Isolation of a Reduced Form of Cyanidin 3-*O*- β -D-Glucoside from Immature Black Soybean (*Glycine max* (L.) Merr.) and Its Reducing Properties

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Abstract: 5,7,3',4'-Tetrahydroxyflav-2-en-3-ol 3-*O*- β -D-glucoside was isolated from the seed coats of immature black soybeans (*Glycine max* (L.) Merr.). This compound is a reduced form of cyanidin 3-*O*- β -D-glucoside (cyanidin 3-G) which was obtained by reaction with hydrochloric acid. The molecule has reducing activity for a tetrazolium derivative (WST-1) in the presence of 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) in a similar manner to NADH. The seed coats of immature black soybeans also contain epicatechin as a major constituent, while cyanidin 3-G and procyanidin B2 are present at lower concentrations. Immature brown soybeans did not contain 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-*O*- β -D-glucoside, but did contain both epicatechin and procyanidin B2. Immature yellow soybeans contained none of them.

Key words: black soybean, flav-2-en-3-ol, anthocyanin, cyanidin 3-*O*- β -D-glucoside

1 INTRODUCTION

Cyanidin 3-*O*- β -D-glucoside (cyanidin 3-G), among the other anthocyanins is widespread in plants¹⁾. Black soybean (*Glycine max* (L.) Merr.) is well known to accumulate cyanidin 3-G as a major anthocyanin constituent in the seed coat²⁻⁴⁾. Analysis of phenolic compounds such as anthocyanins and procyanidins in seed coats of soybeans has been usually performed using mature soybeans as starting material^{2, 3, 5, 6)}.

The analysis of the seed coats of immature soybeans has also been performed to investigate the genotypes and phenotypes of seed coat colors in soybeans^{4, 7)}. Among the different soybean varieties, black soybeans contain both anthocyanins and procyanidins, brown soybeans accumulate only procyanidins, and the majorly cultivated yellow soybeans do not have any pigment. In the report⁴⁾, procyanidins and anthocyanidins obtained by acid hydrolysis of the glycoside bonds in the immature seed coats of black, brown, and yellow soybeans were analyzed by thin layer chromatography (TLC) analysis and procyanidin tests such as the vanillin assay. The seed coat color of soybeans is controlled by 3 independent loci (*I*, *R*, and *T*)⁷⁾. Yellow

soybeans (*I*) do not accumulate anthocyanins or proanthocyanidins, black soybeans (*i*, *R*) contain anthocyanins and proanthocyanidins, and brown soybeans (*i*, *r*, *T*) contain only proanthocyanidins⁴⁾.

We also investigated the composition of the immature seed coats of black and brown soybean varieties using liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS. The immature black soybean (Shintanba-kuro) is bigger than other immature soybeans and is slightly colored because it contains anthocyanins. We discovered that 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-*O*- β -D-glucoside is contained in immature black soybean varieties, and not in brown and yellow soybeans. Here, we report on this compound and its reducing capability in detail.

2 EXPERIMENTAL

2.1 Chemicals

(+)-Catechin and (−)-epicatechin were purchased from Sigma-Aldrich (St. Louis, USA). Procyanidin B1 and B2 were purchased from Funakoshi Co., Ltd (Tokyo,

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Japan). WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]⁸⁾ and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Lab. (Kumamoto, Japan).

2.2 Materials

Three seed color varieties (black, brown, and yellow) of soybeans were used for the analysis of the composition of the seed coats of immature soybeans. The black soybean varieties (Shintanba-kuro, Murasaki-zukin, Natsunoyosooi, and Tankuro) were provided by the Kyoto Prefectural Agriculture, Forestry, and Fisheries Technology Center, while Kurokko-hime was cultivated in the Kyoto Gakuen University field. The brown soybean variety (Dadatya-mame) and yellow soybean variety (Sapporo-midori) were purchased.

