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Quercetin-4′-glucoside Is More Potent than Quercetin-3-glucoside in Protection of Rat Intestinal Mucosa Homogenates against Iron Ion-Induced Lipid Peroxidation

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Quercetin is a typical antioxidative flavonoid found in vegetables, which is more commonly present as its glucosides, quercetin-3-glucoside (Q3G) and quercetin-4′-glucoside (Q4′G). The main aim of this study was to estimate the antioxidant activity of Q3G and Q4′G on iron ion-driven lipid peroxidation of the gastrointestinal mucosa. Q4′G markedly suppressed the lipid peroxidation when rat gastrointestinal mucosa homogenates were incubated with $Fe(NO_3)_3$ and ascorbic acid. Its effectiveness was greater as compared to that of Q3G and comparable to that of quercetin aglycone. Furthermore, Q4′G yielded higher amounts of quercetin aglycone than Q3G on incubation with the homogenates. However, Q4′G showed a lower chelating activity in comparison to Q3G. These results indicate that Q4′G, even though it has a low chelating activity, because of its efficient conversion to antioxidative aglycone on exposure to the mucosa, can act as a powerful antioxidant on iron ion driven lipid peroxidation in the intestinal mucosa. Thus, vegetables rich in Q4′G, such as onion, are likely to serve as favorable antioxidant sources for suppressing iron-induced oxidative stress in the intestinal tract.

KEYWORDS: Quercetin glucoside; intestinal mucosa; lipid peroxidation; chelating activity; rat

INTRODUCTION

Quercetin, 3,3′,4′,5,7-pentahydroxyflavone, is a typical flavonoid widely distributed in vegetables. In recent years, potent antioxidant activity of quercetin and other flavonoids has attracted much attention due to the beneficial effect of vegetables on health (*1*, *2*). Quercetin is well-known as a powerful free radical scavenger and as a chelating agent that inactivates metal iron responsible for the generation of reactive oxygen species. However, dietary quercetin is mostly present as its glycoside, an "inactive" form as an antioxidant in which one or more sugar groups are bound to its phenolic group by a glycosidic linkage (*3*). A variety of flavonoid glycosides are absorbed into the body after deglycosylation by enterobacteria (*4*). Recent studies on intestinal quercetin glucosides metabolism indicate that the enterocyte itself has the metabolic ability to absorb quercetin glucosides, which includes transport and hydrolysis (*5*). Nevertheless, a large part of dietary quercetin is not absorbed by the intestine and is excreted into the feces (*6*), during which they interact with other nutrients and nonnutrients present in the intestinal lumen. Thus, quercetin not only has a beneficial effect when absorbed into the intestine but also in its unabsorbed form in the gastrointestinal lumen (*7*).

The gastrointestinal mucosa are constantly exposed to a variety of prooxidants derived from ingested foods. In addition, the mastication and digestion of lipid-containing foods in the gastrointestinal fluids can induce lipid peroxidation, due to the exposure of unsaturated lipids to catalytic heme and/or nonheme irons (*8*, *9*). Carbonyl compounds, byproducts of lipid peroxidation such as 4-HNE, are potentially toxic compounds (*10*−*12*), and their accumulation in the body can trigger deleterious effects associated with mucosal inflammation and development of cancer (*13*−*15*). The gastrointestinal mucosa are undoubtedly a physiological target for the antioxidant activity of flavonoids, as the concentration of dietary quercetin is much higher in the lumen of gastrointestinal tract than in plasma and other tissues in the body (*6*). Therefore, it is of much interest to study the preventive action of quercetin glycosides on diet-derived lipid peroxidation occurring in the gastrointestinal mucosa.

