Isolation and Characterization of a Novel Flavonoid Possessing a 4,2'-Glycosidic Linkage from Green Mature Acerola (Malpighia emarginata DC.) Fruit

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The novel flavonoid, leucocyanidin-3-O-D-glucoside, possessing a 4,2'-glycosidic linkage was isolated from green mature acerola (Malpighia emarginata DC.) puree and given the trivial name “aceronidin.” To examine the functions of aceronidin, its antioxidative activity and both its α-glucosidase and α-amylase inhibition activities, as a potential inhibitor of the sugar catabolic enzyme, were evaluated against those of taxifolin, catechin, isoquercitrin and quercitrin which each have a similar structure. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching activity of aceronidin was stronger than that of α-tocopherol and comparable to that of flavonoids. In the yeast α-glucosidase inhibitory assay, aceronidin showed significantly greater inhibition than the other flavonoids tested. In the human salivary α-amylase inhibitory assay, aceronidin showed inhibition activity. Taken together, these results indicate aceronidin to be a potent antioxidant that may be valuable as an inhibitor of sugar catabolic enzymes.

Key words: acerola; flavonoid; glycosidic linkage; antioxidative; sugar catabolic enzyme inhibition

Polyphenols, which are ubiquitous components of plants, are well known to have antioxidative properties. Many plant constituents have recently been evaluated for such predicted functions as antioxidative activity, apoptosis induction and some enzyme inhibition abilities.1–4) Acerola, which originally comes from a West Indian island in the Atlantic ocean, is a fruit containing a large amount of vitamin C and abundant fructose and malic acid, and has high nutritional value, making acerola fruit very beneficial to human health. Pino and Marbot have analyzed the volatile flavor constituents of acerola fruit by GC–MS, and elucidated the components of acerola fruit.5) In terms of function, it has been reported that an acerola extract had an inhibitory effect on NO production in mouse macrophage-like cells and an antitumor effect against lung cancer.6,7) Hanamura et al. have also reported the isolation of several flavonoids from red mature acerola fruit and their functional characterization.8)

After acerola fruit has completely matured to about 2 cm in diameter, its color gradually changes from green to bright red in about 4 days. It has been confirmed that this change in color is caused by an increase in the flavonoids, cyanidin-3-rhamnoside (C3R) and pelargonidin-3-rhamnoside (P3R), that are not detectable in green mature acerola fruit. On the basis of these results, it has been clarified that an alteration in part of the polyphenol constituents takes place as acerola fruit ripens.

Green mature acerola fruit contains a higher amount of vitamin C than red mature fruit (data not shown). Although there are few studies on its polyphenol composition, we have confirmed some polyphenols, quercetin (quercetin-3-rhamnoside) and p-coumaric acid by HPLC, its absorption spectrum and TLC analysis. We isolated in this present study a polyphenol to investigate other polyphenols contained in green mature acerola fruit and clarified a novel flavonoid from the result of an NMR analysis. We report here the isolation of this distinctive flavonoid from a puree of green mature acerola fruit and the evaluation of its functions in vitro, including its antioxidative activity against both DPPH radicals and linoleic acid, and its inhibitory activity against both α-glucosidase and α-amylase.

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance spectroscopy; ESI-MS, electrospray ionization mass spectrometry; IR, infrared spectroscopy; CD, circular dichroism; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DEPT, distortionless enhancement by polarization transfer; DQF-COSY, double quantum filtered correlation spectroscopy; HSQC, heteronuclear single quantum coherence; NOE, nuclear overhauser effect; HMBC, heteronuclear multiple bond correlation
Materials and Methods

**Samples.** The green mature acerola (*Malpighia emarginata* DC.) puree was provided by Nichirei do Brazil Agricola (Recife, Brazil) and immediately stored at −24 °C until needed.

