Isolation and characterization of a cyanidin-catechin pigment from adzuki bean (Vigna angularis)

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A B S T R A C T

Adzuki bean is used to prepare many kinds of foods in east Asia, and the seed coat contains water-soluble anthocyanins, catechins, and flavonols. In the present study, ethyl acetate-soluble purplish pigments were isolated from adzuki bean. Pigments of soaked adzuki bean were extracted with 1% HCl in methanol. Ethyl acetate-soluble purple pigments were obtained from the methanol soluble components. Purple pigments 1 and 2 were purified from the ethyl acetate-soluble pigments by Sephadex LH-20 column chromatography and preparative reversed-phase HPLC. NMR and mass spectra suggested that pigment 1 was a condensation product of cyanidin and (+)-catechin, in which 5-hydroxy and C-4 positions of the cyanidin moiety were substituted by the addition of 5-hydroxy and C-6 positions of the (+)-catechin moiety, respectively. Pigment 2 was an isomer of pigment 1. It is suggested that pigments 1 and 2 contribute to the purpurish-red colour of foods prepared using adzuki bean.

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1. Introduction

Adzuki bean, Vigna angularis (Willd.) Ohwi et Ohashi, is used for various dishes in east Asia. The paste prepared from adzuki bean is an important ingredient for Japanese and Chinese sweets, the colour of which is purpurish-red or dark purple. In addition, adzuki bean is cooked together with glutinous rice to prepare purplish-red rice in Japan. The preparation of purplish-red rice is traditional in many lucky events, such as festivals or birthdays. As pigments of adzuki bean paste and glutinous rice, anthocyanins are in the seed coat of which is purplish-red or dark purple. In addition, adzuki beans are an important ingredient for Japanese and Chinese sweets. The paste prepared from adzuki bean is used for various dishes in east Asia. The preparation of purplish-red rice is tradition-
the isolated purple pigment is a novel compound that contains cyanidin and (+)-catechin moieties in the molecule, and it is discussed that the pigment could contribute to make foods purplish-red, when cooked with adzuki bean.

2. Materials and methods

2.1. Plant materials and reagents

Adzuki bean (cultivar Erimoshouz) was obtained from Kawakami Co., Ltd. (Kitakyushu, Japan). Dimethylsulfoxide (DMSO)-d₆ and tetramethylsilane were from Sigma–Aldrich Japan Co. LLC (Tokyo, Japan). Quercetin and all other reagents were from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

2.2. Extraction of purple pigments

Water was added to 900 g of dried adzuki bean to make the total volume of 3 L, and then left at room temperature (about 25 °C) overnight. After removing water by decantation, pigments of soaked bean were extracted with 1.5 L of 1% HCl in methanol for 1–2 h (Fig. 1). The extract (about 1.2 L) was concentrated to 400–450 ml at ca. 40 °C using a rotary evaporator. The concentrated solution was filtered through a filter paper under reduced pressure. The filtrate was extracted 3 times with 200 ml of ethyl acetate. The ethyl acetate extracts were combined, and then washed 3 times with 200 ml of 0.1% HCl in water to make the colour of ethyl acetate layer turn purple by removing water-soluble reddish pigments. Petroleum ether (100 ml) was added to the purple ethyl acetate solution (about 500 ml), and then the mixture of petroleum ether and ethyl acetate was washed 3 times again with 200 ml of 0.1% HCl in water.

The purple precipitate obtained by filtration was extracted with 100 ml of ethyl acetate for 2 h at room temperature (Fig. 1). Petroleum ether (25 ml) was added to the purplish ethyl acetate extract, and then the mixture of ethyl acetate and petroleum ether was washed 3 times with 100 ml of 0.1% HCl in water.

Purple organic solutions, which were prepared from the filtrate and the precipitate as described above, were combined and dehydrated with anhydrous sodium sulfate. The above extraction process (Step 1 in Fig. 1) was repeated twice.

2.3. Column chromatography and preparative HPLC

The purple residue, which was obtained from the dehydrated organic solution, was dissolved in 2 ml of methanol. To prepare a purple fraction from the methanol solution, column chromatography was carried out on a Sephadex LH-20 column (30 × 2 cm i. d.). The eluent used was methanol. After evaporation of methanol of the purple fraction, the residue was dissolved in 8 ml of a mixture of methanol and 25 mM KH₂PO₄ (2:1, v/v). Two purple pigments were isolated from the solution by preparative HPLC as described below.