For this study, we selected Q3G and Q4′G (**Figure 1**), which are the typical monoglucosides in vegetables, and studied their antioxidant effectiveness on ferrous ion-induced lipid peroxidation of the gastrointestinal mucosa in comparison to its aglycone. Q3G is commonly distributed in a wide variety of

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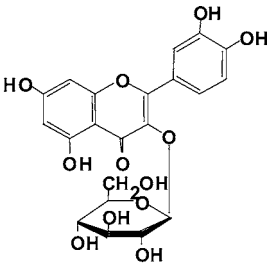
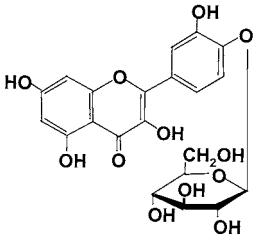


Quercetin 3- *O*-β-glucoside
(Q3G)



Quercetin 4 '-*O*-β-glucoside
(Q4'G)

Figure 1. Structures of Q3G and Q4′G.

vegetables, whereas Q4′G is uniquely present in onion (*16*). The results indicate that Q4′G is superior to Q3G in the suppression of mucosal lipid peroxidation, although free radical scavenging activity of Q4′G is known to be much lower than that of Q3G (*17*).

MATERIALS AND METHODS

Materials. Quercetin, hexanal, 4-HNE, and deferoxamine mesylate salt were obtained from Sigma Chemical Company (St. Louis, MO). Q3G and Q4′G were chemically synthesized from quercetin and 2,3,4,6-tetra-*O*-acetyl-α-glucopyranosyl bromide using the Koenigs−Knorr reaction (*18*); detailed procedures were published elsewhere (*16*). In brief, quercetin and tetraacetyl-α-1-bromoglucose were dissolved in dimethyl sulfoxide and then stirred overnight after the addition of potassium carbonate. The resulting mixture was then adjusted to an acidic condition by adding a few drops of formic acid. The precipitate in the acidic solution was separated by centrifugation. The precipitate was then washed and concentrated in vacuo. Sodium methylate solution was added to the residue and kept at room temperature for 20 min. The solution was then neutralized and filtered. The filtrate was then chromatographed on a column of Toyopearl HW 40 (Tosoh, Tokyo, Japan). The quercetin glucoside fractions were further purified by preparative high-performance liquid chromatography (HPLC) on an ODS column. Each quercetin glucoside peak was collected and identified by SIMS and NMR analyses. The purity of the each preparation was more than 98%. All other chemicals and solvents were of analytical grade and used without purification.

Mucosa Homogenate Preparation. Male Wistar rats (8 weeks old) weighing about 200 g were purchased from Japan SLC Co. (Hamamatsu, Japan). Rats were deprived of food for approximately 20 h and sacrificed by decapitation under anesthesia. The stomach, small intestine (jejunum and ileum), and large intestine were removed and flushed with cold saline to remove intestinal contents. Mucosa were scraped with a glass plate and washed with cold saline (*19*). The mucosa obtained from one or two rats were homogenized with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.135 M KCl (1.0 mL per 0.5 g of the mucosa) using Potter-Elvehjem tissue homogenizer (3000 rpm, 2 min) followed by ultrasonication for 1 min (Branson ultrasonifier). The protein concentration of each homogenate was adjusted to 5.0 mg/mL by diluting with 0.1 M Tris-HCl buffer, pH 7.4. Protein concentration was determined using the Bradford assay, and bovine serum albumin was used as a standard (*20*). All procedures of the homogenate preparation were performed on ice, and the homogenates were stored at −30 °C until use.

Mucosa Homogenate Oxidation and Measurement of Lipid Peroxidation Level. An aliquot (0.6 mL) of each mucosa homogenate was first diluted with distilled water (2.1 mL), to which quercetin or its glucosides was added (15 μL of 5 mM quercetin or its glucoside in methanol solution with a final concentration of 25 μM). For the control, an equivalent volume of methanol (15 μL) was added to the homogenate. Oxidation was started by the addition of 0.3 mL of the mixed solution of $Fe(NO_3)_3$ and ascorbic acid (final concentrations; 100 μM and 1 mM, respectively) at 37 °C with continuous shaking. After 4 h, the peroxidation level was determined with the TBA−fluorometric assay (*21*). Hexanal and 4-HNE were measured as additional lipid peroxidation markers (*22*). In brief, an aliquot of the mixture (0.13 mL) was mixed with 70 μL of freshly prepared dinitrophenylhydrazine reagent (0.35 mg/mL in 1 M KCl) and incubated in the dark for 2 h at room temperature. The reaction mixture was extracted with chloroform and methanol (1:2, v/v) and subjected to centrifugation. The chloroform layer was collected and evaporated in vacuo. The residue was then dissolved in methanol and injected into a Shimadzu HPLC LC-10AS equipped with an ODS-80Ts column (4.5 mm × 150 mm, Tosoh). Methanol and water (31:9, v/v) were used as the eluting solvent with a flow rate of 1.0 mL/min. Eluate was detected at the absorption wavelength of 378 nm. Peak assignment of 4-HNE and hexanal and their quantification were done in comparison with their respective standard compounds.