**Chemicals.** DPPH was purchased from Nacalai Tesque (Kyoto, Japan). Taxifolin, quercetin, catechin, α-glucosidase and α-amylase were purchased from Sigma (St. Louis, MO, USA). Isoqueretin was purchased from Kanto Chemical Co. (Tokyo, Japan), and α-tocopherol was purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Isolation and purification of a novel flavonoid from green mature acerola puree.** The supernatant of the green mature acerola puree (44 kg) was concentrated by evaporation and mixed with Sepabeads SP70 (500 g; Mitsubishi Chemical Corp., Japan) by the batch method. The Sepabeads SP70 resin was then extensively washed with distilled water. The adsorbed extract was eluted with 4000 ml of 10% (v/v) ethanol (EtOH). After evaporation (150 g), the eluted fraction was passed through an ODS-A column (300 g, 40 mm i.d. × 500 mm; YMC, Japan), extensively washed with distilled water and successively eluted with 2500 ml of 10% (v/v) methanol (MeOH) and 2000 ml of 20% (v/v) MeOH. The resulting evaporated fraction (2.47 g) was successively washed with 2500 ml of 10% (v/v) methanol (MeOH) and 2000 ml of 20% (v/v) MeOH. The resulting evaporated fraction (2.47 g) was passed through a Sephadex LH-20 column (130 g, 2.5 cm i.d. × 100 cm; GE healthcare, USA), eluting with 1000 ml of MeOH at a flow rate of 1.0 ml/min. Each fraction (15 ml) was analyzed by TLC (1-butanol:AcOH:H2O at 4:1:5) and reversed-phase HPLC (Inertsil ODS-3 column, 4.6 mm i.d. × 250 mm; GL Sciences, Japan) to ascertain those containing the target flavonoid. HPLC was run at 35 °C and at a flow rate of 1.0 ml/min with a linear gradient of MeOH for 55 min from 5% to 100% MeOH with monitoring at 280 nm. The relevant fractions were pooled and freeze-dried (0.14 g). The target flavonoid was finally crystallized by leaving it to stand at 4 °C in an H2O solution (8.8 mg).

**Structural determination.** The high-resolution ESI-MS data was recorded with an LCT instrument (Waters, USA). Leucine-enkephalin was used as an internal standard. The NMR spectrum was obtained with a UNITY INOVA 500 instrument (Varian, USA). TMS was used as an internal standard, and the isolated flavonoid was measured in CD3OD. The IR spectrum was determined with an FTS-135 spectrometer (Bio-Rad, USA), and the CD spectrum was measured with a JASCO J-820 circular dichroism spectrometer. The specific rotation was measured with a SEPA-300 instrument (Horiba, Kyoto, Japan).

**Isolated flavonoid.** UV−vis $\lambda_{max}$ 278 nm (MeOH); high-resolution ESI-MS: $m/z$ 473.1064 ([M + Na]+, 473.1060 calcd. for C21H22O11Na); $^1$H- and $^{13}$C-NMR data; see Table 1. IR (KBr): 3402, 3314, 2959, 2883, 1633, 1612, 1123 cm$^{-1}$; CD (MeOH) nm (θ): 300 (−55.7926), 290 (394.613), 280 (2714.6), 270 (1366.88), 260 (553.825), 250 (198.409); [α]$D_{25}^\text{20}$ 46.86° (c 1.03, MeOH).

**Assay for DPPH radical quenching activity of flavonoids.** The DPPH radical quenching activity was measured by the method of Shimada et al. with some modifications. In brief, each reaction solution consisted of a 100 mM acetate buffer (pH 5.5), the flavonoid sample in MeOH and 100 μM DPPH in EtOH. The reaction was started by adding a DPPH solution and, after incubating for 30 min at 30 °C, the absorbance was measured by a UV-2400PC spectrometer ( Shimadzu, Kyoto, Japan) at 517 nm. The EC50 value was calculated by using MeOH as a control.

**Assay for linoleic acid autoxidation inhibitory activity.** The antioxidative activity against the autoxidation of linoleic acid was measured by the method of Osaka and Namiki with some modifications. In brief, each assay solution containing 0.5% linoleic acid, 40% (v/v) EtOH and a 20 mM phosphate buffer (pH 7.0) was divided between two glass bottles with screw caps, one being kept at 40 °C and the other of 4 °C for 3 weeks. An aliquot of each solution was taken out and mixed with 75% (v/v) EtOH, a 30% (w/v) ammonium thiocyanate solution and 20 mM FeCl3 solution containing 3.5% (v/v) HCl. After precisely 3 min, the absorbance at 500 nm was measured with a spectrometer. The ΔOD500 value was calculated from the difference between OD500 of the sample kept at 40 °C and that kept at 4 °C.