Preparative HPLC was performed using a Shim-pack PREP-ODS(H) kit (25 cm × 20 mm i. d.; particle size, 5 µm; pore size, 10 nm) (Shimadzu, Kyoto, Japan) combined with a 1 ml sample loop. The mobile phase was a mixture of methanol and 25 mM KH₂PO₄ (2:1, v/v) and the flow rate was 9 ml min⁻¹. Components separated by the preparative HPLC were detected at 210, 360, and 570 nm using a spectrophotometric detector with a photodiode array (SPD-M10Avp; Shimadzu). Peaks 1 and 2 had retention times of about 19 and 25 min, respectively (Fig. 2, top). Ultraviolet–visible (UV–vis) absorption spectrum of the component of peak 1 (pigment 1) had an absorption maximum at 568 nm in the

![Fig. 1. Isolation processes for pigments 1 and 2 from adzuki bean. For details, see text. MeOH, methanol; EtOAc, ethyl acetate.](image)

![Fig. 2. Preparative HPLC. (Top) HPLC profile at 360 nm. Sample for HPLC analysis was prepared using Sephadex LH-20 column chromatography. (Bottom) Absorption spectra of peaks a (quercetin), 1 (pigment 1), and 2 (pigment 2) in the mobile phase for preparative HPLC.](image)
mobile phase, and the spectrum was the same as that of the component of peak 2 (pigment 2) (Fig. 2, bottom). The fraction of each peak was collected, and the pigments were extracted from the mobile phase with ethyl acetate after removing methanol.

The purple residue was obtained from each ethyl acetate extract after evaporating the solvent. Each residue was dissolved in 8 ml of a mixture of acetonitrile and 25 mM KH$_2$PO$_4$ (1:2, v/v) to further purify pigments 1 and 2 using the above HPLC column. The mobile phase was a mixture of acetonitrile and 25 mM KH$_2$PO$_4$ (2:1, v/v), and its flow rate was 9 ml min$^{-1}$. Purified pigments 1 and 2 (retention time, about 17 and 23 min, respectively) were extracted after removing acetonitrile from the mobile phase. The residue obtained from each ethyl acetate extract was dissolved in 1 ml of 1% HCl in ethanol, and then water was added to each acidic ethanol solution to make a final volume of 10 ml. A purple precipitate was generated after each solution was left in the dark at 4°C overnight. The precipitate was collected by centrifugation (5,000g, 3 min), washed with 10 ml of water by centrifugation, and then lyophilized. From 1.8 kg of dried adzuki bean, 2.6 mg of pigment 1 and 0.6 mg of pigment 2 were obtained. Pigment 1 was used for structural analysis.

2.4. Analytical HPLC and liquid chromatography-mass spectra (LC-MS)

The purity of isolated pigments 1 and 2 was examined using a Shim-pack CLC-ODS column (15 × 6 mm i. d.) (Shimadzu) combined with the above spectrophotometric detector with a photodiode array. The mobile phase was a mixture of methanol and 25 mM KH$_2$PO$_4$ (3.2 or 2:1, v/v) and the flow rate was 1 ml min$^{-1}$. Electrospay ionisation (ESI) and atmosphere-pressure chemical ionisation (APCI) mass spectra were obtained with a LCMS QP8000x quadrupole mass spectrometer (Shimadzu). LC was done with a TSKgel-ODS 80TS column (15 cm × 2 mm i. d.) (Tosoh, Tokyo, Japan). The mobile phase was a 15-min linear gradient from 65% to 100% methanol (containing 0.2% formic acid) and the flow rate was 0.2 ml min$^{-1}$. Positive-ion mass spectra were obtained at an ESI or APCI prove voltage of +4.5 kV.