Quantification of Quercetin and Its Glucosides in the Reaction Mixture. Quercetin and its glucosides in the reaction mixture were quantified after incubating the mucosa homogenates with or without $Fe(NO_3)_3$ by reversed phase HPLC as reported previously (*23*). Ascorbic acid (the final concentration, 1 mM) was added to the mucosa either with or without $Fe(NO_3)_3$ in order to prevent the autoxidative decomposition of quercetin and its glucosides during incubation. Briefly, they were extracted from the reaction mixture with methanol and injected into HPLC with a C18 column after the concentration by evaporation. The mobile phase of methanol/water/acetic acid (46:52:2, v/v/v) was used. The eluate was monitored with a UV detector at 365 nm. Kaempferol was used as an internal standard for calculating their concentrations.

Interaction of Quercetin and Its Glucosides with Ferrous Ion. Solutions of quercetin or its glucosides (10 mM) in methanol were prepared and diluted to 25 μM with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.135 M KCl. Their absorption spectra were recorded between 300 and 550 nm (*24*). Additional scans were taken at 30 and 180 min after the addition of $Fe(NO_3)_3$ and ascorbic acid (final concentrations, 25 and 250 μM) to the diluted 25 μM quercetin or its glucosides. Furthermore, the effect of deferoxamine salt on the absorption spectra was measured by adding 250 μM deferoxamine salt to the 25 μM quercetin or its glucoside solution 10 s after the addition of $Fe(NO_3)_3$ and ascorbic acid.

Data Analysis. Data were represented as the means ± SD of three independent experiments. Results in **Figure 2B**,**C** were shown as the means of two independent experiments. Statistical analysis was evaluated by one way analysis of variance or the Kruskal−Wallis nonparametric test when appropriate, followed by the Student−Newman−Keuls or Bonferroni/Dunn posthoc multiple comparison. Differences with $p < 0.05$ were considered significant.

RESULTS

Effect of Quercetin Glucosides on Lipid Peroxidation of Mucosa Homogenates. TBARS and its aldehydes, hexanal and 4-HNE, were measured as indices of lipid peroxidation level in the gastrointestinal mucosa. As seen in **Figure 2A−C**, addition of $Fe(NO_3)_3$ and ascorbic acid extensively elevated the degree of lipid peroxidation, which increased in the order of stomach < large intestine < small intestine. Thus, the small intestinal mucosa were most susceptible to $Fe(NO_3)_3$ and ascorbic acid-induced lipid peroxidation under the experimental conditions used in this study. Furthermore, quercetin aglycone

