Antioxidative Activity of an Isoflavonoid, 2″-O-Glycosylisovitexin Isolated from Green Barley Leaves

Kazumi Kitta, Yoshihide Hagiwara, and Takayuki Shibamoto*

Department of Environmental Toxicology, University of California, Davis, California 95616

A flavonoid, 2″-O-glycosylisovitexin (2″-O-GIV), with antioxidative activity previously proved by the thiobarbituric acid method, was isolated from young green barley leaves. Antioxidative activity of 2″-O-GIV was determined by using gas chromatography to measure amounts of malonaldehyde (MA) formed from squalene and ethyl linoleate upon UV irradiation or with Fenton's reagent. 2″-O-GIV suppressed the formation of MA from squalene upon oxidation with Fenton's reagent 32% at the level of 150 μM/75 μL of squalene. 2″-O-GIV decreased MA formation from ethyl linoleate upon UV irradiation to 40% at pH 7.4; no significant effect was observed at pH 3.5 and 11. 2″-O-GIV (100 μM) decreased MA formation from ethyl linoleate (10 μL) with Fenton's reagent to 59% and 10% at pH 3.5 and 7.4, respectively. 2″-O-GIV showed antioxidative activities almost equal to that of α-tocopherol in all of the experiments.

INTRODUCTION

Flavonoids occur throughout the entire plant kingdom from fungi to angiosperms. Numerous flavonoids have been characterized, and new structures are being reported at an increasing rate. The largest group of flavonoids has a γ-pyrone moiety (flavonols and flavonones). The position of the benzenoid substituent differentiates isoflavonoids (3-position) from flavonoids (2-position). Flavonoids occur as aglycons and glycosides. C-Glycosyl flavonoids, in which the sugar moiety is attached by a carbon–carbon bond directly to the aromatic ring, occur predominantly in the leaves of various cereal crops as derivatives of apigenin and luteolin (Herrmann, 1976). Vitexin, a glucose derivative of apigenin, is the best known of this group (Robinson, 1983). Isovitexin was found in rice hull (Ramarathnam et al., 1989). Recently, 2″-O-glycosylisovitexin (2″-O-GIV) was isolated from young green barley leaves (Osawa et al., 1992).

Many flavonoids and their related compounds reportedly possess strong antioxidative activities. Some flavonoids have demonstrated considerable protection against lipid peroxidation (Pratt, 1979; Pratt and Birac, 1979). Their antioxidative activities are due to their ability to chelate metal ions by means of either the 3-hydroxy, 4-keto grouping or the 5-hydroxy, 4-keto grouping and to scavenge free radicals, deriving from the phenolic moiety of the structure (Pratt, 1976).

In the present study, 2″-O-GIV isolated from green barley leaves was examined for antioxidative activities using lipid peroxidation systems.

EXPERIMENTAL PROCEDURES

Chemicals. Sodium dodecyl sulfate (SDS), hydrogen peroxide, malonaldehyde bis(diethyl acetal), 2-methylpyrazine, and α-tocopherol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethyl linoleate, squalene, Trizma-HCl, Trizma base, and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO). N-Methylhydrazine was bought from Fluka Chemical Co. (Ronkonkoma, NY). N-Methylpyrazole was synthesized according to the method described by Umano et al. (1988).

2″-O-Glycosylisovitexin (2″-O-GIV). 2″-O-GIV was isolated from young green barley leaves (*Hordeum vulgare* L. var. nudum Hook) harvested 2 weeks after germination according to a previously reported method (Osawa et al., 1992). The freeze-dried barley leaves were extracted with 80% ethanol and the extract was then fractionated with column chromatography. The fraction containing the most 2″-O-GIV (60% methanol eluate) was recrystallized with methanol. Pure light yellow 2″-O-GIV was obtained.

UV Irradiation of Squalene and Ethyl Linoleate in the Presence of 2″-O-GIV. An aqueous solution (5 mL) containing 75 μL of squalene or 10 μL of ethyl linoleate and 2% SDS was uniformly emulsified with a Super-mixer (Lab-Line Instrument, Inc.) in test tubes with various amounts of 2″-O-GIV or α-tocopherol; the sample tubes were then irradiated with a Rayonet RPR-100 chamber reactor (Southern New England Ultraviolet Co., Homden, CT) equipped with eight UV lamps (λ = 300 nm) for 6 h. The temperature in the reactor was kept at approximately 35 °C during irradiation. After irradiation, 50 mL of 4% BHT ethanol solution was added to the samples to prevent further oxidation. One sample tube was wrapped with aluminum foil throughout the experiment to obtain a control.