**Assay for α-glucosidase inhibitory activity.** The α-glucosidase inhibition assay was done according to the method of Kim et al. with some modifications. Each reaction solution consisted of 0.3 units/ml of α-glucosidase from *Saccharomyces cerevisiae* in a 10 mM phosphate buffer containing 0.2% BSA (pH 7.0), a 100 μM flavonoid sample in 10% (v/v) DMSO and 5.0 mM p-nitrophenyl-α-D-glucopyranoside in a 100 mM phosphate buffer (pH 7.0). The enzyme reaction was started by adding the substrate solution and, after incubating for 10 min at 30 °C, the absorbance at 405 nm was measured with a microplate reader (model 550, Bio-Rad, USA). The inhibition rate of α-glucosidase was calculated by using a 10% DMSO solution as the control.

**Assay for α-amylase inhibitory activity.** The α-amylase inhibition assay was carried out according to the method of Kim et al. with some modifications. Each reaction solution consisted of 0.3 units/μl of α-amylase from human saliva in a 10 mM phosphate buffer containing 0.2% BSA (pH 7.0), a 333 or 167 μM flavonoid sample in 20% (v/v) DMSO and 1.67 mM 4-nitrophenyl-α-D-penta-(1→4)-glucopyranoside in a 100 mM phosphate buffer (pH 7.0). The enzyme reaction was started...
amylase was calculated by using a 20% DMSO solution to be \( \frac{1}{2} \) signal was observed at \( m \). Results

\[ /C_{14} \]

\[ 60 \text{ min at } 30^\circ \text{C} \]

by adding the substrate solution and, after incubating for 60 min at 30 \(^\circ\)C, the absorbance at 405 nm was measured with a microplate reader. The inhibition rate of \( M. K. \text{AWAGUCHI} \)

thus suggested that the molecular formula was \( C_{138} \) mg of a colorless flavonoid was isolated from 44 kg of green mature acerola. From the results of HPLC and TLC, by treating the puree with Sepabeads SP70 and ionization with MeOH at a flow rate of 1.0 ml/min then led to the partial structure of \( \text{–CH (5.31 ppm)–CH (4.87 ppm)–} \). Furthermore, 4.25 ppm (J = 11.0 Hz, J = 3.4 Hz) determined the axial-axial form between H-1" and H-2". The signal at 3.27 Hz, \( J = 9.5 \) Hz, \( J = 8.4 \) Hz (dd)

by adding the substrate solution and, after incubating for 60 min at 30 \(^\circ\)C, the absorbance at 405 nm was measured with a microplate reader. The inhibition rate of \( \alpha \)-amylase was calculated by using a 20% DMSO solution as the control.

Results

Isolation and structural determination of a novel flavonoid from green mature acerola puree

We have speculated that some flavonoids are present in green mature acerola from the results of HPLC and TLC. By treating the puree with Sepabeads SP70 and an ODS-A column, the component flavonoids were substantially purified and a proportion of these flavonoids with UV absorption at 280 nm was subsequently fractionated by stepwise elution. Sephadex LH-20 separation with MeOH at a flow rate of 1.0 ml/min then led to the selective isolation of the target flavonoid. As a result, 8.8 mg of a colorless flavonoid was isolated from 44 kg of the green mature acerola puree.

In the high-resolution ESI-MS data, the [M + Na]+ signal was observed at m/z 473.1064 (Fig. 1). It was thus suggested that the molecular formula was \( C_{52}H_{22}O_{12} \). A signal observed at m/z 923.2102 was speculated to be [2M + Na]+. Details of the chemical structure of this flavonoid were established from \(^1\)H- and \(^13\)C-NMR measurements (Table 1). The \(^1\)H-NMR spectrum indicated that the isolated compound contained a 1, 2, 4-trisubstituted (6.79, 6.84 and 6.92 ppm) and a 1, 2, 3, 5-tetrasubstituted (5.8 and 5.99 ppm) benzene. In the \(^13\)C-NMR spectrum, twenty-one signals were observed, this agreeing with the result from the high-resolution ESI-MS data measurements. Twelve signals appeared in the benzene ring region, six signals in the sugar region and three signals in another region. The DQF-COSY spectrum led to the partial structure of –CH (5.31 ppm)–CH (4.25 ppm)–CH (4.87 ppm)–CH (4.87 ppm)–CH (4.87 ppm)–CH (4.87 ppm)–CH (4.87 ppm)–CH (4.87 ppm)–. Furthermore, 4.25 ppm (J = 11.0 Hz, J = 3.4 Hz) and 4.87 ppm (J = 3.4 Hz) \(^1\)H-NMR chemical shifts and the HSQC spectral data indicated a flavan-3,4-diol skeleton.

