined in a range of solvents and the effect of the reaction conditions on the reactivity of this system was determined. Finally, we proposed and confirmed a plausible mechanism for the oxidation of **1** under H_2O_2 conditions.

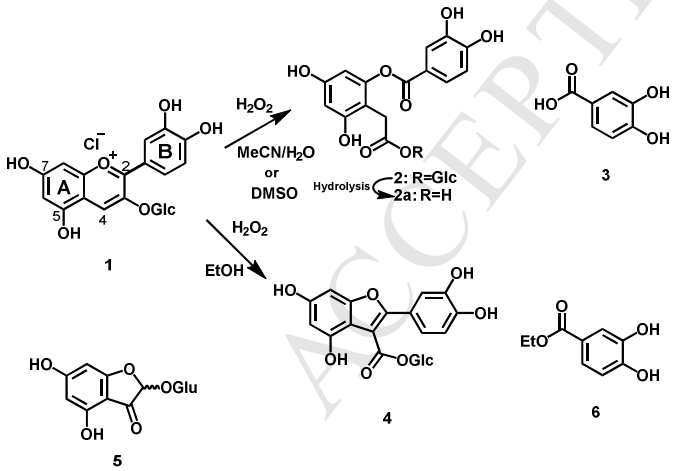

Fig. 1. Oxidation of **1** in various solvents under H_2O_2 conditions.

2. Results and discussion

*2.1. Reaction of **1** with H_2O_2*

Anthocyanins are known to exhibit radical scavenging ability, as mentioned in Section 1. To clarify the radical scavenging mechanism, the reaction of **1** with H_2O_2, a reactive oxygen species, was investigated. Compound **1** was treated with H_2O_2 in 50 mM phosphate buffer (pH 6.8), and the reaction mixture was analyzed using HPLC. Although the amount of compound **1** was observed to decrease, no product peaks were detected. The same result was obtained in phosphate buffer without H_2O_2. Anthocyanins are known to be less stable under neutral and basic conditions, and the obtained results suggest that the reaction of **1** with H_2O_2 cannot be evaluated under these conditions. Therefore, the reaction of **1** with H_2O_2 was performed under acidic conditions (pH 2.4), in which anthocyanins are expected to be stable. Under these conditions, the HPLC signal corresponding to **1** decreased significantly, and the appearance of three new peaks was observed. Additionally, the area of these peaks increased when an 80% aqueous solution of acetonitrile (MeCN) was used instead of water (Fig. 2A).

The obtained compounds were isolated by preparative HPLC and analyzed by NMR spectroscopy and MS. As a result, the reaction products were identified as protocatechuic acid (**3**, 9% yield), which was derived from the B-ring, and compound **2** and its aglycone **2a** (57% yield), which were produced by opening the C-ring (Fig. 1). The partial conversion of compound **2** to its aglycone **2a** during the concentration process was attributed to the facile hydrolysis of the glucose ester under acidic conditions. These three compounds were identical to those reported previously in the reaction of **1** with AMVN (Fig. 2B).[14] In contrast, the reaction of **1** with H_2O_2 in ethanol (EtOH) produced compound **4** as the main product. The formation of this product occurred through a rearrangement reaction accompanied by ring contraction, and this product was also obtained in the reaction of **1** with AMVN in EtOH (Fig. 1).