2.5. Nuclear magnetic resonance (NMR) spectra

$^1$H and $^{13}$C NMR spectra were recorded at room temperature with an ECX-400P FT-NMR spectrometer (JEOL, Ltd., Tokyo, Japan) with DMSO$_d_6$ as the solvent and tetramethylsilane as the internal standard. $^1$H NMR was performed at 399.78 MHz, and the $^1$H-$^1$H chemical shift correlated (COSY) technique was employed to assign $^1$H shifts and couplings. $^{13}$C NMR was at 100.53 MHz with proton decoupling. Heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC) techniques were used to assign correlations between $^1$H and $^{13}$C signals.

2.6. Presence of pigment 1 in boiled adzuki bean

Water was added to 30 g of adzuki bean to make the total volume of 100 ml, and then boiled for 20 min. After cooling, adzuki bean was separated from the brownish-red boiling water. As pigment 1 was extractable from the water solution by the use of ethyl acetate (see above) and the direct extraction of the pigment by ethyl acetate avoided its acid hydrolysis, both the boiled adzuki bean (43 g) and the boiling water fraction (about 60 ml, pH 5.9) were extracted with 30 and 50 ml of ethyl acetate, respectively. The boiling enhanced the release of pigment 1 from adzuki bean and increased the extractability of pigment 1 by ethyl acetate from the adzuki bean. Each residue obtained after evaporating ethyl acetate was dissolved in 0.5 ml of a mixture of methanol and 25 mM KH$_2$PO$_4$ (2:1, v/v), and analysed by the above analytical HPLC systems.

2.7. Spectrophotometric studies

Effects of organic solvent and pH on UV–vis absorption spectra of pigment 1 were studied using a spectrophotometer (UV-2450, Shimadzu) equipped with an integrating sphere (ISR-240A, Shimadzu). The path length of the measuring beam was 4 mm. Isolated pigment 1 was dissolved in DMSO at a concentration of 10 mM, and the concentration in each organic solvent was 20 μM. Buffer solutions of pH 1–3 and pH 3–9 were prepared using 50 mM KCl–HCl and 0.1 M Na$_2$PO$_4$–0.1 M NaH$_2$PO$_4$, respectively, and the concentration of pigment 1 in the buffer solutions were also 20 μM.

3. Results and discussion

3.1. Structural analysis of pigments 1 and 2

HPLC analysis of isolated pigment 1 showed a single peak in a wavelength range from 200 to 800 nm, and the absorption spectrum was the same as that of peak 1 in Fig. 2. The positive ESI-MS of pigment 1 showed the molecular ion (M$^+$) at m/z 557.1. The positive APCI-MS showed fragment ions at m/z 435.1 ([M – C$_8$H$_8$O$_3$]+), 405.1 ([M – C$_9$H$_8$O$_3$]+), and 393.1 ([M – C$_8$H$_8$O$_3$]+) in addition to the molecular ion at m/z 557.1 (Fig. 3). An ion at m/z 557 has been reported to be the major fragment ion of anthocyanin–flavanol pigments by the ESI-MS$^+$ analyses (Macz-Pop et al., 2006; Sentandreau et al., 2010; Sentandreau et al., 2012). According to the references, the fragment ion at m/z 557 seems to be generated by deglycosidation and dehydration of anthocyanin–flavanoid pigments. If we assume that the ion detected by ESI- and APCI-MS (m/z 557.1) is the molecular ion of pigment 1, then it probably represents an anthocyanidin–flavanoid adduct. The following NMR data of pigment 1 support this ion as the molecular ion.