Oxidation of Squalene and Ethyl Linoleate with Fenton's Reagent (Fe^{2+}/H_2O_2) in the Presence of 2″-O-GIV. An aqueous solution (5 mL) containing 75 μL of squalene or 10 μL of ethyl linoleate, 0.25 mmol of Trizma buffer (pH 7.4), 0.75 mmol of potassium chloride, surfactant SDS (2%), 1 μmol of ferrous chloride, and 0.5 μmol of hydrogen peroxide was stirred in test tubes with different amounts of 2″-O-GIV or α-tocopherol, and then the samples were incubated at 55 °C for 16 h. The oxidation of samples was stopped by adding 50 mL of 4% BHT ethanol solution. The incubation system was covered with aluminum foil to avoid any influence of light on the lipid peroxidation system.

Effects of pH on Antioxidative Activity of 2″-O-GIV. Three buffer solutions were prepared to maintain the pH of the lipid peroxidation system: acetic acid/sodium acetate buffer for pH 3.5, Trizma buffer for pH 7.4, and potassium phosphate dibasic/potassium phosphate tribasic for pH 11.0. The lipid peroxidation system containing ethyl linoleate and 2″-O-GIV was irradiated by UV or oxidized by Fenton's reagent at pH 3.5 or 11.0 according to the method described above.

Measurement of Antioxidative Activity. Antioxidative activity was determined by analyzing malonaldehyde (MA) formed from squalene or ethyl linoleate upon oxidation. The gas chromatographic method for MA analysis reported previously (Tamura et al., 1991) was used. MA was reacted with N-methylhydrazine, and then the resulting derivative, 1-methylpyrazole, was analyzed by gas chromatography with a nitrogen–phosphorus specific detector.

RESULTS AND DISCUSSION

Table I shows the effect of 2″-O-GIV and α-tocopherol on formation of MA from squalene upon UV irradiation

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Table I. Effect of 2″-*O*-GIV or α-Tocopherol on Formation of MA from Squalene upon UV Irradiation or with Fenton's Reagent at pH 7.4

	amt of MA formed,[a] nmol/mg of squalene			
	UV irradiation		Fenton's reagent at pH 7.4	
antioxidant concn, μM	2″-*O*-GIV	α-tocopherol	2″-*O*-GIV	α-tocopherol
0	1.08 ± 0.03	1.08 ± 0.03	0.94 ± 0.05	0.94 ± 0.05
20	1.23 ± 0.06	1.27 ± 0.04	0.77 ± 0.02	0.75 ± 0.02
50	0.89 ± 0.03	1.05 ± 0.05	0.72 ± 0.07	0.71 ± 0.04
100	1.03 ± 0.06	1.17 ± 0.07	0.69 ± 0.05	0.59 ± 0.01
150	0.99 ± 0.07	0.99 ± 0.04	0.64 ± 0.01	0.66 ± 0.03

[a] Values are mean ± sD (n = 4).

Table II. Effect of 2″-*O*-GIV or α-Tocopherol on MA Formation from Ethyl Linoleate upon UV Irradiation at Different pH Values

	amt of MA formed,[a] nmol/mg of ethyl linoleate					
	pH 3.5		pH 7.4		pH 11	
antioxidant concn, μM	2″-*O*-GIV	α-tocopherol	2″-*O*-GIV	α-tocopherol	2″-*O*-GIV	α-tocopherol
0	6.29 ± 0.61	6.29 ± 0.61	12.17 ± 1.12	12.17 ± 1.21	4.70 ± 0.56	4.70 ± 0.56
20	6.70 ± 0.13	6.96 ± 0.45	9.17 ± 0.89	8.25 ± 1.09	4.62 ± 0.18	4.73 ± 0.98
50	5.81 ± 0.48	6.56 ± 0.63	7.20 ± 0.17	7.08 ± 1.01	4.65 ± 0.72	4.64 ± 0.78
100	6.78 ± 0.03	6.43 ± 0.68	7.53 ± 0.25	7.46 ± 0.40	4.68 ± 0.57	5.05 ± 0.78

[a] Values are mean ± sD (n = 4).