Data in the \(^1\)H-NMR spectrum show that sugar protons were in a high magnetic field (3.27–4.63 ppm). The signal at 4.63 ppm was attributed to C-1", and its coupling constant (J = 7.9 Hz) determined the axial-axial form between H-1" and H-2". The signal at 3.27 ppm was attributed to H-2' and H-3'.
The antioxidative activity of aceronidin, α-tocopherol natural antioxidant, and the related flavonoids, taxifolin, catechin, isoquercitrin and quercitrin, was examined by a DPPH radical quenching system (Fig. 3). The DPPH radical quenching activity of aceronidin was stronger than that of taxifolin, catechin, isoquercitrin and quercitrin. In addition, the hydrolysis of this flavonoid with trifluoroacetic acid produced D-glucose. These results enabled the glycoside to be determined as β-D-glucose.

The HMBC signals showed C-1′, C-2′ and C-4 to be respectively correlated with H-3, H-4 and H-2′. It was thus suggested that there were two glycosidic linkages between C-3 and C-1′ and between C-4 and C-2′. NOEs were observed between H-2′ and H-4, between H-3 and H-4 and between H-2 and H-1′. The molecular structure of the compound shown in Fig. 2 was determined from these results. This flavonoid, possessing two glycosidic linkages between C-3 and C-1′ and between C-4 and C-2′, was identified as a novel flavonoid by this investigation and we have therefore named it “aceronidin.”

**DPPH radical quenching activity of aceronidin**

The antioxidative activity of aceronidin, α-tocopherol, and the other flavonoids, as compared with the other flavonoids and was thus inferred to be an effective α-glucosidase inhibitor.

**Inhibitory effect on α-glucosidase**

The inhibitory effect of aceronidin on yeast α-glucosidase is shown in Fig. 5. At a concentration of 100 μM, aceronidin exhibited significant inhibitory activity as compared with the other flavonoids and was thus inferred to be an effective α-glucosidase inhibitor.

**Inhibitory effect on α-amylase**

The inhibitory activity of aceronidin on human salivary α-amylase is shown in Fig. 6. The inhibition rates for aceronidin were 24.5% and 34.0% at 167 μM and 333 μM, respectively (IC₅₀ value of 820 μM). These results confirm that aceronidin had α-amylase inhibitory properties. However, it showed weak inhibitory activity as compared with isoquercitrin and quercitrin.

**Discussion**

We isolated in this study a novel flavonoid (named aceronidin) from green mature acerola puree and evaluated its functions of antioxidative activity and sugar catabolic enzyme inhibitory activity. Acoronidin,
whose structure was determined for the first time, is a relatively minor component in green acerola fruit.

We confirmed that aceronidin had antioxidative activity equivalent to that of the other flavonoids tested in assays for DPPH radical quenching and inhibition of linoleic acid autoxidation (Table 2 and Fig. 4). These two types of assay revealed aceronidin to exhibit antioxidative activity against water- and lipid-soluble substances. The antioxidative intensity of a flavonoid is attributed to a 2,3-double bond, which possesses a catechol group in the B-ring and a 3-hydroxyl group and glycoside. Due to its glycosidic moiety and its 2,3-single bond, we expected that aceronidin would not have strong antioxidative activity in comparison to the other flavonoids tested.

In our /C11-glucosidase assay, aceronidin showed the strongest inhibitory effect among the five flavonoids tested (Fig. 5). /C11-Glucosidase inhibition activity has been reported for several polyphenols. It was assumed from these findings that both the glycoside site and the aglycon site of the flavonoid would function in some roles of the enzyme. It is therefore postulated for aceronidin that a specific structure must participate in /C11-glucosidase inhibition and additionally enhance its inhibition activity.

Concerning its inhibitory effect against /C11-amylase, the activities of luteolin and ellagitannin have been reported, although the details remain to be elucidated. The result of this study showed that aceronidin had low inhibitory activity (Fig. 6), suggesting that the discriminative structure of aceronidin was not involved in the level of its inhibitory effect against /C11-amylase.

In conclusion, we isolated the novel flavonoid, aceronidin, from green mature acerola puree. Acroronidin showed antioxidative activity, as well as inhibitory activity against both /C11-glucosidase and /C11-amylase. The inhibition of /C11-glucosidase and /C11-amylase would lead to a retardation of carbohydrate digestion. These results therefore indicate that aceronidin might be a potent antioxidant and might have potential value as an inhibitor of sugar catabolic enzymes.
References


