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Chapter 17

Possible Inhibition of Atherosclerosis by a Flavonoid Isolated from Young Green Barley Leaves

Takashi Miyake[1], Yashihide Hagiwara[1], Hideaki Hagiwara[2], and Takayuki Shibamoto[1,3]

[1]Department of Environmental Toxicology, University of California, Davis, CA 95616

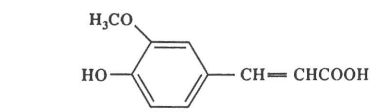
[2]Hagiwara Institute of Health, 1173 Maruyama, Asazuma-cho, Kasai 679-01, Japan

Young green barley leaves are known to possess potent pharmacological properties, including antioxidative, anti-inflammatory, antimutagenic, and antiallergic activities. In particular, an flavonoid, 2"-O-glycosylisovitexin (2"-O-GIV), isolated from an ethanol extract of young green barley leaves, possesses a strong inhibitory effect toward lipid peroxidation. 2"-O-GIV inhibited acetaldehyde formation from LDL by 76% at a level of 1 μmol/50 μg, whereas ferulic acid inhibited by 66% at the same level. In a case of a blood plasma system, 2"-O-GIV and probucol inhibited acetaldehyde formation by 89% and 94%, respectively, at a level of 3 μmol. 2"-O-GIV and vitamin C inhibited MDA formation by 54% and 32%, respectively, at a level of 0.1 μmol. A synergetic effect between 2"-O-GIV and vitamin C was observed.

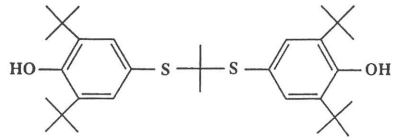
Barley has been cultivated and fed to livestock since ancient times. Essence extracted from young green barley leaves has been reported to exhibit many biological characteristics including anti-aging, anti-carcinogenesis, anti-diabetic, and anti-arteriosclerosis (*1*). However, no scientific proof of these characteristics existed until a potent anti-oxidant was isolated and identified in a green barley leaf essence (*2*). This novel natural antioxidant, which is a flavonoid, was identified as 2"-O-glycosyl isovitexin (2"-O-GIV, Figure 1). Since the discovery of this flavonoid, the antioxidative activities of 2"-O-GIV examined in various lipid peroxidation systems including squalene/UV (*3*), ethyl ester of fatty acids/Fenton's reagent (*4*), phospholipids or cod liver oil/Fenton's reagent (*5*), and ω-3 fatty acids/Fenton's reagent have been reported(*6*).

Lipid peroxidation model systems have been used most commonly to investigate biological activities of chemicals because lipid peroxidation is associated with many biological complications such as carcinogenesis, mutagenesis, aging, and atherosclerosis (*7-9*) as well as with human immunodeficiency virus (HIV) progression (*10*). However, its mode of toxic action is not yet clearly understood. Lipids produce many low molecular weight carbonyl compounds upon oxidation (*11*). Therefore, these carbonyl compounds such as malondialdehyde (MDA), glyoxal, acrolein, acetaldehyde, and formaldehyde which directly crosslink to proteins and bind

[3]Corresponding author.



Ferulic acid



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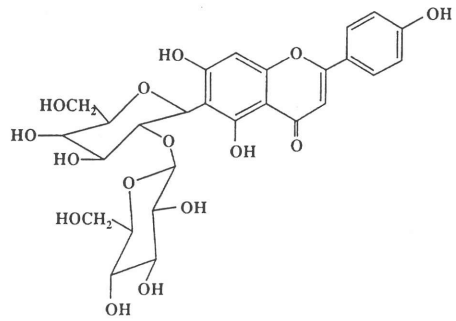

2"-O-Glycosylisovitexin (2"-O-GIV)

Figure 1. Structure of ferulic acid, probucol, and 2"-O-glycosyl isovitexin.

covalently to nucleic acids (*12*) may possibly play an important role in the toxic effects caused by lipid peroxidation (*13, 14*).

In addition to initiating these adverse effects, low molecular weight aldehydes formed from lipid peroxidation can be used as indicators to detect oxidation in lipids. Therefore, many studies have been conducted using these aldehydes as indicator, in particular, malondialdehyde (MDA). The formation of acetaldehyde was also used to monitor oxidative reaction mechanisms of L-ascorbic acid (*15*).

In the present study, the antioxidative activity of 2"-O-GIV was examined using low density lipoprotein and blood plasma oxidized with Fenton's reagent.