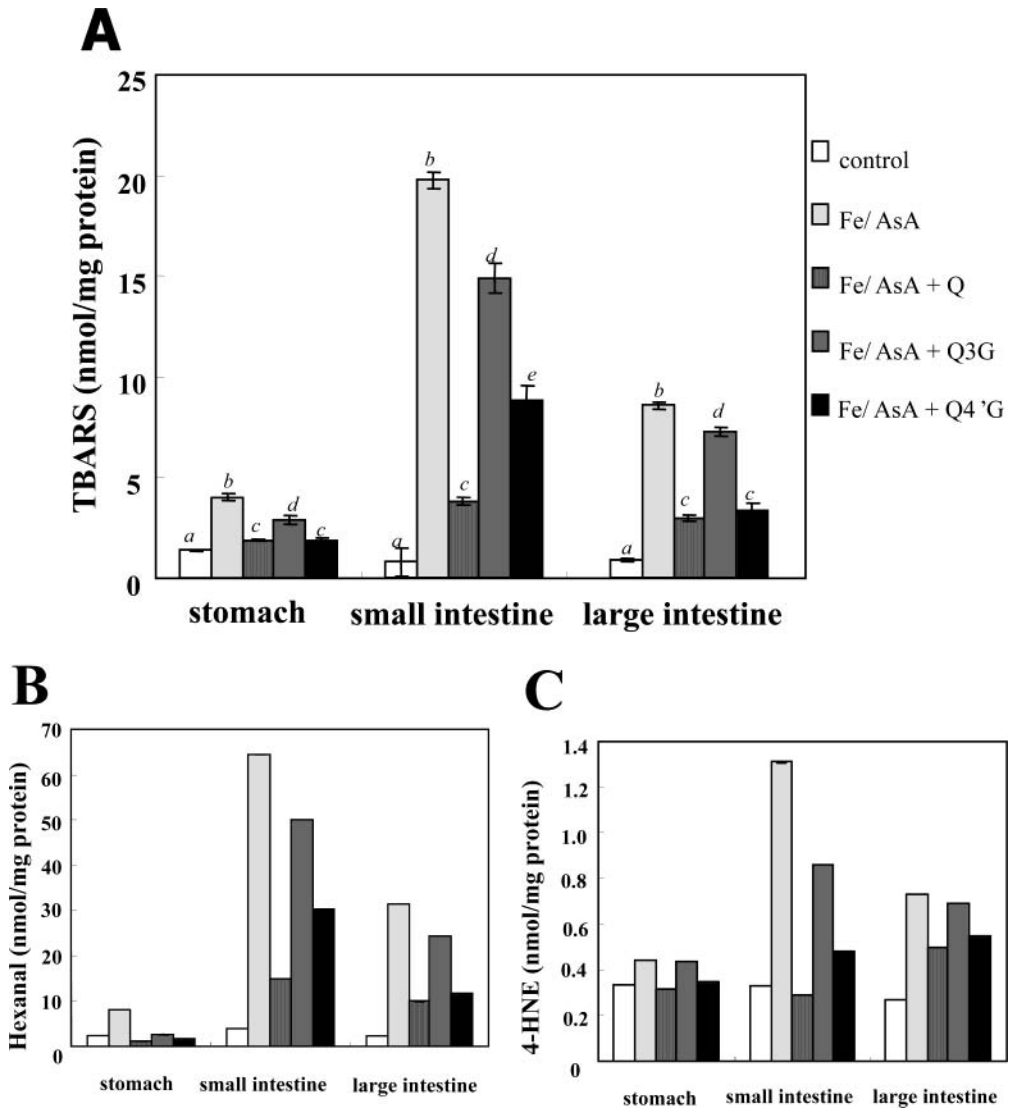


Figure 2. Effect of quercetin glucosides on $Fe(NO_3)_3$ and ascorbic acid induced lipid peroxidation of gastrointestinal mucosa. (**A**) TBARS, (**B**) hexanal, and (**C**) 4-HNE. Each mucosal homogenate (5 mg/mL) was diluted (5-fold) with distilled water, and then, lipid peroxidation was started by addition of $Fe(NO_3)_3$ and ascorbic acid (final concentrations: 100 μM and 1 mM, respectively) in the presence or absence of 25 μM quercetin aglycone/25 μM Q3G/25 μM Q4′G at 37 °C with continuous shaking. Fe, $Fe(NO_3)_3$; AsA, ascorbic acid; and Q, quercetin aglycone. Results in panel **A** are the means ± SD of three independent experiments. Means with different letters for each mucosa are significantly different. Results in panels **B** and **C** are the means of two independent experiments.

and its glucosides, Q3G and Q4′G, significantly suppressed the accumulation of TBARS (**Figure 2A**). A similar suppressive effect of quercetin aglycone and its glucosides on accumulation of hexanal (**Figure 2B**) and 4-HNE (**Figure 2C**) was observed. The order of the effectiveness of lipid peroxidation suppression increased in the order of Q3G < Q4′G ≤ quercetin aglycone.