Table III. Effect of 2″-*O*-GIV or α-Tocopherol on MA Formation from Ethyl Linoleate with Fenton's Reagent at Different pH Values

	amt of MA formed,[a] nmol/mg of ethyl linoleate			
	pH 3.5		pH 7.4	
antioxidant concn, μM	2″-*O*-GIV	α-tocopherol	2″-*O*-GIV	α-tocopherol
0	68.30 ± 5.95	68.30 ± 5.95	28.92 ± 2.57	28.92 ± 2.57
20	49.77 ± 5.17	49.59 ± 5.91	2.43 ± 0.43	2.54 ± 0.23
50	40.57 ± 3.95	42.93 ± 4.29	2.42 ± 0.18	2.51 ± 0.25
100	40.21 ± 7.43	42.74 ± 1.80	2.51 ± 0.08	2.53 ± 0.13

[a] Values are mean ± sD (n = 4).

and Fenton's reagent at pH 7.4. A significant effect of 2″-*O*-GIV upon UV-irradiated squalene was not observed in its concentration range of 20–150 μM. Compared with the control sample, α-tocopherol also did not significantly alter this system. The lack of antioxidative activity in this system may be due to the solubility of squalene in aqueous solution. Squalene was not uniformly suspended in the solution even with a surfactant. It is possible that this resulted in the insufficient absorption of UV light. The MA level arising from squalene had an inverse relationship to the concentration of the antioxidants with Fenton's reagent. 2″-*O*-GIV demonstrated the maximum oxidation suppression of 32% at the level of 150 μM. 2″-*O*-GIV and α-tocopherol showed almost equal antioxidative activity in the present test system.

Table II shows the effect of 2″-*O*-GIV and α-tocopherol on MA formation from ethyl linoleate upon UV irradiation at different pH values. No significant effects were observed when the samples were irradiated with 2″-*O*-GIV or α-tocopherol at pH 3.5 and 11. However, with 100 μM at pH 7.4, 2″-*O*-GIV or α-tocopherol suppressed approximately 40% MA formation.

In the control samples MA formation from UV-irradiated ethyl linoleate was suppressed by 48% in an acidic solution, compared to the solution adjusted at pH 7.4. Under basic conditions, MA formation decreased to 61%. The difference may be due to the pH dependence of singlet oxygen production. It has been reported that the yield of singlet oxygen formation is a function of pH, owing to the various ionic species present (Pottier et al., 1975). Changes in pH possibly cause the alteration of the electron distribution in both the lipid and the photosensitizer molecules, which may affect the rate of reactions such as excitation of sensitizers, the formation of singlet oxygen, and the oxidation initiation step.

Table III shows the effect of 2″-*O*-GIV and α-tocopherol on MA formation from ethyl linoleate with Fenton's reagent at pH 3.5 and 7.4. In the case of control samples, the formation of MA at pH 3.5 was much greater than that at pH 7.4. However, Fenton's reaction was completely inhibited under the basic conditions. This is quite reasonable because Fenton's reagent produces 1 mol each of $OH^{\bullet}$ and OH^{-} as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$$

Therefore, in an acidic condition, the reaction proceeds in the forward direction to produce more hydroxy radical; in a basic condition, the reaction proceeds in the opposite direction. Because MA formation in the basic condition was completely inhibited, the effects of only low pH over antioxidative activity were studied. MA levels gradually decreased as the concentration of the antioxidant increased and the maximum suppression, a 41% decrease, was obtained by 100 μM 2″-*O*-GIV or α-tocopherol at pH 3.5, whereas over 90% suppression was obtained by 20–100 μM 2″-*O*-GIV or α-tocopherol at pH 7.4. 2″-*O*-GIV is less active in a low pH aqueous solution. This may be due to protonation of 5-hydroxy and 4-keto groups on 2″-*O*-GIV under acidic conditions; consequently, these groups lose the chelating ability toward metal ions.

Although biological systems require active oxygen species, such as hydroxy radical, superoxide, and singlet oxygen, those species may be involved in functional damage to organisms and may lead to pathological complications. Besides intrinsic oxidation-protecting enzyme systems,

such as superoxide desmutase, catalase, and glutathione peroxidase, certain food-derived substances, such as tocopherols, ascorbic acid and carotenes, have been shown to diminish the adverse effects of oxidation in organisms. Investigation of the effectiveness of 2″-O-GIV as an in vivo antioxidant is in order.

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