Experimental

Chemicals. L Ascorbic acid (reagent grade), butylated hydroxytoluene (BHT), Trizma® hydrochloride, Trizma® base, fatty-acid-free bovine serum albumin, probucol, and fat red 7B were purchased from Sigma Chemical Co. (St. Louis, MO). Cysteamine hydrochloride, 2,4,5-trimethylthiazole, ferulic acid, sodium dodecyl sulfate (SDS), hydrogen peroxide, and ferrous chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hydrogen peroxide was obtained from Fisher Scientific Co., Ltd. (Fair Lawn, NJ). The standard stock solution of 2,4,5-trimethylthiazole was prepared by adding 10 mg of 2,4,5-trimethylthiazole to 1 mL of dichloromethane and was stored at 5 °C. Authentic 2-methylthiazolidine was synthesized according to the method reported previously (*16*). The structures of probucol and ferulic acid are shown in Figure 1.

2"-O-Glycosylisovitexin (2"-O-GIV) was isolated from young green barley leaves (*Hordium vulgare L. var. nudum Hook*) harvested two weeks after germination by a method previously reported (*2*) using column chromatography with Amberlite XAD-2 nonionic polymeric absorbent. The structure of 2"-O-GIV is shown in Figure 1.

Preparation of Low-Density Lipoprotein (LDL). LDL was prepared from blood sample obtained from a male quarter horse (5 yeare old) according to the method reported previously (*15*). After filtration sterilization (0.45 μm; Nalge Stbron) of the LDL, the protein concentration was determined by the Coomassie Blue dye-binding assay (*17*). A 10-μL aliquot of appropriately diluted LDL was added to the dye reagent, the solution mixed, and absorbance at 594 nm measured versus a reagent blank, using a Hewlett-Packard 8452A diode array UV spectrophotometer. A standard curve using bovine serum albumin was used to calculate the LDL concentration.

Preparation of Blood Plasma. The blood from a male quarter horse (5 years old) was collected in a sterile, 3.5-mL tube containing 60 μL of a 7.5% EDTA solution (4.5 mg EDTA). Plasma and red blood cells were separated following centrifugation (5000 rpm for 30 min at 4 °C) and immediately frozen on dry ice. The plasma was stored at - 80 °C until use.

Oxidation of LDL and Blood Plasma With Fenton's Reagent With or Without Antioxidants. A 5-mL aqueous solution containing 23 μL of LDL (final concentration, 2.17 μg/mL), 0.25 mmol of Trizma® buffer (pH 7.4), 0.75 mmol of potassium chloride, 1 mmol of ferrous chloride, and 0.5 mmol of hydrogen peroxide was incubated with 2"-O-GIV (amounts ranging from 0 to 1 μmol, with 0.1 μmol increments) or ferulic acid (amounts ranging from 0 to 1 μmol, with 0.1 μmol increments) at 37 °C for 15 h.

In a separate experiment, samples containing blood plasma (516 μg of protein), 0.25 mmol of Trizma buffer (pH 7.4), 0.75 mmol of potassium chloride, 1 mmol of ferrous chloride, 0.5 mmol of hydrogen peroxide, and 10% of surfactant SDS were incubated with 2"-O-GIV (0, 0.05, 0.07, 0.1, 0.2, and 0.3 μmol) or probucol (0, 0.05, 0.07, 0.1, 0.2, and 0.3 μmol) at 37 °C for 15 h. In another experiment, samples containing blood plasma (860 μg of protein), 0.25 mmol of Trizma® buffer (pH 7.4), 0.75 mmol of potassium chloride, 1 mmol of ferrous chloride, and 0.5 mmol of hydrogen peroxide were incubated with 2"-O-GIV (amount ranging from 0 to 0.1 μmol with 0.01 μmol increment), vitamin C (amounts ranging from 0 to 0.1 μmol, with 0.02 μmol increments), or a mixture of 2"-O-GIV and vitamin C (total amounts ranging from 0 to 0.1 μmol, with 0.01 μmol increments), in which molar ratio of 2"-O-GIV and vitamin C was 1/1.

While the incubation continued, the mixtures were covered with parafilm. A 5-mL solution containing exactly the same materials except for the ferrous chloride and the hydrogen peroxide was incubated in the same manner as a control sample. Oxidation of the samples was stopped by adding 50 μL of 4% BHT ethanol solution. The incubation system was covered with aluminum foil to avoid any influence of light on the LDL and blood plasma peroxidation systems. The all experiments were replicated three times.

