Antioxidative Activity of Nasunin in Chouja-nasu (Little Eggplant, *Solanum melongena* L. 'Chouja')

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Antioxidative activities of nasunin, isolated from Chouja-nasu (little eggplant, *Solanum melongena* L. 'Chouja'), and of some other anthocyanins were studied by measuring their inhibitory effect on carotene oxidation induced by linoleic acid-lipoxygenase system (carotene bleaching method) and on autoxidation of linoleic acid. Nasunin showed the strongest antioxidative activity followed by malvin and rubrobrassicin when their activities were measured by carotene bleaching method at pH 7, and by linoleic acid autoxidation system at pH 2.8. Delphinidin, malvidin and cyanidin, which are aglycones of nasunin, malvin and rubrobrassicin, respectively, also showed antioxidative activities. The antioxidative activity of nasunin was stronger than that of delphinidin at pH 2.8, and the activity of malvin and rubrobrassicin showed slight difference from that of malvidin and cyanidin, respectively. These results presumably indicate that the *p*-coumaric acid moiety of nasunin at least partly contributes to its strong antioxidative activity.

Chouja-nasu (little eggplant, *Solanum melongena* L. 'Chouja') is cultivated in Shonai district of Yamagata, Japan, and mainly utilized as raw materials for pickles. However, it has not yet been examined, what kind of anthocyanin is contained in the peels of Chouja-nasu, despite the fact that nasunin (delphinidin-3-[4-(*p*-coumaroyl)-L-rhamnosyl (1→6) glucoside]-5-glucoside) is known to be contained in the peels of several varieties of eggplant other than Chouja-nasu¹⁾²⁾. On the other hand, some flavonoids contained in vegetables are known to show antioxidative activity³⁾⁻⁹⁾, suggesting the possibility that other phenolics such as anthocyanins may also show antioxidative activity. In the previous paper, we have reported that malvin, an anthocyanin isolated from wild grapes, showed antioxidative activity¹⁰⁾. This finding suggested the possibility that other anthocyanins may also have antioxidative activity. In this study, antioxidative activity of nasunin isolated from Chouja-nasu was examined and compared with the activities of other anthocyanins.

Materials and Methods

Preparation of anthocyanins

Peels of Chouja-nasu collected in Shonai district of Yamagata Prefecture were immersed in 3% trifluoro acetic acid solution at 5°C, and filtered. The residue was re-extracted in the same way and the combined extracts was passed through a column of XAD-7. After washing with H₂O and 30% MeOH, the column was eluted with MeOH, and the eluate was evaporated. The residue was dissolved in MeOH-AcOH-H₂O (10:1:9, v/v) and chromatographed on a Sephadex LH-20 column, using the same solvent. The evaporated effluents corresponding to a major reddish-purple band on the column was dissolved in a small amount of 0.01% HCl-MeOH, and the anthocyanin (I) was precipitated by the addition of diethyl ether.

FABMS measurement of the isolated anthocyanin (I) showed a molecular ion at *m/z* 919, an [M-162]⁺ ion at *m/z* 757 (loss of glucose moiety), an [M-292]⁺ ion at *m/z* 627 (loss of *p*-coumaroylrhamnose moiety), an [M-