Recovery of Quercetin Aglycone and Its Glucosides after Incubation with Mucosal Homogenates. Recovery of quercetin and its glucosides after incubation with mucosa homogenate in the presence and absence of $Fe(NO_3)_3$ is shown in **Figure 3**. The levels of quercetin and its glucosides, Q3G and Q4′G, were adjusted to 25 nmol/mg protein prior to incubation for each experiment. The recovery of quercetin aglycone on addition of $Fe(NO_3)_3$ was significantly reduced in the stomach, small intestine, and large intestine mucosa, indicating that the aglycone was being used as an antioxidant during the incubation with $Fe(NO_3)_3$.

Both Q3G and Q4′G yielded quercetin aglycone in the absence of $Fe(NO_3)_3$. Interestingly, aglycone was formed at a significantly higher ratio from Q4′G than from Q3G. Total recovery of Q4′G in the presence of $Fe(NO_3)_3$ decreased significantly as compared to that in the absence of $Fe(NO_3)_3$ in all of the mucosa homogenates, whereas total Q3G recovery showed a significant decrease only in the small intestinal mucosa homogenate. A large proportion of Q4′G was converted into its aglycone in the small intestinal mucosa homogenate as compared to those in the gastric and large intestinal mucosa homogenates (97.9 ± 1.8, 53.0 ± 10.0, and 79.9 ± 5.6%, respectively, $p < 0.05$). Furthermore, quercetin aglycone derived from Q4′G in the presence of $Fe(NO_3)_3$ was significantly decreased as compared with that in the absence of $Fe(NO_3)_3$ in all of the mucosa homogenates. In contrast, glucoside forms incubated with $Fe(NO_3)_3$ did not decrease in either mucosa homogenate.

Chelating Activity of Quercetin Aglycone and Its Glucosides. Figure 4 exhibits the absorption spectra of quercetin aglycone (**A**), Q3G (**B**), and Q4′G (**C**) in the absence or presence of $Fe(NO_3)_3$ and ascorbic acid. The maximum wavelength at nearly 380 nm is derived from the inherent structure of the B-ring of quercetin, whereas that in the range from 420 to 450