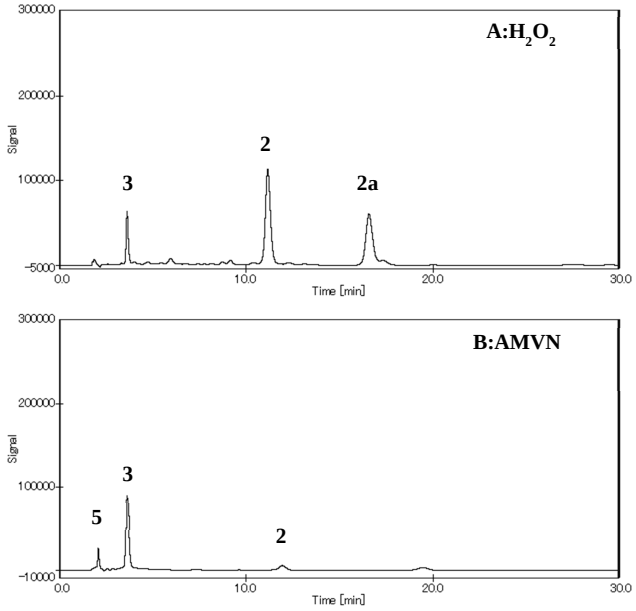

Fig. 2. HPLC chromatograms of the products formed in the reaction of **1** with (A) H_2O_2 in H_2O and (B) AMVN in MeCN/H_2O.

It has been reported that the alkyl radical generated by heating AMVN reacts with oxygen to yield an alkyl peroxide radical,[15] indicating that the reaction with AMVN does not involve the direct reaction of an alkyl radical. Interestingly, when **1** was treated with H_2O_2 in an 80% aqueous solution of MeCN, compound **5**, which was produced in 15% yield in the reaction with AMVN,[14] was not observed. It should be noted that the difference between the chemical structures of compounds **5** and **2** is the position of oxidation, as compound **5** is formed by oxidation at the 4-position of **1**, whereas **2** is produced by oxidation at the 3-position. Considering the reaction mechanism,

oxidation at the 4-position of compound **1** requires either the loss of the aromaticity of the C-ring or ring opening owing to the nucleophilic addition of water at the 2-position. Conversely, it is assumed that oxidation at the 3-position of **1** occurs when the aromaticity of the C-ring is maintained. Thus, compound **5** was not formed in the presence of H_2O_2 because the rate of oxidation was higher than that of the nucleophilic addition of water to the 2-position owing to the greater reactivity of H_2O_2. This result is consistent with compound **1** being consumed faster in the presence of H_2O_2 than in the AMVN system.

*2.2. Reaction of **1** with hydroxyl radicals*

Among reactive oxygen species, hydroxyl radicals exhibit the highest oxidative ability. They are produced from H_2O_2 under UV irradiation conditions or by the Fenton reaction. To investigate differences from the reaction under H_2O_2 conditions, **1** was reacted with hydroxyl radicals produced by the Fenton reaction. First, to confirm the production of hydroxyl radicals, a qualitative analysis was performed using the ESR spin-trapping technique. Following the addition of H_2O_2 to a $FeSO_4 \cdot 7H_2O$/diethylenetriaminepentaacetic acid (DTPA) aqueous solution containing 2-(5,5-dimethyl-2-oxo-2λ5-[1,3,2]dioxaphosphinan-2-yl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (CYPMPO), an ESR signal corresponding to the CYPMPO–hydroxyl radical adduct (a spin adduct of the hydroxyl radical) was detected. Although the quantities were slightly lower than that in water, hydroxyl radicals were also observed in 80% MeCN and 80% EtOH aqueous solutions under the same conditions, confirming the generation of hydroxyl radicals under these conditions. In contrast, no signal was detected in the presence of **1** under the same conditions (Fig. S1). This result suggests that the generated hydroxyl radicals were trapped by **1**.

Next, compound **1** was treated under the Fenton reaction conditions in acidic water (pH 2.4), and the solution was analyzed using HPLC. Under these conditions, the HPLC signal corresponding to **1** decreased and several new peaks appeared. The areas of these peaks increased when an 80% aqueous solution of MeCN was used instead of water. However, the chromatographic pattern observed for this reaction solution was the same as that observed for the reaction under H_2O_2 conditions, as described in Section 2.1. Thus, both reactions are assumed to produce the same products. This result suggests that the products generated by the treatment of **1** under Fenton reaction conditions were the same as those obtained by the reaction of **1** with H_2O_2, not with hydroxyl radicals.