p-coumaroylrutinoside moiety]⁺ ion at *m/z* 465 and an aglycine ion at *m/z* 303, indicating the possibility that I is nasunin. Absorption maxima of I in 0.1% HCl-MeOH (280, 306 and 538 nm), and the ratio of the absorption at 306 nm to that of the visible absorption maximum (72%) were in good agreement with those of nasunin as described in the literature¹¹⁾. After saponification of I with 10% sodium hydroxide at room temperature, the resultant was neutralized with HCl and extracted in diethyl ether. Silica gel TLC of the extract (benzene-dioxane-AcOH = 100 : 25 : 5, v/v) showed the presence of *p*-coumaric acid at *R_f* = 0.46 by comparison with an authentic specimen. The ether-extracted hydrolysate was adjusted to 20% acetic acid by addition of glacial acetic acid and hydrolyzed at 100°C for 4 h. The hydrolysate containing sugars was desalted through ion exchange resin columns (Amberlite IR 45, OH⁻ type, subsequently Dowex 50 W × 4, H⁺ type) and subjected to paper chromatography (n-BuOH-AcOH-H₂O = 4 : 1 : 1, v/v). Rutinose besides L-rhamnose and D-glucose were identified in comparison with authentic samples. The absorption maxima of aglycone obtained by hydrolysis of I with 12% HCl in 0.1% HCl-MeOH (277 and 547 nm), and the ratio of the absorption at 440 nm to that of the visible absorption maximum (17%) were in good agreement with those of delphinidin¹²⁾. From these data, I was identified as nasunin with a yield of 0.15 g from 1 kg of Chouja-nasu.

Rubrobrassicin (cyanidin-3-diglucoside-5-monoglucoside) and malvin (malvidin-3, 5-diglucoside) were prepared as described previously¹⁰⁾¹³⁾. Aglycones were obtained from their glycosides by hydrolysis with 15% HCl.

Antioxidative activity

Carotene bleaching method: Anthocyanins and α -tocopherol (V.E) were separately dissolved in EtOH to a 1 mmole final concentration, and β -carotene was dissolved in acetone at a final concentration of 2.5 mg/10 mL. Linoleic acid solution was prepared by dissolving 1.2 mg in 1 mL of pH 6 or 7 McIlvaine buffer containing Tween 80 at a concentration of 0.8 mg/mL. Lipoyxygenase (Oriental Co., 126 500 units/mg) was used after diluting with H₂O to a concentration of 1 mg/mL for reaction at pH 6 and of 0.2 mg/mL for reaction at pH 7. An aliquote of 0.1 mL of lipoyxygenase solution was added to a mixture containing 2.4 mL of buffer solution (pH 6 or 7), 0.5 mL of linoleic acid solution, 0.6 mL of β -carotene solution and 0.2 mL of anthocyanin solution. During incubation of this mixture at 20°C, the rate of carotene bleaching was measured at 460 nm. The amount of β -carotene remaining in the reaction mixture at a fixed time after the start of incubation was expressed as percent of the optical density (O.D.) of the reaction mixture at the fixed time to that at 0 min (% of initial). The inhibitory effect of each anthocyanin on carotene bleaching was expressed in terms of inhibitory ratio (I.R.). The I.R. at 5 or 20 min after the start of

Fig. 1 Structure of delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside (nasunin)

incubation was calculated as follows, based on the data at 5 or 20 min after the addition of lipoxygenase: $I.R. (\%) = 100 \times [(100 - P_c) - (100 - P_a)] / (100 - P_c)$, where P_c is the percent of the O.D. at 20 or 5 min to that at 0 min (% of initial) for control (without anthocyanin or V.E.), P_a is that for reaction mixture containing anthocyanin or V.E.

Ferric thiocyanate method: A mixture of 0.9 μ mole of anthocyanin in 2.5 ml 99.5% EtOH, 0.1 ml EtOH and 0.5 ml of linoleic acid solution, prepared by diluting with twice volumes of EtOH, contained in a screw capped test tubes was mixed with previously aerated 1.0 ml of 0.67 M citrate buffer (pH 2 and 4) and 0.4 ml of H₂O. A mixture containing 0.9 μ mole V.E instead of anthocyanin was also

prepared. pH values of these mixtures as determined by glass-electrode pH meter were 2.8 and 4.6, respectively. During incubation of these mixtures at 70°C, 0.1 ml aliquots were drawn at intervals and their hydroperoxide content was determined by ferric thiocyanate method¹⁴⁾. The inhibitory ratio (I.R.) of each anthocyanin on autoxidation of linoleic acid was calculated as follows, based on the data at 24 h after start of the incubation: $I.R. (\%) = 100 \times (D_c - D_a) / D_c$, where D_c is the O.D. for control (without anthocyanin or V.E.), D_a is that for reaction mixture containing anthocyanin or V.E.