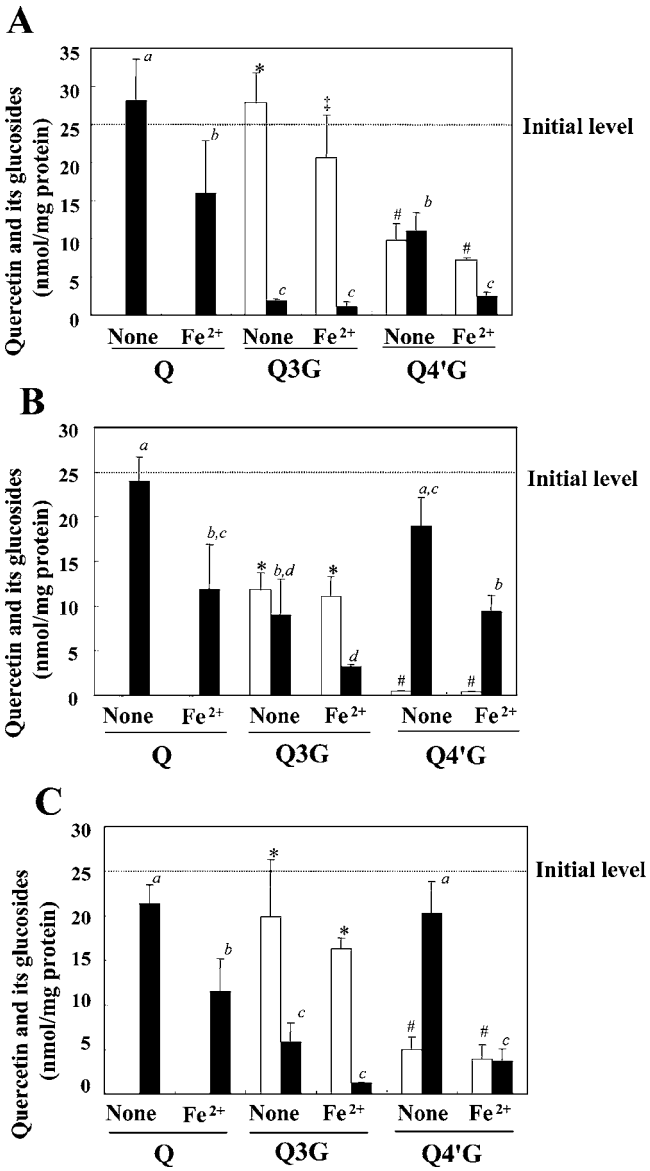


Figure 3. Recovery of quercetin and its glucosides after incubation with gastrointestinal mucosa homogenate. (**A**) Stomach, (**B**) small intestine, and (**C**) large intestine. Bars represent quercetin aglycone (filled bar) and its glucosides (open bars). Incubation conditions were the same as those in **Figure 2**. None, absence of $Fe(NO_3)_3$; Fe^{2+}, presence of $Fe(NO_3)_3$. In both cases, ascorbic acid was added to the incubation mixture for preventing the oxidative decomposition of quercetin or quercetin glucosides. Results are the means ± SD of three independent experiments. Means with different letters (a–d) for quercetin aglycone and symbols (*, ‡, #) for quercetin glucosides are significantly different ($p < 0.05$).

nm is from the chelation of ferrous ion by its B-ring structure (*24*, *25*). In all compounds, the maximum wavelength at nearly 380 nm was found to be shifted to the higher region by the addition of $Fe(NO_3)_3$ and ascorbic acid. Absorption spectra were obtained after 30 min and 3 h of incubation with deferoxamine salt, a strong cheating agent. As aglycone, Q3G, and Q4′G compete with deferoxamine salt in chelating ferrous ion during the incubation, the rate of the change in the maximum wavelength is dependent on the strength of its chelation. For quercetin aglycone, after addition of deferoxamine salt, the maximum wavelength stayed shifted to the higher region for the 30 min incubation, whereas after 3 h, it was shifted to its original maximum wavelength [seen prior to the addition of $Fe(NO_3)_3$]. In the case of Q3G, the recovery of the original maximum wavelength was faster than that of quercetin after the 30 min incubation with deferoxamine salt. In contrast, the maximum wavelength of Q4′G was completely returned to the original maximum wavelength after 30 min of incubation with deferoxamine salt. Therefore, it is apparent that the chelating activities of quercetin aglycone and its glucosides were different and increased in the order of Q4′G < Q3G < quercetin aglycone.

DISCUSSION

We previously examined the antioxidant activity of quercetin and quercetin glucosides on azo radical-induced lipid peroxidation of liposomal membranes and found that they decreased the lipid hydroperoxide formation in the order of Q4′G < Q3G < quercetin aglycone (*26*). Furthermore, Q4′G in comparison with Q3G or quercetin aglycone showed very little inhibition of either azo radical- or ferrous ion-induced lipid peroxidation of human plasma LDL (*27*). The *o*-dihydroxyl group in the B-ring structure of quercetin is responsible for a greater proportion of its free radical scavenging activity. The decreased inhibition of azo radical-induced lipid peroxidation by Q4′G is because of the loss of the *o*-dihydroxyl group in the B-ring due to the introduction of a sugar group (*28*, *29*). While comparing quercetin with kaempferol, Brown et al. (*25*) observed that the *o*-dihydroxyl structure mainly takes part in chelating copper ion during the prevention of copper ion-induced LDL oxidation by quercetin. In this study, we compared the chelating spectrum of quercetin aglycone, Q3G, and Q4′G and found that the iron ion chelating activity of Q4′G was much lower than that of Q3G.