*2.3. Effect of solvent on the reaction of **1** with H_2O_2*

To determine the effect of different solvents on the reactivity of **1** with H_2O_2, the quantity of **1** in the reaction mixture was monitored by HPLC. The transformations were conducted under acidic conditions to eliminate the effects of degradation reactions not caused by H_2O_2. As shown in Fig. 3A, the greatest decrease in the concentration of **1** was observed when the reaction was performed in EtOH, with no HPLC signals corresponding to **1** observed after 2 h. In contrast, the lowest reactivity was observed in 80% aqueous MeCN, with ~30% of **1** remaining after 4 h. The reactivity of **1** in the various solvents followed the order EtOH > EtOH/H_2O > DMSO = DMF = H_2O > MeCN/H_2O. The oxidation of **1** by H_2O_2 under acidic conditions was presumed to involve a bimolecular nucleophilic substitution reaction (S_N2 reaction) that occurs *via* the formation of $H_3O_2^+$, which is generated by the protonation of H_2O_2.[18] This species forms easily in acidic H_2O_2 solutions and is extremely reactive, indicating that the reaction is dominated by diffusion control.[16,17] Furthermore, in the context of protonation, competition exists between H_2O_2 and the reaction solvent. The pK_a values (measured in water) for the conjugate acids of the various solvents are EtOH (−2.4) < DMSO (−1.8) < H_2O (−1.7).[18–20] However, the inverse order was observed for the reaction rate. As solvent protonation is difficult for solvents with low conjugate acid pK_a values, the protonation of H_2O_2 is expected to be promoted. In fact, a high reaction rate was observed when the reaction was conducted in EtOH, which is the solvent with the lowest conjugate acid pK_a value. In addition, this rate decreased upon the addition of water. Furthermore, little difference in reactivity was observed between DMSO and H_2O, which have similar conjugate acid pK_a values. These results suggest a direct relationship between the pK_a value and solvent reactivity in this system. However, although the conjugate acid pK_a value of MeCN was the lowest among the various solvents examined (i.e., −10),[20] the reaction rate in MeCN/H_2O was the lowest observed and inconsistent results were obtained. It should be noted that owing to the insolubility of compound **1** in MeCN, 20% water was added. Under these conditions, the reactivity was likely altered by the addition of water, which affected solvent protonation. As the pK_a value of the conjugate acid of MeCN is significantly smaller than that of water, the protonation of H_2O_2 is favored over MeCN, indicating that the generation of $H_3O_2^+$ species is affected by the addition of water.