2.3 Analysis of seed coat extract of “edamame (edible immature soybeans)” samples

After shelling the immature “edamame” seeds of each soybean variety, seed coats (2 g, wet weight) were immersed in 20 mL of methanol (MeOH) for 3 h. Ten microliters of the supernatant was analyzed by a 20AD high-performance liquid chromatography (HPLC) system equipped with a 4.6×150 mm 5C₁₈ ARII reverse-phase column (Nacalai Tesque, Japan). Elution solvents were constituted by water containing 0.1% formic acid (solvent A) and 90% acetonitrile containing 0.1% formic acid (solvent B). The gradient consisted in the elution of solvent B from 5 to 50% in 10 min followed by 10 min of isocratic elution with 50% B. The flow rate was 1.0 mL/min, and the detection was achieved with a photo diode array detector (Shimadzu Ltd.).

For LC-MS, the same HPLC system described above was used with a variation of the column dimensions (2.0×150 mm). Two microliters of sample was injected. The flow rate was 0.2 mL/min and the detection occurred at 280 and 254 nm. Mass spectrometry was conducted on a LCMS-2010EV instrument (Shimadzu Ltd., Japan) equipped with an electron-spray ionization (ESI) module.

2.4 Isolation and purification from black soybean (Shintanba-kuro) seed coat

Seed coats (72.2 g) obtained from 417 seeds were immersed in MeOH (600 mL) for 24 h, and the extract was then stored at -30°C .

The extract (200 mL) was concentrated under vacuum at 30°C to a final volume of about 30 mL and the concentrated solution was filtered by a Millex®-GP Filter Unit (hydrophilic, 0.22 μm , d: 33 mm). The filtrate was concentrated to 4 mL. Eighteen 0.1 mL sample fractions were purified by preparative HPLC on a 5C₁₈ ARII column (20.0×300 mm) at a flow rate of 8 mL/min using an isocratic elution scheme (20% acetonitrile). The fraction corresponding to the peak

with 9.9 min retention time (RT) was collected and freeze-dried to give 8.6 mg of amorphous powder. The product was analyzed by LC-MS/MS using a LCMS-IT-TOF mass spectrometer module (Shimadzu Ltd., Japan). The product which was termed M450 was also analyzed using an AVANCE-750 nuclear magnetic resonance (NMR) instrument (Bruker Biospin, Germany).

2.5 Purification of the acetylated product by preparative HPLC

The “edamame” extract (150 mL) was evaporated under vacuum at 30°C and gave a residue (800 mg) that was acetylated in acetic anhydride (1.96 mL) and pyridine (3 mL). The reaction solution was poured into water and extracted with ethyl acetate. The extract was washed 3 times with 1.0 M HCl solution and water, and then dried over anhydrous Na₂SO₄. The resulting solution was evaporated to dryness. The sample dissolved in 1 mL of dichloromethane was then chromatographed on 20 g of silica gel (Wakogel C-200, Wako Pure Chemical Ind. Ltd., Japan) and eluted with an *n*-hexane/ethyl acetate mixture (3:1, then 1:1). The obtained fraction was evaporated to dryness and gave 169 mg of an oily product. The product was dissolved in 0.84 mL of acetonitrile to give 200 mg/mL final concentration. Eight 0.1 mL sample fractions were chromatographed by preparative HPLC. The experimental conditions were the same as the preparative chromatography described above with the exception that 70% acetonitrile isocratic elution was used. The fraction corresponding to the peak with 11.9 min RT was collected, concentrated at 30°C , and then freeze-dried to give 4.7 mg of amorphous powder. The product was measured by LC-MS/MS and NMR.

2.6 Structure elucidation by NMR

The structure of the isolated product was elucidated by assignment of all the protons and carbons on the basis of the DQF-COSY and TOCSY ¹H-¹H correlation spectra, and the HSQC and HMBC ¹H-¹³C correlation spectra.

2.7 Acid conversion of the isolated product

Ten microliters of a 3.0 M solution of HCl were added to MeOH solution of M450 (0.38 mM, 190 μL), and the sample was immediately analyzed by HPLC. Following that, the sample was analyzed at 30 min intervals for 2.5 h, followed by 1 h intervals for other 2 h. The area of M450 at 254 nm and cyanidin 3-G at 520 nm were used to obtain their acid conversion time course. The experiment was performed in duplicate and the results are discussed in terms of average values.