$Fe(NO_3)_3$- and ascorbic acid-induced lipid peroxidation are initiated by the generation of ferrous ion from the reduction of ferric ion by ascorbic acid, followed by the peroxyl radical-driven free radical chain reaction. Both chelating and radical scavenging activities are responsible for the inhibition of this kind of peroxidation. Thus, Q4′G being a poor ferric ion chelator should have a lesser lipid peroxidation inhibitory effect when compared to Q3G. However, Q4′G was more effective than Q3G in the inhibition of $Fe(NO_3)_3$ and ascorbic acid-induced lipid peroxidation of the gastrointestinal mucosa.

Q4′G yielded more amount of quercetin aglycone than Q3G on incubation with the gastrointestinal mucosa. β-Glucosidase activity that released quercetin aglycone from its monoglucoside was observed in the rat intestinal mucosa by Ioku et al. (*30*) and later seen in human intestinal epithelial cells (*31*). Thus, quercetin glucosides are likely to be converted into quercetin aglycone before transport into the body. Interestingly, it was reported that β-glucosidase activity of small intestinal mucosa from both rat and human prefers the sugar group at the 4′-position to that at the 3-position (*30*, *31*). It is therefore rational that Q4′G is hydrolyzed to its aglycone more efficiently than Q3G in rat small intestine. In addition, we found that large intestinal mucosa prefer Q4′G to Q3G. The predominance of Q4′G in hydrolysis can explain its higher antioxidant activity in intestinal mucosa despite its lower free radical scavenging and chelating activity. Furthermore, lactose phlorizin hydrolase (LPH; EC 3.2.1.62), a membrane-bound enzyme known to primarily hydrolyze lactase, was rediscovered as a membrane-bound β-glucosidase, which is capable of hydrolyzing quercetin glucosides effectively (*32*). The Q4′G mucosal hydrolysis was 10 times greater than Q3G hydrolysis in a rat-everted jejunum sac model (*33*), suggesting that Q4′G was a better substrate for LPH in the rat intestine when compared to Q3G. Thus, it is reasonable to assume that Q4′G is hydrolyzed to its aglycone before absorption and acts as a strong antioxidant against ferrous ion-induced oxidation in the intestinal lumen.

