Flavonoid Glucosides Are Hydrolyzed and Thus Activated in the Oral Cavity in Humans1,2

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ABSTRACT Increasing epidemiological evidence supports the view that dietary flavonoids have protective roles in oral diseases, including cancer. However, the dietary forms of flavonoids, the flavonoid glycosides, must first be hydrolyzed to the aglycones, which is thought to occur mainly in the intestine. In the present study we tested whether this hydrolytic activity occurs in the oral cavity. Saliva was collected from human subjects, incubated with flavonoid glycosides, and analyzed for aglycone formation by HPLC. When quercetin 4'-glucoside or genistein 7'-glucoside was incubated with human saliva, hydrolysis to quercetin and genistein, respectively, was detected within minutes. Studies of additional flavonoid glycosides demonstrated that glucose conjugates were rapidly hydrolyzed, but not conjugates with other sugars, i.e., rutin, quercitrin, and naringin. In a limited study of 17 subjects, the interindividual variability in the hydrolysis of genistein 7'-glucoside was >20-fold. This supports the contention that salivary hydrolysis of certain flavonoid glucosides may be important in some individuals but not in others. Support for a bacterial contribution to this hydrolysis was obtained from the inhibitory effect of antibiotics in vivo and in vitro and from experiments with subcultured oral bacterial colonies. However, cytosol isolated from oral epithelial cells was also capable of effective hydrolysis. Dietary flavonoid glucosides may thus be hydrolyzed in the oral cavity by both bacteria and shedded epithelial cells to deliver the biologically active aglycones at the surface of the epithelial cells. The aglycones quercetin and genistein both potently inhibited proliferation of oral cancer cells. The large interindividual variability in this hydrolytic activity may be a factor that should be taken into consideration in future studies. J. Nutr. 135: 48–52, 2005.

KEY WORDS: ● flavonoid glycosides ● salivary hydrolysis ● β-glucosidase ● oral cancer

Epidemiological studies have clearly demonstrated a protective role of fruits and vegetables in oral cancers, presumably mediated by their content of polyphenols (1–3). Numerous mechanisms for these effects have been suggested, mainly based on in vitro and cellular studies (4–6). The dietary sources of flavonoids, except for the tea flavonoids (7), are flavonoid glycosides, which in most cases first must undergo hydrolysis to their aglycones to be able to produce effects. Still, serious questions remain regarding how these dietary components gain access to proposed cellular sites of action in the human body. For the tea flavonoids, which are gallic acid esters rather than glycosides, the access to oral epithelial cells may be less complex, as very recently noted (7).

In the past, it was strongly believed that flavonoid glycosides could not be absorbed per se but only after hydrolysis by the bacterial flora in the lower part of the intestine (8,9). In 1995 researchers proposed that flavonoid glycosides can be absorbed intact, presumably via the sodium-dependent glucose transporter SGLT1 (10). Although this was later confirmed (11), it was also shown that many glycosides are not absorbed due to efficient efflux transport by multidrug resistance-associated protein-2 (12). Other studies suggested that hydrolysis of flavonoid glucosides can occur in the small intestine (13), maybe by the broad-specific enterocyte β-glucosidase (14) and/or the lactase phloridzin hydrolase (15). Once this hydrolysis occurs, the aglycones formed are efficiently absorbed, although the bioavailability may be extremely low due to extensive presystemic metabolism (16). Thus, the potential protective effects of dietary flavonoids against cancers of the oral cavity are not understood.

Saliva has been suggested to be able to hydrolyze flavonoid glucosides (17–19), but it has never been considered an important factor. In the present study we attempted to establish the importance of salivary hydrolysis, using as substrates 4 different glycosides of quercetin, i.e., quercitrin (the 3-rhamnoside), rutin (the 3-rhamnoglucoside), isorquercitrin (the 3-glucoside), and spiraeoside (the 4'-glucoside), which previously have been shown to be hydrolyzed by β-glucosidases of human (14,15) and/or bacterial origin (9). We also tested genistin (genistein 7-glucoside), naringin (naringenin 7-rham-
noglucoside), and phlorizin (phloretin 2'-glucoside) as substrates, all commonly present in the human diet.

MATERIALS AND METHODS

Materials. Genistin, genistein, naringin, phloridzin, quercitrin (quercetin 3-rhamnoside), rutin (quercetin 3,7-rhamnoglucoside), quercetin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma; spiraeoside (quercetin 4'-glucoside) and isoquercitrin (quercetin 3-glucoside) were purchased from Indofine Chemicals. Colgate manufactured Chlorhexidine Gluconate Oral Rinse (PerioGard), and Listerine was distributed by Warner-Lambert Consumer Health Care. Dimethyl sulfoxide (DMSO), glacial acetic acid, and methanol were purchased from Fisher Scientific; trifluoroacetic acid was obtained from Aldrich Chemical Company. Fetal bovine serum was produced by Atlanta Biologicals, and other cell culture medium components were obtained from Cellgro Mediatech, Fisher Scientific.