2.8 Reaction of the isolated product with WST-1 in the presence of 1-methoxy PMS⁹⁾

A mixture of MeOH solution of M450 (15 mM, 10 μL) and Tris-HCl buffer, pH 8.5, (90 μL) was added to the same

buffer (5 mL). The solution (100 μ L) containing 10 mM WST-1 and 0.25 mM 1-methoxy-PMS and 4 mL of deionized water were successively added. The absorbance at 440 nm and 520 nm was monitored at 1 min intervals after the addition of the WST-1 and 1-methoxy-PMS solutions. The experiments were also conducted using 1 mM NADH instead of M450. These experiments were performed in duplicate and the results are discussed in terms of average values.

3 RESULTS

3.1 Structure elucidation of the product from the seed coat extract and its acetylated form.

The extract from immature black soybean seed coats yielded a predominant peak at a RT of about 7.7 min in the chromatogram at both 254 and 280 nm. That species exhibited an experimental positive m/z of 451.1227. The isolated product, M450, had a molecular weight of 450 associated with the chemical formula $C_{21}H_{22}O_{11}$, whose theoretical positive m/z (451.1235) is in excellent agreement with the experimental value. In the $^1\text{H-NMR}$ spectrum, two doublet signals at 3.53 and 3.61 ppm had the same large coupling constant ($J = 19.7$ Hz) were generated by protons bonded at the same carbon in the HSQC spectrum (C_4 carbon, 22.8 ppm). Protons were identified as geminal methylene protons (H_4 protons). Given the characteristic chemical shift, the five protons at 5.93, 6.00, 6.74, 7.21, and 7.33 ppm were identified as aryl protons. Protons at 5.93 and 6.00 ppm had the same J value ($J = 2.0$ Hz), which demonstrated meta coupling (H_6 and H_8). The proton at 6.74 ppm gave a doublet signal having with $J = 8.3$ Hz, the proton at 7.33 ppm exhibited a doublet signal with $J = 1.8$ Hz, while the proton at 7.21 ppm showed a double doublet signal with $J = 1.8$ and 8.3 Hz. Therefore, these protons showed an ABX pattern (H_2 at 7.33, H_5 at 6.74, and H_6 at 7.21 ppm). The signals of C_2 , C_5 , and C_6 were assigned in the HSQC spectrum to the peaks at 116.3, 115.6, and 121.0 ppm, respectively. In the HMBC spectrum, the proton at 5.93 ppm showed cross peaks at 97.7, 100.1, 153.5, and 158.2 ppm with the attached carbon, while the proton at 6.00 ppm showed cross peaks at 95.1, 100.1, 157.2, and 158.2 ppm with the attached carbon. C_5 and C_7 carbons should resonate at similar chemical shifts at much lower magnetic fields (e.g., around 150 ppm). Therefore, the peaks at 5.93 and 6.00 ppm were conclusively assigned to H_8 and H_6 , respectively, while the peaks at 95.1, 97.7, 100.1, 153.5, 157.2, and 158.2 ppm were assigned to C_8 , C_6 , C_{10} , C_9 , C_5 , and C_7 , respectively. Using the same logic, the resonances and the cross peaks of the other protons in the HSQC and HMBC spectra were analyzed. In the sugar moiety, H_2 , H_3 , H_4 protons were located in the sharp region comprised between 3.3 and 3.4 ppm with coupling constants of about 9 Hz, thus demonstrating an equatorial

configuration (Table 1). In the HMBC spectrum, the H_1 proton at 4.80 ppm showed a cross peak with C_3 carbon at 132 ppm. Thus, we discovered that the structure of M450 corresponded to 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-O- β -D-glucoside (Fig. 1). The assignments of the $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra are reported in Table 1.

The acetylated product of M450 was identified to be 5,7,3',4'-tetra-*O*-acetylflav-2-en-3-ol 3-O-(2",3",4",6"-tera-*O*-acetyl)- β -D-glucoside following the same strategy used for M450 itself. The compound showed an experimental positive m/z of 809.1943 associated with the chemical formula $C_{37}H_{38}O_{19}Na$, whose theoretical positive m/z (809.1937) is in excellent agreement with the experimental value. The assignments of the relative $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra are listed in Table 2.