Analysis of Acetaldehyde and MDA. The amount of acetaldehyde was determined as a thiazolidine derivative according to the method reported previously (*18*). The amount of MDA was measured as a pyrazole derivative by the method developed previously (*19*). A Hewlett-Packard Model 5890 GC equipped with a nitrogen phosphorus detector (NPD) and a 30 m x 0.25 mm i.d. (d_f = 1 μm) DB-1 bonded-phase fused silica capillary column (J & W Scientific, Folsom, CA) was used for quantitative analysis of acetaldehyde and MDA. The detector and injector temperatures were 250 °C. The linear velocity of the helium carrier gas was 30 cm/sec with a split ratio of 21:1. The oven temperature was programmed from 60 to 180 °C at 4 °C/min and held for 10 min. GC peak areas were integrated with a Tsp SP 4400 series integrator. An HP Model 5890 series II GC interfaced to a HP 5971 mass spectrometer was used to confirm the identity of the thiazolidine derivative of acetaldehyde, 2-methylthiazolidine, and pyrazole derivative of MDA, 1-methylpyrazole, in the samples. The GC conditions were the same as for the GC with NPD. The mass spectra were obtained by electron impact ionization at 70 eV with an ion source temperature of 250 °C.

Results and Discussion

Oxidative damages caused by reactive oxygen species have been known to initiate and to promote many diseases, such as cancer (*20*), cardiovascular disease (*21*), and atherosclerosis (*22*). A strong relationship between atherosclerosis and amounts of lipid peroxidation products in the inside wall of arteries has been reported (*23, 24*). Recently, oxidative damage of LDL has received much attention as a process which implicates the development of human atherosclerosis (*22*). Therefore, LDL has been widely used to investigate the relationship between lipid peroxidation and atherosclerosis (*25*).

LDL is generally prepared from blood lipoprotein using a centrifuge. Figure 2 shows fractions of lipoprotein prepared according to their density (*26*). Figure 3 shows compositions of various lipoprotein fractions (*26*). LDL contains greatest

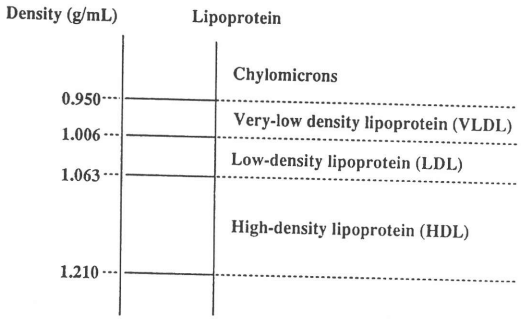

Figure 2. Fractions of lipoprotein.

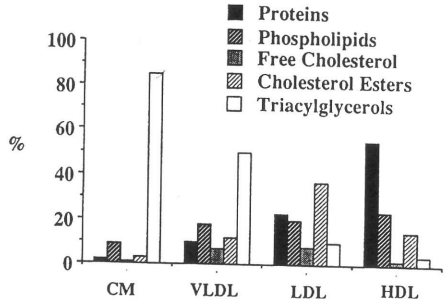

Figure 3. Compositions of various lipoprotein fractions.

amount of cholesterol esters which may be associated with the development of atherosclerosis. Figure 4 shows the inhibitory effect of 2"-O-GIV and ferulic acid against LDL oxidation. Acetaldehyde was used to monitor the formation and inhibition of lipid peroxidation because MDA tends to be trapped with proteins. Over 2 nmol of acetaldehyde was formed from 50 μg of LDL. Formation of acetaldehyde decreased when the amount of either 2"-O-GIV or ferulic acid was increased. Ferulic acid inhibited acetaldehyde formation by 50% at the level of 0.3 μmol/50 μg of LDL) whereas 2"-O-GIV required 0.7 μmol/50μg of LDL to obtain the same level of inhibition. On the other hand, 2"-O-GIV inhibited acetaldehyde formation by 76% at the level of 1 μmol/50 μg whereas ferulic acid inhibited by 66% at the same level.

Figure 5 shows the antioxidative activities of 2"-O-GIV and probucol measured in a blood plasma system. Probucol was used to examine the relative antioxidative activity of 2"-O-GIV because it is a commercial product with a million dollar sales in Japan and has been used to treat atherosclerosis. However, probucol produced a significant amount of MDA by oxidation (T. Miyake and T. Shibamoto, unpublished data). Therefore, acetaldehyde was used as an indicator of oxidation of blood plasma. The antioxidative activities of 2"-O-GIV and probucol were almost identical. When blood plasma (516 μg) was oxidized without an antioxidant, 135 nmol of acetaldehyde was recovered. Inhibitory activity of both 2"-O-GIV and probucol toward acetaldehyde formation increased greatly when their levels increased over 0.7 μmol. 2"-O-GIV and probucol inhibited acetaldehyde formation by 89% and 94%, respectively, at the level of 0.3 μmol). The results indicate that 2"-O-GIV may inhibit atherosclerosis.