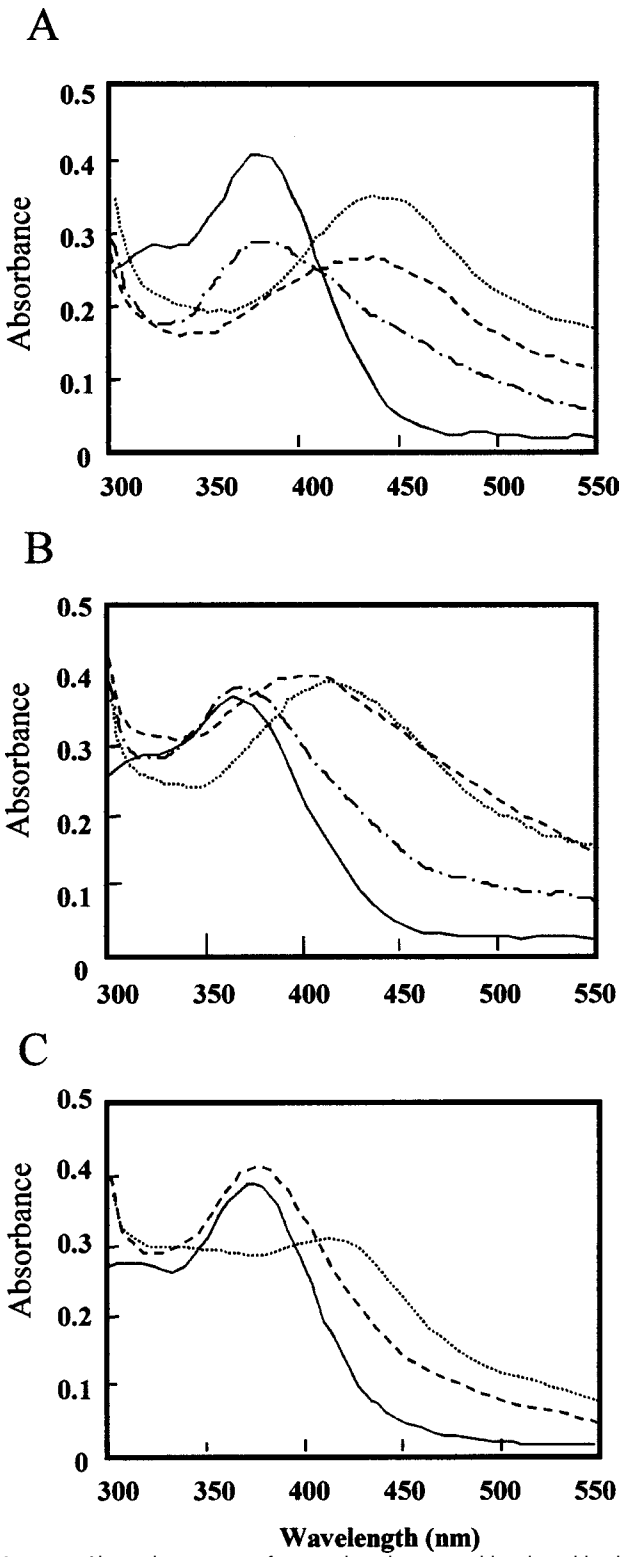


Figure 4. Absorption spectra of quercetin aglycone and its glucosides in the absence and presence of $Fe(NO_3)_3$ and ascorbic acid. (**A**) Quercetin, (**B**) Q3G, and (**C**) Q4′G. Lines represent original compounds' spectra without any additives (solid lines); with $Fe(NO_3)_3$ and ascorbic acid (dotted lines); with $Fe(NO_3)_3$, ascorbic acid, and deferoxamine salt measured after 30 min of incubation (dashed lines); and after 3 h of incubation (chain lines). Spectra of 25 μM quercetin or its glucosides in Tris-HCl buffer (0.1 M, pH 7.4) containing 0.135 M KCl were measured prior to the addition of $Fe(NO_3)_3$ and ascorbic acid (final concentrations: 25 and 250 μM, respectively) and at 30 min and 3 h after addition of $Fe(NO_3)_3$ and ascorbic acid. Deferoxamine salt was added 10 s after the addition of $Fe(O_3)_3$ and ascorbic acid in the experiment with deferoxamine salt.

Lately, a number of studies have suggested the involvement of free iron ions in the development of gastrointestinal diseases, such as inflammation and colon cancer (*34*−*37*). Dietary iron remains substantially unabsorbed in the intestine and, therefore, could be involved in the generation of hydroxyl radical by the Fenton type reaction in conjugation with colonic microflora. Previously, we have demonstrated that phytic acid hydrolysis products (*38*) and phosphatidylserine (*39*) exert a unique protective effect on iron ion-induced lipid peroxidation. Iron ion and ascorbic acid used for the initiators of lipid peroxidation in this study are common food factors present in the digestive tract. Thus, the protective effect of quercetin glucosides against lipid peroxidation of the intestinal mucosa indicates the importance of quercetin glucoside-containing foods for protection of intestinal mucosa against oxidative stress originating from dietary prooxidants.

In conclusion, Q4′G is superior to Q3G in protection of the rat intestinal mucosa from iron ion-induced lipid peroxidation, despite its lower free radical and iron ion-chelating activity. This is corroborated by the effective hydrolysis of Q4′G to its active aglycone in the intestinal mucosa. Thus, vegetables rich in Q4′G such as onion may be beneficial in preventing oxidative damage to the intestinal tract. The physiological role of quercetin glucoside-rich vegetables on the digestive tract needs to be further explored.

ABBREVIATIONS USED

4-HNE, 4-hydroxynonenal; Q3G, quercetin 3-O-β-glucoside; Q4′G, quercetin 4′-O-β-glucoside; TBA, thiobarbituric acid; TBARS, TBA reacting substances; LDL, low-density lipoprotein.

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