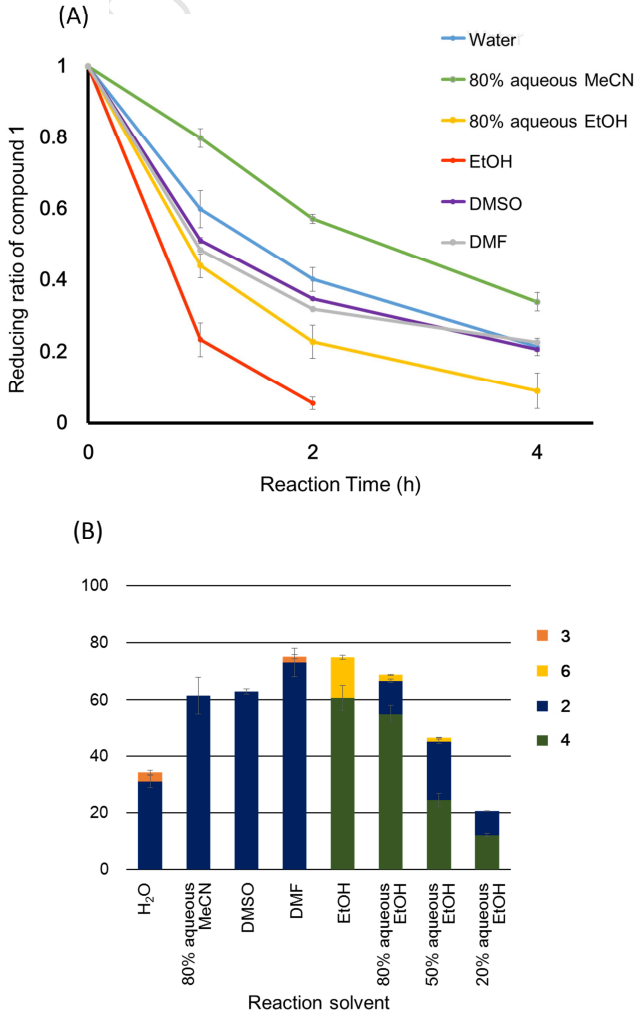

Fig. 3. Influence of reaction solvent on reactivity and product yield. (A) Reduction in the content of compound **1**. (B) Product yields of compounds **2**, **3**, **4**, and **6** in various reaction solvents. The yields of compounds **2** and **4** include their aglycone yields.

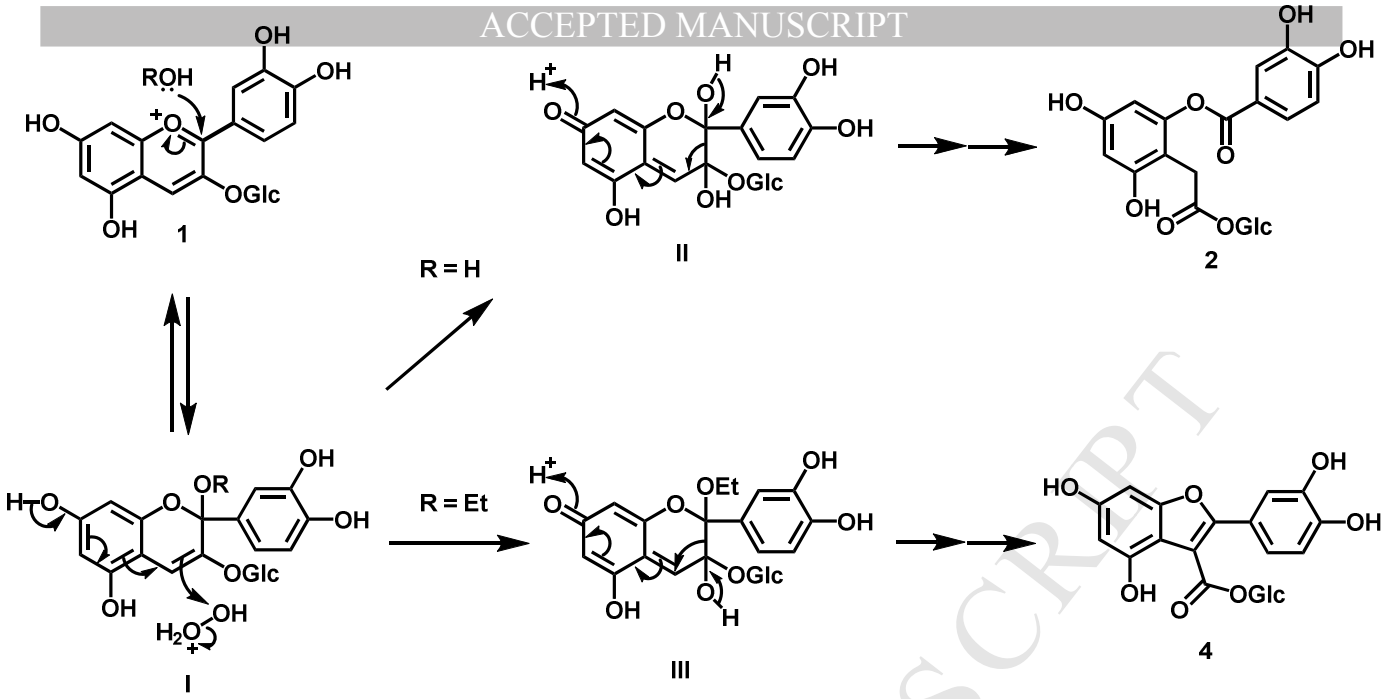

Fig. 4. Hypothetical oxidation mechanism for compound **1**.