Collection of human saliva. The study was approved by the Institutional Review Board for Human Research. The limited population study was conducted using 17 volunteers recruited from 1 high school science class (ages 15–17 y). Other saliva samples were from adult subjects (23–64 y). Unstimulated saliva (2 mL) was collected in the morning with the subject abstaining from toothbrushing since the previous evening. In some adult subjects saliva was collected before and after brushing (no toothpaste) or before and after rinsing with an antibacterial mouthrinse (chlorhexidine or Listerine) for 30 s. In the latter experiments, saliva was collected at 6 min and 1, 2, 6, and 24 h after the mouthrinse.

Flavonoid hydrolysis by saliva. The saliva (2 mL) was diluted 1:1 with distilled water and shaken vigorously to reduce viscosity. In some experiments, diluted saliva was centrifuged at 10,000 × g and filtered with 1- and 0.2-μm filters to remove oral cells and bacteria, respectively. Other saliva samples (1 mL) were incubated on a shaking bath at 37°C for 24 h with an equal volume of DMEM (containing 10% fetal bovine serum) with or without 200,000 U/L penicillin and 0.2 g/L streptomycin prior to incubation with flavonoid glycosides.

Flavonoid glycosides dissolved in DMSO (final concentration < 0.1%) were added to two 1-mL aliquots of the saliva mixture to a final flavonoid concentration of 25 or 50 μM/L. After vigorous shaking and vortexing, argon was added to the samples before incubation for specified times at 37°C. The pH of the saliva samples was 6.7 ± 0.2 (mean ± SEM) before and 6.4 ± 0.1 after incubation. An equal volume of methanol was added to the samples following incubation. The samples were centrifuged at 16,000 × g for 2 min and the supernatant was analyzed by HPLC with flavonoid-specific UV detection.

Results. The hydrolysis of all 7 of the flavonoid glycosides studied to their respective aglycones by saliva was examined using HPLC separation with flavonoid-specific UV detection. This was shown for quercetin 4'-glucoside (spiraoside), one of the most important flavonoid glycosides in the human diet, present in large amounts in onions, for example (22). Both the glucoside and its aglycone quercetin had excellent chromatographic properties, and there was little interference from other salivary components at the 370-nm wavelength used. The hydrolytic reaction was linear with time for at least 3 h. There was no evidence of other products formed under these conditions. The reactions were carried out under argon to avoid degradation of any of the aglycones formed. For a given saliva sample, the reproducibility of the reaction was acceptable with a CV of about 15%.

![Image of flavonoid hydrolysis](https://example.com/flavonoid-hydrolysis-graph.png)

The hydrolysis of all 24 flavonoid glycosides studied was examined using saliva from 1 subject in replicate analyses (Table 1). For estimation of the rates of hydrolysis, the percentage conversion to the aglycones, taking into account the molar extinction coefficients, was determined after 2-h incubations at 37°C. Four of the glycosides were hydrolyzed efficiently, spiraeoside, phlorizin, genistin, and isoquercitrin, with spiraeoside being by far the best substrate. On the other hand, rutin was hydrolyzed very little, and quercitin and naringin were not hydrolyzed at all. The same pattern was seen with saliva from 3 other subjects, although the interindividual variability in the hydrolysis rate was large (see below).

We next examined the hydrolysis of one flavonoid glu-
TABLE 1

<table>
<thead>
<tr>
<th>Flavonoid glycoside</th>
<th>Aglycone formation</th>
<th>% of added glycoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringin (naringenin 7-rhamnoglucoside)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Quercitrin (quercetin 3-rhamnoside)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rutin (quercetin 3-rhamnoglucoside)</td>
<td>9.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Isoquercetin (quercetin 3-glucoside)</td>
<td>40.5 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>Genistin (genistein 7-glucoside)</td>
<td>44.3 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Phloridzin (phloretin 2-glucoside)</td>
<td>67.8 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Spiraeoside (quercetin 4'-glucoside)</td>
<td>85.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Genistin (genistein 7-glucoside)</td>
<td>44.3 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Isoquercetin (quercetin 3-glucoside)</td>
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</tbody>
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1 Values are means ± SEM, n = 3–4.
2 Saliva from 1 volunteer was incubated for 2 h at 37°C with 25 μmol/L flavonoid glycosides and analyzed by HPLC for the aglycone products and the parent flavonoid glycosides.

The bacterial flora, we compared the hydrolysis of genistin to genistein by raw saliva and raw saliva preincubated with cell culture medium (without antibiotics) for 24 h. There was a dramatic increase consistent with the anticipated bacterial growth in cultured saliva (Fig. 3). In the presence of penicillin and streptomycin, this increase was abolished (Fig. 3). To further confirm the importance of bacteria in the hydrolysis of flavonoid glycosides, blood agar and chocolate agar plates were inoculated with saliva from 1 volunteer who had previously been shown to have high salivary hydrolytic activity. Eight different oral bacterial colonies subcultured to homogeneity were tested for their ability to hydrolyze genistin. Two of the cultures showed a high level of activity, about 25% hydrolysis after a 1-h incubation with 25 μmol/L genistin, whereas the others were totally inactive. The experiment was repeated with the same results. The 2 active strains were classified as gram-positive cocci in tetrads and gram-negative diplococci, both common nonpathogenic oral bacteria.