The structural assignment of pigment 1 was confirmed by $^1$H- and $^{13}$C NMR analyses (Table 1). The $^1$H NMR spectrum indicates the presence of cyanidin moiety ($\delta$ 6.69 ($d$, $J$ = 1.8 Hz, 1H, H-6), 6.85 ($d$, $J$ = 2.3 Hz, 1H, H-8), 6.92 ($dd$, $J$ = 8.7 Hz, 1H, H-5), 8.02 ($dd$, $J$ = 2.4, 8.7 Hz, 1H, H-6), and 8.15 ($d$, $J$ = 2.3 Hz, 1H, H-2')) (Chiroli & Jay, 1995), and flavanol moiety ($\delta$ 2.47 ($dd$, $J$ = 16.5, 6.9 Hz, 1H, H-4'$_ax$), 2.66 ($dd$, $J$ = 16.4, 4.6 Hz, 1H, H-4'$_eq$), 3.97 ($dt$, $J$ = 5.5, 6.8 Hz, 1H, H-3'), 4.80 ($d$, $J$ = 6.4 Hz, 1H, H-2'), 6.12 ($s$, 1H, H-8'), 6.62 ($dd$, $J$ = 1.8, 8.2 Hz, 1H, H-6'), 6.73 ($d$, $J$ = 8.6 Hz, 1H, H-5'), and 6.74 ($d$, $J$ = 2.3 Hz, 1H, H-2')) (Shen, Chang & Ho, 1993). On the $^1$H-H COSY spectrum of the flavanol moiety, cross peaks appeared at resonances at $\delta$ 2.47 and 2.66, at $\delta$ 2.47 and 3.97, at $\delta$ 2.66 and 3.97, and at $\delta$ 3.97 and 4.80 (data not shown). The $^1$H NMR data of H-2', H-3' and H-4' in the flavanol moiety resembled to those in (+)-catechin rather than (−)-epicatechin: the relative 2,3-stereochemistry was trans, as the proton signal at $\delta$ 4.80 (H-2') appeared as a doublet ($J_{2,3}$ = 6.4 Hz) corresponding to (+)-catechin (Shen, Chang & Ho, 1993).

The $^{13}$C NMR spectrum showed 30 carbon signals including flavanol carbons at $\delta$ 27.4 (C-4'), 65.8 (C-3'), and 81.8 (C-2'). The structural assignment was supported by HMBC cross peaks as shown in Table 1: the correlations were observed between the proton signal at $\delta$ 6.69 (H-6) and carbon signals at $\delta$ 105.1 (C-4a), 150.1 (C-5), 162.1 (C-7), and 96.3 (C-8); between the proton signals at $\delta$ 6.85 (H-8) and carbon signals at $\delta$ 105.1 (C-4a), 98.5 (C-6), 162.1 (C-7), and 150.8 (C-8a); between the proton signals at $\delta$ 8.15 (H-2') and carbon signals at $\delta$ 152.0 (C-2'), 145.0 (C-3'), 149.4 (C-4'), and 122.5 (C-6'); between the proton signals at $\delta$ 6.92 (H-5') and carbon signals at $\delta$ 122.9 (C-1'), 145.0 (C-3), and 149.4 (C-4'); between the proton signals at $\delta$ 8.02 (H-6') and carbon signals at $\delta$ 152.0 (C-2'), 116.5 (C-2'), and 149.4 (C-4'); between the proton signals at $\delta$ 4.80 (H-2') and carbon signals at $\delta$ 65.8 (C-3'), 27.4 (C-4'),
161.9 (C-8′a), 129.6 (C-1′′), 114.2 (C-2′′), and 118.1 (C-6′′); between the proton signals at δ 3.97 (H-3′′) and carbon signals at δ 106.2 (C-4′); between the proton signals at δ 81.8 (C-2′′), 65.8 (C-3′), 106.2 (C-4′), and 167.4 (C-5′), and 161.9 (C-8′a); between the proton signals at δ 2.66 (H-4′) and carbon signals at δ 81.8 (C-2′′), 65.8 (C-3′), 81.2 (C-4′), 106.2 (C-4′a), 167.4 (C-5′), and 161.9 (C-8′a); between the proton signals at δ 2.66 (H-4′′) and carbon signals at δ 81.8 (C-2′′), 65.8 (C-3′), 106.2 (C-4′), and 167.4 (C-5′), and 161.9 (C-8′a); between the proton signals at δ 6.12 (H-6′) and carbon signals at δ 81.2 (C-2′′), 103.3 (C-6′), 153.6 (C-7′), and 106.2 (C-4′a); between the proton signals at δ 6.73 (H-5′′) and carbon signals at δ 81.8 (C-2′′), 65.8 (C-3′), 106.2 (C-4′), and 145.1 (C-4′′); and between the proton signals at δ 6.62 (H-6′′) and carbon signals at δ 81.8 (C-2′′), 114.2 (C-2′′), and 145.1 (C-4′′). From these spectral data, we assigned that pigment 1 was a condensation product of cyanidin and (+)-catechin, in which the 5-hydroxy and C-4 positions of the cyanidin moiety were substituted by the addition of the 5-hydroxy and C-6 positions of the (+)-catechin moiety, respectively (Fig. 4). We named this cyanidin/(+)-catechin adduct vignacyanidin, based on the generic name of the adzuki plant.