3.2 Analysis of the seed coat extract of "edamame" samples

All the black soybeans contained cyanidin 3-G (RT = 7.6 min), procyanidin B2 (RT = 8.6 min), M450 (RT = 8.8 min), and epicatechin (RT = 8.9 min). Brown soybeans contained both procyanidin B2 and epicatechin, but neither cyanidin 3-G nor M450. Yellow soybeans did not contain any of the above. It can be concluded that M450 is exclusively contained in the seed coat of black soybeans.

3.3 Acid conversion of M450

The time course of the acid conversion of M450 into cyanidin 3-G is shown in Fig. 2. As the peak area of M450 decreased, that of cyanidin 3-G increased. This result suggests that M450 is converted to cyanidin 3-G by the acid catalyst. A 1:1 mixture of M450 solution and 20% aqueous citric acid solution produced the same result (data not shown).

3.4 Reducing capability of M450

The reducing capability of M450 was measured by the reaction with WST-1 in the presence of 1-methoxy-PMS. As shown in Fig. 3, although it requires longer reaction times, M450 can reduce WST-1 with the same efficiency of NADH.

4 DISCUSSION

M450, which was isolated from the seed coat of immature black soybeans, was identified as 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-O- β -D-glucoside (Fig. 1). This compound has never been isolated before as a natural product. However, the flav-2-en-3-ol moiety was found as an intermediate in the chemical synthesis of anthocyanin. Quercetin 3-rutinoside (rutin) was reduced with zinc powder in HCl/MeOH to give a mixture of flav-2-en-3-ol, flav-3-en-3-ol, and cyanidin derivatives, which were purified by HPLC¹⁰. In that report, the carbonyl group at position 4 of

Table 1 Chemical shifts in ^1H - and ^{13}C -NMR spectrum of M450 in CD_3OD .

	Position	^1H -NMR 750 MHz; ppm	J; Hz	^{13}C -NMR 187.5 MHz; ppm
aglycone	1	—	—	—
	2	—	—	138.5
	3	—	—	132
	4	3.464	d, J = 19.0	22.8
	4	3.629	d, J = 19.0	22.8
	5	—	—	157.2
	6	5.995	d, J = 2.0	97.7
	7	—	—	158.2
	8	5.928	d, J = 2.0	95.1
	9	—	—	153.5
	10	—	—	100.1
	1'	—	—	126.4
	2'	7.326	d, J = 1.8	116.3
	3'	—	—	145.4
	4'	—	—	146
	5'	6.736	d, J = 8.3	115.6
	6'	7.205	dd, J = 8.3, 1.8	121
glucoside	1''	4.798	d, J = 7.4	100.6
	2''	3.384	dd, J = 7.4, 8.9	75.1
	3''	3.402	dd, J = 8.9, 8.6	78.1
	4''	3.372	dd J = 8.6, 10.2	71.4
	5''	3.311	ddd J = 2.0, 5.3, 10.2	78.3
	6''	3.838	dd, J = 12.1, 2.0	62.5
	6''	3.689	dd, J = 12.1, 5.3	62.5

Fig. 1 The chemical structure of M450 (5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-O- β -D-glucoside).

rutin was reduced by Zn to give these compounds. The product mixture in HCl/MeOH was modified by blowing air into the sample to give cyanidin 3-rutinoside¹⁰. Pelargonidin 3-O- (6''-O-acetyl)- β -D-glucopyranoside was synthesized through the kaempferol 3-O- (6''-O-acetyl)- β -D-glucopyranoside intermediate¹¹. The flav-2-en-3-ol derivative generated by the reduction of zinc amalgam was changed to pelargonin derivative by trifluoroacetic acid treatment. Apparently, M450 is a reduced form of cyanidin 3-G that was changed to cyanidin 3-G itself by an acid catalyst (Fig. 2). Therefore, it is clear that flav-2-en-3-ol derivatives can be oxidized by acid or air under acid conditions to generate their corresponding anthocyanins.