2.4. Effect of solvent on the reaction products

The effect of the solvent on the product selectivity of the reaction between H_2O_2 and **1** was examined using EtOH, EtOH/H_2O, 80% aqueous MeCN, DMSO, and DMF (Fig. 3B). Upon addition of H_2O_2, compound **2** was obtained as the major product when aqueous MeCN, DMSO, and DMF were used, whereas compound **4** was obtained as the major product in addition to trace amounts of compound **6** when EtOH was used. In the EtOH/H_2O solvent system, the production of compounds **3** and **4** was inhibited as the water ratio increased, whereas the formation of compound **2** was promoted.

With the exception of the water-based systems, a total yield of 60–75% was obtained for compounds **2**, **3**, and **4**. However, upon increasing the water content in the EtOH/H_2O system, this yield decreased sharply. We expect this to be due to the protic nature and high polarity of water, which likely complicates the reaction by promoting nucleophilic addition to the product and the reaction intermediate.

Additionally, the generation of ethyl protocatechuate (**6**) from the B-ring was observed in EtOH, although the amount formed decreased with increasing water content. The formation of trace amounts of protocatechuic acid (**3**) was also observed under all solvent conditions examined. Initially, we assumed that compound **3** was generated *via* the hydrolysis of compound **2**, as this product was formed in aqueous solution at pH 4. However, compound **3** was generated when the reaction mixture obtained by reacting **1** with H_2O_2 in the presence of water (and containing compounds **2**, **3**, and **4** in low yields) was treated under strongly oxidizing conditions (data not shown). This result suggests that compound **3** was generated from **1** *via* a more complex route.

2.5. Confirmation of the proposed oxidation mechanism

A mechanism for the oxidation of **1** by H_2O_2 was then proposed based on the chemical structures of the reaction products, as outlined in Fig. 4. To confirm the proposed mechanism, **1** was oxidized using $H_2{}^{18}O$ or $H_2{}^{18}O_2$ and the reaction products were analyzed using negative ion mode UPLC–ESI-MS/MS (Fig. S2).

Compound $\mathbf{2_A}$, obtained by treatment of **1** with H_2O_2 in MeCN/$H_2{}^{18}O$, gave a pseudo molecular ion peak ($[M-H]^-$) at *m/z* 321, which is 2 Da higher than the original signal and confirms the incorporation of the ^{18}O atom. To identify the position of the ^{18}O oxygen atom, MS^2 analysis at *m/z* 321 was performed. In this case, two major fragment ions were observed at *m/z* 155 and 183. By comparison with the MS^2 data of compound **2**, the fragment ion peak at *m/z* 155 was identified as ^{18}O-protocatechuic acid, suggesting that the carbonyl oxygen atom at the 2-position of the C-ring was derived from $H_2{}^{18}O$ (Fig. 4). Anthocyanins are known to exist as mixtures of several structures in chemical equilibrium because of reversible hydration at the 2-position. The production of compound $\mathbf{2_A}$, which has ^{18}O at the 2-position of the C-ring, was presumed to occur through this hydration reaction.