The direct condensation between anthocyanins and flavonols takes place during processing and storage of red wines (Fulcrand et al., 1996; Fulcrand et al., 2006; Lee et al., 2004; Mateus et al., 2002). Such anthocyanin–flavanol adducts have been synthesized in model systems (Dueñas, Fulcrand, & Cheynier, 2006; Es-Safi, Cheynier, & Moutounet, 2002). In the synthesized adducts, the 7-hydroxy and C-8 positions of the catechin moiety substituted by the addition of 5-hydroxy and C-4 positions of malvidin 3-O-glucoside moiety is included (Dueñas et al., 2006). In addition, small amounts of anthocyanin–flavanol condensed pigments have been found in some plants, as described in the introduction. In these anthocyanin–flavanol adducts, the C-8 position of the anthocyanin moieties bind to the C-4 position of flavonol moieties.

Pigment 2 showed the molecular ion (M⁺) at m/z 557.1 with the same fragmentation pattern as vignacyanidin: ESI-MS, m/z 557.1 (M⁺, 100%); APCI-MS, m/z 393.1 ([M – C9H8O3]+, 88%), 405.1 ([M – C8H8O3]+, 3%), and 435.1 ([M – C7H6O2]+, 17%) and 557.1 (M⁺, 100%). Furthermore, the UV–vis absorption spectrum of pigment 2 was the same as that of vignacyanidin (Fig. 2). These data suggest that pigment 2 is an isomer of vignacyanidin, such as a cyanidin/(−)-epicatechin adduct, where the (+)-catechin moiety is replaced by the (−)-epicatechin moiety. Since 2R,3S compounds [such as (+)-catechin] elute earlier than 2R,3R compounds [such as (−)-epicatechin] in reversed-phase columns (Fosn et al., 2004), the order of elution in reversed-phase HPLC supports that pigment 2 might be a cyanidin/(−)-epicatechin adduct. In the present study, we could not resolve the detailed structure of pigment 2 due to its low yield.

Anthocyanins are normally present as glycosides in plants, and anthocyanin–flavanol adducts found in red wine and plants are also glycosides (Dueñas et al., 2008; Fulcrand et al., 2006; Macz-Pop et al., 2006; Sentandreu et al., 2010; Sentandreu et al., 2012), but vignacyanidin and its isomer isolated in the present study were aglycones. The use of acidic solutions during the isolation of vigna-
The following results. Namely, both pigments were extracted with cyanidin and its isomer, might suggest these were produced from their glycosides by hydrolysis. This possibility was excluded by the following results. Namely, both pigments were extracted with ethyl acetate from boiling-water extract of adzuki bean (pH 5.9) and boiled adzuki bean (data not shown), which indicates (i) that vignacyanidin and its isomer are not compounds formed by acid hydrolysis nor enzymatic hydrolysis and (ii) that these pigments can remain in foods prepared using adzuki bean. Two mechanisms are possible for the synthesis of vignacyanidin and its isomer in seed coat of adzuki bean; one is the reaction of (epi)catechins with cyanidin aglycones, and the other is the removal of sugars from the glycosides of vignacyanidin and its isomer, which are formed from (epi)catechins and cyanidin glycosides. Further studies are required to elucidate the mechanism of synthesis of vignacyanidin and its isomer in seed coat of adzuki bean during ripening.

In addition to vignacyanidin and its isomer, the quercetin aglycone was found as an ethyl acetate-soluble component (Fig. 2, peak a). Quercetin was identified by comparing the retention time and the absorption spectrum with those of standard quercetin. Since quercetin is formed from its glycosides during drying of onion scales (Takahama & Hirota, 2000) and quercetin glycosides are present in the adzuki bean (Yoshida, Kondo, Ito, & Kondo, 2005), quercetin in adzuki bean may also be formed from quercetin glycosides in the seed coat during ripening. An aglycone with a xanthylum skeleton has been isolated from the adzuki bean (Yanase, Nishimoto, Kamatari, & Nakatsuka, 2012), suggesting that phenolic aglycones can coexist with their glycosides in the dried seed coat of the adzuki bean. Furthermore, an aglycone of pyranocyanidin has been reported to be present in rose petals (Fukui et al., 2002).