M450 also reduced WST-1 in the presence of 1-methoxy-PMS to generate a formazan dye (Fig. 3). 1-Methoxy-PMS is usually used to detect NADH or NADPH and, since viable cells produce both NADH and NADPH, to measure cell viability indirectly. As shown in Fig. 3, in the WST-1 reduction experiment the absorbance maxima at 440 nm and 520 nm,

Table 2 Chemical shifts in ^1H - and ^{13}C -NMR spectrum of acetylated M450 in CDCl_3 .

	Position	^1H -NMR 750 MHz; ppm	J; Hz	^{13}C -NMR 187.5 MHz; ppm
aglycone	1	—	—	—
	2	—	—	138.2
	3	—	—	131.7
	4	3.528	d, J = 19.7	23.6
	4	3.612	d, J = 19.7	23.6
	5	—	—	151.5
	6	6.715	d, J = 1.8	107.7
	7	—	—	149.9
	8	6.647	d, J = 1.8	110.4
	9	—	—	148.8
	10	—	—	110.6
	1'	—	—	141.9
	2'	7.545	d, J = 1.5	122.8
	3'	—	—	130.4
	4'	—	—	141.6
	5'	7.175	d, J = 8.5	122.8
	6'	7.62	dd, J = 8.5, 1.5	126.2
glucoside	1"	4.839	d, J = 7.8	99.1
	2"	5.124	dd, J = 7.8, 9.3	71.1
	3"	5.172	dd, J = 9.3, 9.4	72.8
	4"	5.109	dd, J = 9.4, 10.2	68.1
	5"	3.692	ddd = 1.9, 4.6, 10.2	72.1
	6"	4.107	dd, J = 12.2, 1.9	61.7
	6"	4.205	dd, J = 12.3, 4.6	61.7
CH_3CO		1.85, 2.02, 2.05, 2.28, 2.30, 2.32, 2.34		168.0, 168.1, 168.4, 168.8, 169.3, 169.3, 170.2, 170.6

the formazan and anthocyanin absorption wavelengths, respectively, increased suggesting that M450 can reduce WST-1 in the presence of 1-methoxy-PMS and transforms into cyanidin 3-G. However, the reaction was slower than NADH. In fact, in contrast to M450, NADH may more readily transfer a hydrogen atom to 1-methoxy-PMS. Nonetheless, M450 exhibits a reducing capability similar to that of NADH or NADPH.

M450 was found in all the seed coats of immature black soybeans examined. However, it was not detected in brown soybeans, although both black and brown soybeans contain epicatechin and procyanidins. Additionally, immature yellow soybeans did not contain any of these constituents.

Immature black soybeans were slightly colored by anthocyanin. At that time, M450 was dominantly contained in seed coats. The color became deeper as black soybeans grew and cyanidin 3-G also increased in the seed coat. It

appears that M450 changes to cyanidin 3-G in seed coats.

It has been proposed that anthocyanidins are biosynthetically derived from (+)-naringenin through (2*R*,3*R*)-dihydroflavonol and (2*R*,3*S*,4*S*)-leucocyanidin, which are oxidized by anthocyanidin synthase (ANS), and that anthocyanidins are then glycosidated to generate anthocyanins^{12, 13}. In the currently accepted anthocyanin biosynthesis scheme, M450 does not appear. However, in a report where ANS from *Gerbera hybrida* catalyzed the conversion of (+)-catechin to cyanidin, it was proposed that the flav-2-en-3-ol derivative, which is the aglycone of M450, is an intermediate for generating cyanidin or cyanidin 3-G after glucosidation¹⁴. M450 may be accumulated in the seed coats of immature black soybeans because further oxidation of M450 does not occur.

Although it is not clear whether M450 (5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-O- β -D-glucoside) is an intermediate

Fig. 2 The time course of M450. After aqueous HCl was added to the M450 solution, the sample was immediately analyzed by HPLC. Following that, the sample was analyzed at 30 min intervals for 2.5 h, followed by 1 h intervals for other 2 h. The area of M450 at 254 nm and cyanidin 3-G at 520 nm were used to obtain their acid conversion time course.