Results and Discussion

The relative decrease of O.D. at 460 nm with

Table 1 Antioxidative activity of anthocyanins on carotene bleaching by linoleic acid-lipoxygenase

At pH 6, Incubation time	O.D. at 460 nm (% of initial)					Inhibitory ratio after 20 min (%)
	0	5	10	15	20 min	
Control	100	82	67	56	47	0
+ Nasunin	100	96	93	90	86	74
+ Malvin	100	92	84	78	73	49
+ Rubrobrassicin	100	91	85	80	77	57
+ Delphinidin	100	99	95	92	89	79
+ Malvidin	100	95	92	90	89	79
+ Cyanidin	100	95	93	91	88	77
+ V.E	100	99	98	97	97	94

At pH 7, Incubation time	O.D. at 460 nm (% of initial)					Inhibitory ratio after 5 min (%)
	0	1	2	3	4 5 min	
Control	100	84	68	52	39 28	0
+ Nasunin	100	94	89	82	77 72	61
+ Malvin	100	92	86	78	72 65	51
+ Rubrobrassicin	100	85	73	61	50 42	28
+ Delphinidin	100	92	84	76	67 59	43
+ Malvidin	100	96	92	88	83 79	71
+ Cyanidin	100	98	96	92	88 83	76
+ V.E	100	99	98	97	97 97	96

Reaction mixture containing linoleic acid, β -carotene, lipoxygenase and buffer solution (pH 6 or 7) was incubated at 20°C. The amount of β -carotene remained after fixed times was expressed as percent of the O.D. of reaction mixture at fixed times to that at 0 min (% of initial). Inhibitory ratio (I.R.): $I.R. (\%) = 100 \times [(100 - P_c) - (100 - P_a)] / (100 - P_c)$, where P_c is the percent of initial for control, P_a is that for reaction mixture containing anthocyanin or V.E.

reaction time measured by carotene bleaching method is shown in Table 1. Nasunin, malvin and rubrobrassicin showed antioxidative activities at pH 6 and 7, and the strongest activity among the three anthocyanins was observed in nasunin. The inhibitory ratios of nasunin, malvin and rubrobrassicin were 61, 51 and 28% at pH 7, respectively, and 74, 49 and 57% at pH 6, respectively. These results indicate that the antioxidative activities of nasunin and rubrobrassicin are stronger at pH 6 than at pH 7, and the activity of malvin is almost equal at both pH values. Particu-

larly, the inhibitory ratio of rubrobrassicin sharply decreased along with increasing pH compared with the other two anthocyanins. On the other hand, delphinidin, malvidin and cyanidin, which are the respective aglycones of the three anthocyanins, mentioned above also showed antioxidative activities. Their inhibitory ratios were 43, 71 and 76% at pH 7, respectively, indicating that the antioxidative activities of aglycones except for delphinidin is stronger than those of their respective glycosides at pH 7 as measured by carotene bleaching method.

Fig. 2 Effect of various anthocyanins on autoxidation of linoleic acid

Reaction mixture (pH 2.8 or 4.6) containing linoleic acid and anthocyanin was incubated at 70°C. After fixed times, hydroperoxide content of the reaction mixture was measured by ferric thiocyanate method.

(a) and (b): ●, control without anthocyanin or V.E.; ○, with nasunin; △, with malvin; □, with rubrobrassicin; ▲, with V.E.
(c) and (d): ●, control without anthocyanin or V.E.; ○, with delphinidin; △, with malvidin; □, with cyanidin; ▲, with V.E.