Figure 6 shows the antioxidative activities of 2"-O-GIV and vitamin C in a blood plasma system. When blood plasma was oxidized with Fenton's reagent, 1.11 nmol/μg (blood plasma) of MDA was recovered. However, no MDA was recovered from unoxidized blood plasma. In this experiment, MDA was used as an indicator of oxidation because vitamin C itself produced a significant amount of acetaldehyde by oxidation with Fenton's reagent (*15*). 2"-O-GIV and vitamin C inhibited MDA formation by 54% and 32%, respectively, at the level of 0.1 μmol. On the other hand, when equal mols of 2"-O-GIV and vitamin C were mixed, a 75% inhibitory effect was obtained at the level of 0.1 μmol (total). A synergetic effect between 2"-O-GIV and vitamin C was observed. The antioxidative activities of flavonoid such as 2"-O-GIV are due to their ability to chelate metal ions (such as Fe2+) by means of either the 3-hydroxy, 4-keto grouping or the 5-hydroxy, 4-keto grouping, in addition to scavenging free radicals deriving from the phenolic moiety of the structure (*27*).

Conclusion

Atherosclerosis is one of the most common diseases in developed countries, such as Japan and the U.S., where people tend to eat more fatty foods. Detail mechanisms of atheroscrelosis are not yet clearly understood but there is a strong evidence that lipid peroxidation plays an important role in the initiation of atheroscrelosis. Therefore, antioxidants such as probucol have been used to treat atheroscrelosis. However, use of synthetic antioxidants (e.g., butylated hydroxy toluene) in human foods has been restricted because of their possible chronic toxicity. 2"-O-GIV is a natural plant product and large amounts of barley leaves (which contain 0.5-0.7% of 2"-O-GIV) have been consumed by livestock for many years without any evidence of adverse effects. 2"-O-GIV may be useful in the treatment of atherosclerosis. Therefore, further investigation of its physiological effects should be undertaken.

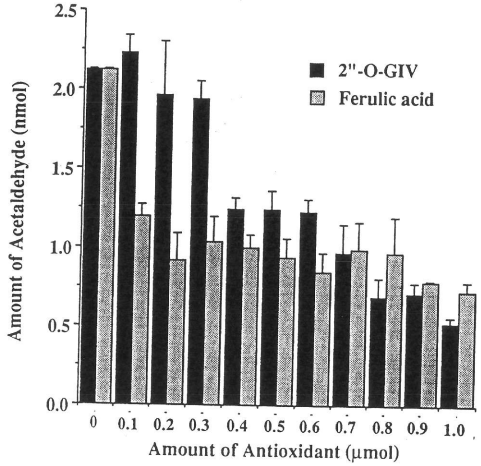

Figure 4. Inhibitory effect of 2"-O-GIV and ferulic acid on LDL oxidation.

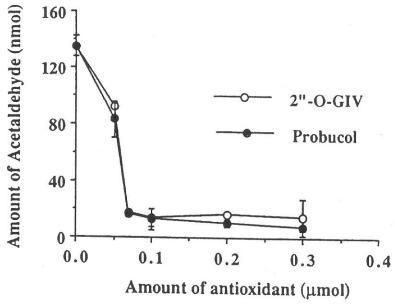

Figure 5. Antioxidative activities of 2"-O-GIV and probucol measured in blood plasma.

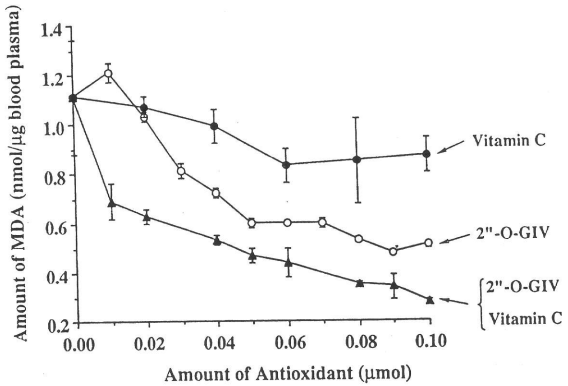

Figure 6. Antioxidative activities of 2"-O-GIV and vitamin C in blood plasma.

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MEASUREMENT OF FUNCTIONAL SUBSTANCES