Fig. 3 The reducing capability of M450. The absorbance at 440 nm and 520 nm was monitored at 1 min intervals after the addition of the WST-1 and 1-methoxy-PMS solutions. Filled triangle: WST-1+1-methoxy PMS at 440 nm, filled square: WST-1+1-methoxy PMS at 520 nm, open circle: NADH (without M450)+WST-1+1-methoxy PMS at 440 nm, open triangle: Without 1-methoxy PMS at 440 nm, open square: Without 1-methoxy PMS at 520 nm.

in the anthocyanin biosynthesis, it is obvious that M450 is a reduced form of cyanidin 3-G and can be converted to it by oxidation.

5 Conclusions

M450 isolated from seed coats of immature black soybeans was identified as 5,7,3',4'-tetrahydroxyflav-2-en-3-ol 3-O- β -D-glucoside. This is a reduced form of cyanidin 3-G, and, similarly to NADH or NADPH, possesses reducing activity against WST-1.

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REFERENCES

- Andersen Ø. M. Recent Advances in the Field of Anthocyanins-Main Focus on Structures. In *Recent Advances in Polyphenol Research*, first edition; Daayf, F., Lattanzio, V., Eds; Wiley-Blackwell: Oxford, UK, 2008; 1, 167-201.
- Kuroda, C.; Wada, M. The Colouring Matter of "Kuro-mame". *Proc. Imp. Acad. Japan (Tokyo)* **9**, 17-18 (1933).
- Kuroda, C.; Wada, M. The constitution of natural colouring matters, kuromamin, shisonin, and nasunin. *Bull. Chem. Soc. Japan.* **11**, 272-287 (1936).
- Todd, J. J.; Vodkin, L. O. Pigmented soybean (*Glycine max*) seed coats accumulate proanthocyanidins during development. *Plant Physiol.* **102**, 663-670 (1993).
- Choung, M. G.; Baek, I. Y.; Kang, S. T.; Han, W. Y.; Shin, D. C.; Moon, H. P.; Kang, K. H. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *J. Agric. Food Chem.* **49**, 5848-5851 (2001).
- Takahata, Y.; Ohnishi-Kameyama, M.; Furuta, S.; Takahashi, M.; Suda, I. Highly polymerized procyanidines in brown soybean seed coat with a high radical-scavenging activity. *J. Agric. Food Chem.* **49**, 5843-5847 (2001).
- Zabala, G.; Vodkin, L. Cloning of the pleiotropic T locus in soybean and two recessive alleles that differentially affect structure and expression of the encoded flavonoid 3' hydroxylase. *Genetics* **163**, 295-309 (2003).
- Ishiyama, M.; Shiga, M.; Sakamoto, K.; Mizoguchi, M.; He, P. G. A New sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. *Chem. Pharm. Bull.* **41**, 1118-1122 (1993).
- Ishiyama, M.; Miyazono, Y.; Sasamoto, K.; Ohkura, Y.

- Ueno, K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* **44**, 1299-1305 (1997).
- 10) Yoshida, K.; Kondo, T.; Oyama, K. *Japan Kokai Tokkyo Koho* 2009137904 (2009).
- 11) Oyama, K.; Kawaguchi, S.; Yoshida, K.; Kondo, T. Synthesis of pelargonidin 3-O-6'-O-acetyl- β -D-glucopyranoside, an acylated anthocyanin, via the corresponding Kaempferol glucoside. *Tetrahedron Lett.* **48**, 6005-6009 (2007).
- 12) Holton, T. A.; Cornish, E. C. Genetics and Biochemistry of Anthocyanin Biosynthesis. *The Plant Cell* **7**, 1071-1083 (1995).
- 13) Nakajima, J.; Tanaka, Y.; Yamazaki, M.; Saito, K. Reaction mechanism from leucoanthocyanidin to anthocyanidin 3-glucoside, a key reaction for coloring in anthocyanin biosynthesis. *J. Biol. Chem.* **276**, 25797-25803 (2001).
- 14) Wellmann, F.; Griesser, M.; Schwabb, W.; Martens, S.; Eisenreich, W.; Materna, U.; Lukačina, R. Anthocyanidin synthase from *Gerbera hybrida* catalyzes the conversion of (+)-catechin to cyanidin and a novel procyanidin. *FEBS Lett.* **580**, 1642-1648 (2006).
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