In contrast, upon treatment of **1** with $H_2{}^{18}O_2$ in MeCN/H_2O, compound $\mathbf{2_B}$ with a pseudo molecular ion peak ($[M-H]^-$) at *m/z* 321 was observed. In this case, the MS^2 analysis suggested that the carbonyl oxygen atom at the 3-position of the C-ring was derived from $H_2{}^{18}O_2$. Furthermore, treatment of compound **1** with $H_2{}^{18}O_2$ in EtOH yielded compound **5** bearing an ^{18}O-label on the carbonyl moiety at the 4-position of the C-ring. Oxidation of tautomeric species I occurs at the 3-position owing to electron donation from the phenolic hydroxyl group at the 5- or 7-position of the A-ring, resulting in the formation of species II (Fig. 4). In MeCN/H_2O, species II is converted to **2** by cleavage of the C2–C3 bond, driven by aromatization of the A-ring. The production of $\mathbf{2_B}$, in which the carbonyl oxygen at the 3-position is labeled, supports this mechanism. In EtOH, oxidation of species I, which is formed by the nucleophilic addition of EtOH at the 2-position, generates species III in the same manner. Species III is converted to **4** by a rearrangement reaction.

3. Conclusion

Our investigation of the reaction of **1** with H_2O_2 revealed that the reaction products were identical to those formed in the reaction with AMVN. A potential mechanism for this transformation, proposed based on the chemical structures of the

obtained products, was confirmed by UPLC-MS/MS experiments using ^{18}O-labeled reagents. This mechanism was further validated by observing the influence of various reaction conditions on the reaction rate and product distribution.

As H_2O_2 is a reactive oxygen species that is present in many biological systems, it is likely that the degradation products formed by reactions between anthocyanins and H_2O_2 are also present in anthocyanin-containing foods. However, the potential benefits of these products to human health remain under debate. In addition, as the contents of these products in various plant species are expected to increase post-harvest, they could potentially be used to determine the freshness of anthocyanin-containing fruits and vegetables. Furthermore, although anthocyanins are important plant-derived pigments, they are rather unstable. We therefore expect that the results presented herein will aid in the development of novel methods for improving the stability of the anthocyanin structure. Further studies on anthocyanin stability are now in progress.

4. Experimental section

4.1. General

Cyanidin-3-*O*-glucoside (**1**) was isolated from a black bean extract, which was a kind gift from Nagara Science Co. (Gifu, Japan), and recrystallized from a mixture of 5–10% HCl in methanol (MeOH).[21,22] H_2O_2 solution (30% (w/w) in H_2O, 9.8 M) and all organic solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

ESR analysis was performed using a JEOL JES-FA100 spectrometer (Tokyo, Japan). The spin-trapping reagent CYPMPO was purchased from Shidai Systems (Saitama, Japan). NMR spectra were recorded on a JEOL ECA-500 or a JEOL ECA-600 instrument (Tokyo, Japan) using TMS as an internal standard. UPLC–ESI-MS was performed using a Waters Xevo G2 QTOF mass spectrometer (Waters, Milford, MA, USA) equipped with a C_{18} analytical column (ACQUITY UPLC BEH C_{18} column, 2.1 mm id × 100 mm; Waters, Milford, MA, USA). The mobile phase was composed of 0.5 vol% acetic acid, 10 vol% aqueous MeCN, or 15 vol% aqueous MeCN. All analyses were conducted at 35 °C, and the flow rate was set at 0.2 mL/min. HPLC analyses were performed using a JASCO PU-2089 intelligent pump equipped with a JASCO MD-2010 PDA detector and a JASCO CO-2065 column oven (Tokyo, Japan). A COSMOSIL 5C_{18}-MS-II column (4.6 mm id × 150 mm; Nacalai Tesque Inc., Kyoto, Japan) and a NB-ODS-9 column (10 mm id × 250 mm; Nagara Science Co., Ltd., Gifu, Japan) were used for analytical and preparative HPLC, respectively.