Since lipoxygenase was inactive at pH value below 5, the antioxidative activity of anthocyanin at pH 2.8 and 4.6 was measured by ferric thiocyanate method. Time-courses of the autoxidation of linoleic acid at pH 2.8 and 4.6 in the presence or absence of anthocyanin are shown in Fig. 2 (a)~(d), and the inhibitory ratio of each anthocyanin after 24 h of incubation is shown in Table 2. When the inhibitory ratios of nasunin, malvin and rubrobrassicin after 24 h of incubation at pH 2.8 were compared to one another, nasunin showed the strongest anitoxidative activity, followed by malvin and then, rubrobrassicin (Table 2), indicating that nasunin had the strongest activity at pH 2.8, as well as at pH 6 and 7, in which the activity was measured by carotene bleaching method. The inhibitory ratios of nasunin, malvin and rubrobrassicin, and of their respective aglycones decreased when the pH value of the reaction mixture was increased from pH 2.8 to 4.6 (Fig. 1 (a)~(d), Table 2), indicating that the antioxidative activity decreases along with increasing pH. This trend was the same with that obtained by carotene bleaching method in which the pH value increased from 6 to 7, except for malvin and cyanidin in which the activity was almost equal when measured at both pH 6 and 7 by

Table 2 Inhibitory ratios of various anthocyanins on autoxidation of linoleic acid

	Inhibitory ratio (%)	
	pH 2.8	pH 4.6
Control	0	0
+ Nasunin	98	65
+ Malvin	87	64
+ Rubrobrassicin	81	61
+ Delphinidin	82	39
+ Malvindin	90	73
+ Cyanidin	88	61
+ V.E	93	91

Inhibitory ratio was calculated from the O.D. after 24 h of incubation showed in Fig. 1. See the text for the formula of calculation.

carotene bleaching method. The activity of nasunin at pH 2.8 was slightly stronger than that of V.E.

The inhibitory ratios of aglycones, delphinidin, malvidin and cyanidin after 24 h of incubation at pH 2.8 were 82, 90 and 88%, respectively, and the value of delphinidin (82%) was lower in comparison with that of its glycoside, nasunin (98%) at pH 2.8. This indicates that the antioxidative activity of nasunin considerably decreased by liberation of both *p*-coumaroylrutinoside moiety at 3-position and glucosyl moiety at 5-position of nasunin. On the other hand, the inhibitory ratios of malvidin and cyanidin were not much different from those of their respective glycosides, malvin and rubrobrassicin, (inhibitory ratio : 90 vs 87% for malvidin vs malvin, 88 vs 81% for cyanidin vs rubrobrassicin), in which both malvin and rubrobrassicin have sugar moiety at both 3- and 5- positions. This result, taking into account the fact that the chemical structure of nasunin has sugar and *p*-coumaric acid moieties at 3-position in addition to sugar moiety at 5-position, suggested that *p*-coumaric acid moiety of nasunin may significantly contribute to enhance the activity of nasunin.

Both glycosides and their aglycones showed stronger activities at pH 2.8 than at pH 4.6, indicating that flavylum cation form of anthocyanin may be more effective than colorless carbinol pseudobase, chalcone and quinoidal anhydrobase forms¹⁵⁾. However, demonstration of the antioxidative activity of anthocyanin at pH 6 and 7, measured by carotene bleaching method, shows the possibilities that colorless carbinol pseudobase, chalcone and quinoidal anhydrobase of anthocyanin may also have considerable antioxidative activities.

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長者ナスに含まれるナスニンの 抗酸化効果

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長者ナス（小ナス）から単離したナスニン、および他の2~3のアントシアニン色素が、リノール酸-リポキシダーゼによる β -カロチンの退色、およびリノール酸の自動酸化に及ぼす影響について、それぞれ、カロチン退色法、ロダン鉄法を用いて検討した。pH 7においてカロチン退色法で測定した抗酸化効果はナスニンで最も強く、次いで、マルビン、ルプロブラッシンの順であった。pH 2.8においてロダン鉄法で測定した抗酸化効果も同じ傾向を示した。また、抗酸化効果はアグリコンのディルフィニジン、マルビジンおよびシアニジンにも認められたが、後2者の活性はそれらの配糖体と大差がなかった。ナスニンはディルフィニジンに比べて高い活性を示した。この結果はリノール酸の自動酸化系におけるナスニンの強い活性にそのp-クマール酸部分に関わっている可能性を示した。