### 3.2. Effects of solvent and pH on absorption spectra of vignacyanidin

UV–vis absorption spectra of vignacyanidin (20 μM) were recorded in methanol, 1-butanol, and ethyl acetate. Absorption maximum (λ_{max}) of the spectra were observed at 572, 581, and 591 nm in methanol, 1-butanol, and ethyl acetate, respectively, indicating that λ_{max} shifted to longer wavelength with the increase of solvent hydrophobicity. Molar extinction coefficients at their λ_{max} in the above solvents were similar to each other, and the value in methanol was calculated to be 31 mM \(^{-1}\) cm\(^{-1}\). The value of cyanidin 3-O-glucoside dissolved in various acidic solutions such as 1% HCl in methanol and 0.1 M HCl in aqueous ethanol has been reported to range from 19 to 34 mM \(^{-1}\) cm\(^{-1}\) (Lee, Durst, & Wrolstad, 2005; Yoshida et al., 1996).

It is well known that the absorption of anthocyanin is dependent on pH. UV–vis absorption spectrum of vignacyanidin had a λ_{max} at 551 nm and a shoulder at approximately 600 nm at pH 1 (Fig. 5, top). The λ_{max} shifted to 520 nm when pH was increased to 5 without affecting the absorbance significantly. Accompanying the increase in pH from 6 to 9, λ_{max} shifted to a longer wavelength, increasing the λ_{max} absorbance (Fig. 5, bottom). Such changes in λ_{max} and absorbance with the increase in pH from 6 to 9 have been reported for pelargonidin 3-glucopyranoside and 5-carboxyypyrano-pelargonidin 3-glucopyranoside isolated from strawberry (Fragariaananassa) (Andersen et al., 2004), supporting the presence of the anthocyanin structure in vignacyanidin.

The absorbance around 500 nm of the pelargonidin glucosides at pH 5.1 is less than one tenth and one fifth of that at pH 3 and 6, respectively (Andersen et al., 2004), which is normally common for anthocyanins (Wrolstad et al., 2005), whereas the absorbance around 500 nm of the carboxyypyrano-pelargonidin glucoside is not largely affected when pH is decreased from 6 to 1 (Andersen et al., 2004). The effect of pH on the absorbance for the carboxyypyrano-pelargonidin glucoside and vignacyanidin was similar (Fig. 5), supporting the presence of a pyran structure in vignacyanidin. Absorption maximum of vignacyanidin was observed around 560 nm at pH 8.9. The difference might be explained by the difference in the number of conjugated double

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**Table 1**

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<th>Position</th>
<th>(^{1}H) Shift (\delta^b)</th>
<th>(^{13}C) Shift (\delta^b)</th>
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<td>H-6</td>
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*a* Measured in DMSO-\(d_6\).

*b* Shifts in parts per million downfield relative to tetramethylsilane.

*c* Multiplicity: \(s\), singlet; \(d\), doublet; \(t\), triplet.

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**Fig. 4.** Proposed structure of pigment 1 (vignacyanidin).
bonds and the number of hydroxyl groups in B ring of the anthocyanin moiety.

3.3. Concluding remarks

In this study, two cyanidin–catechin adducts were isolated from the adzuki bean. In pigment 1 (vignacyanidin), the 5-hydroxy and C-4 positions of cyanidin were substituted by the addition of 5-hydroxy-3-glucoside, and its isomer in the boiling water extract of adzuki bean and the adzuki bean suggests that these pigments can contribute to the coloration of the adzuki bean paste and glutinous rice.

The coloration is probably due to the binding of the pigments to starch in the adzuki bean paste and glutinous rice. The binding of the vagnacyanidin and its isomer is deduced from the report that flavonoids such as quercetin and epicatechin-O-(4,5-dimethyl)-gallate can bind to starch (Takahama & Hirota, 2010). Because the binding of quercetin and epicatechin-O-(4,5-dimethyl)-gallate to starch results in suppression of α-amylase-catalysed digestion of starch, the next study will deal with the interactions between vagnacyanidin and starch under various conditions.

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