*4.2. Reaction of **1** with H_2O_2 in 80% aqueous MeCN*

Compound **1** (30.0 mg) was dissolved in 80% aqueous MeCN (6 mL) prior to the addition of aqueous H_2O_2 (24.5 mM, 3 mL) that had been diluted 400 times using 80% aqueous MeCN. The resulting solution was stirred at 40 °C for 8 h. Subsequently, the reaction mixture was cooled to room temperature, MeCN was removed under reduced pressure, and the resulting residue was subjected to column chromatography using HP20SS resin (Mitsubishi Chemical Co., Tokyo, Japan). Elution was achieved using MeOH after washing with two column volumes of water to remove H_2O_2. The sample was purified by HPLC (column: NB-ODS-9, 10 mm id × 250 mm) using MeCN/H_2O (10:90 v/v) containing 0.5% trifluoroacetic acid (TFA) to yield compounds **2** (57%) and **3** (9%). The chemical structure of compound **2** was confirmed by comparison with literature NMR data.[14]

4.3. ESR spectra

To an aqueous solution of $FeSO_4 \cdot 7H_2O$/DTPA (1 mM/1 mM, 100 µL) was added water, 80% MeCN/H_2O, or 80% EtOH/H_2O (100 µL) and CYPMPO aqueous solution (310 mM, 10 µL). The spectra were recorded in a 25 µL glass capillary 15 min after mixing. Typically, the instrumental settings were as follows: center field, 326.4 mT; sweep width, ±7.5 mT; field modulation frequency, 100 kHz; field modulation width, 0.2 mT; amplitude, 500; sweep time, 1 min; time constant, 0.03 s; microwave frequency, 9163.310 MHz; and microwave power, 0.998 mW. The ESR spectrum of manganese held in the ESR cavity was used as an internal standard.

*4.4. Comparison of the reactivity of **1** in various solvents*

To a solution of compound **1** (1 mg) in H_2O (200 µL, 10 mM) was added aqueous H_2O_2 (100 µL), which was prepared from 30% aqueous H_2O_2 by diluting 400 times with H_2O. The resulting solution was stirred at 40 °C and HPLC analyses were performed after 0, 1, 2, and 4 h. The HPLC analysis conditions were as follows: COSMOSIL 5C_{18}-MS-II (4.6 mm id × 150 mm; Nacalai Tesque Inc., Kyoto, Japan); mobile phase, 0.5% TFA/12% aqueous MeCN; flow rate, 1.0 mL/min; system temperature, 35 °C; and detection wavelength, 520 nm. The reaction was monitored using the peak area of compound **1**. The above reaction was repeated using DMSO, DMF, EtOH, and MeCN/H_2O as the solvent instead of H_2O.

4.5. Confirmation of the oxidation mechanism using $H_2{}^{18}O$ or $H_2{}^{18}O_2$

To confirm the proposed oxidation mechanism, the reaction was performed according to the procedure described in Section 4.4 using $H_2{}^{18}O$ or $H_2{}^{18}O_2$ instead of H_2O or H_2O_2, respectively. All reaction mixtures were analyzed by UPLC–TOF-MS. For the analysis of compound **2**, the mobile phase was composed of 0.5% formic acid/10% MeCN in H_2O, whereas for the analysis of compound **4**, 0.5% formic acid/15% MeCN in H_2O was used. The flow rate was set at 0.2 mL/min and the ESI interface was operated in negative ion mode. The MS parameters for the analysis were as follows: capillary potential, 0.6 kV; sampling cone, 13 V; desolvation temperature, 500 °C; source temperature, 150 °C; desolvation gas flow, 1000 L Ar/h; and cone gas flow, 50 L Ar/h. The mass spectrometer was operated in MS/MS mode with a collision cell energy of 13 V.

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Declarations of interest: none

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/

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ACCEPTED MANUSCRIPT

Highlights

The hydrogen-peroxide-mediated oxidation of cyanidin-3-*O*-glucoside was investigated.

The effect of solvent on the reaction rate and product distribution was examined.

A plausible mechanism for oxidation under hydrogen peroxide conditions was proposed.

The reaction mechanism was validated by UPLC-MS/MS using ^{18}O-labeled reagents.