These observations, although highly supportive of a bacterial source for the β-glucosidase activity, did not rule out a
contribution from shedded oral epithelial cells. When saliva samples were examined by microscopy, they all contained variable numbers of scaly oral epithelial cells, about half of which appeared to be intact. To examine the effect of these cells on hydrolysis, 3 subjects brushed their cheeks, gums, and tongue with a toothbrush before providing saliva samples, an established procedure to collect loosely attached epithelial cells. Microscopy confirmed a substantial increase in cell numbers. The quercetin 4'-glucoside to quercetin hydrolysis in these samples increased substantially compared to samples obtained before brushing ($P < 0.05; n = 3$), consistent with a contribution by shed epithelial cells. The aglycone/glucoside ratios after a 30-min incubation were $0.12 \pm 0.03$ before brushing vs. $0.55 \pm 0.17$ after brushing and after a 60-min incubation were $0.33 \pm 0.08$ vs. $4.42 \pm 0.34$. To further test the hydrolytic activity of oral epithelial cells without the confounding presence of oral bacteria we used cytosols prepared from oral squamous carcinoma SCC-9 cells (24). Hydrolysis of quercetin 4'-glucoside to quercetin by this cytosol was efficient (Fig. 4). The reaction was clearly saturable over the 5–100 μmol/L quercetin 4'-glucoside concentration range. Nonlinear regression analysis of the data yielded an apparent $K_m$ of 34 μmol/L and a $V_{max}$ of 64 nmol/(h \cdot mg protein).

To obtain a quantitative estimate of the effectiveness of the oral hydrolysis of flavonoid glucosides irrespective of mechanisms, we conducted experiments in situ. Thus, 15 mL of a 10 μmol/L solution of quercetin-4'-glucoside was held in the mouth of 1 volunteer for 5 min, and glucoside and aglycone quercetin content were assayed by HPLC. In 3 experiments, a consistent 57–63% of the glucoside disappeared during this short time period, taking into account volume changes. Also, 17–24% of the glucoside was hydrolyzed to quercetin.

To determine the potential biological importance of the collective findings, we examined the antiproliferative effects of quercetin and genistein, the 2 flavonoids demonstrating efficient salivary formation from their precursor glucosides, on the oral squamous carcinoma SCC-9 cells using the MTT assay and a wide range of flavonoid concentrations (0.1–200 μmol/L) (Fig. 5). Both flavonoids produced potent inhibition with a minimum effective concentration (MEC) of 5 and 10 μmol/L for quercetin and genistein, respectively. Thus, the aglycones formed in the oral cavity may have anticancer and antibacterial (3) effects or more generally scavenge hydrogen peroxide and other reactive oxygen species (19).

**FIGURE 4**  Michaelis-Menten kinetics of the hydrolysis of quercetin 4'-glucoside to quercetin by cytosol from human oral epithelial SCC-9 cells. The incubation time was 1 h.

**FIGURE 5**  Antiproliferative effects of quercetin (Q) and genistein (G) in human oral squamous carcinoma SCC-9 cells. MEC, minimum effective concentration. Values are means ± SEM, $n = 12–18$. *Different from the flavonoid-free (0 μmol/L) DMSO control; $P < 0.05$.

**DISCUSSION**

Although several previous studies have demonstrated that saliva can hydrolyze certain flavonoid glucosides (17–19), those studies were limited in scope. From the present study it appears that this hydrolysis is limited to glucose conjugates, because other glucosides, such as the rhamnosides (quercitrin) and rhamnoglucosides (rutin and naringin), either were hydrolyzed very slowly or were resistant to salivary hydrolysis. Interestingly, these latter types of glucosides are easily hydrolyzed by the human fecal flora (9). Quercetin 4'-glucoside in particular, but also genistin, i.e., genistein 7-glucoside, can be rapidly hydrolyzed by saliva. These are 2 of the more abundant flavonoids in the human diet (22,25). Thus, the formation of quercetin and genistin from their dietary sources may be rapid enough in certain individuals to be important also in vivo. Holding a solution of quercetin 4'-glucoside in the mouth for 5 min resulted in a 60% loss of glucoside. About 20% of that loss could be accounted for as quercetin in the saliva, with the remaining 40% being absorbed by the oral epithelial cells, presumably as quercetin.

Still, whether this oral hydrolysis will be of importance for local effects on the oral epithelium, considering the relatively short residence time of most foods in the oral cavity, is difficult to assess. Our study of 17 high school students demonstrated a remarkable interindividual variability in hydrolysis rate. These observations suggest that oral hydrolysis may be quantitatively important in some individuals but not in others. Such variability may have a genetic origin, but could also involve environmental factors.

Hydrolysis of flavonoid glucosides by β-glucosidases in the oral cavity is also likely to be influenced by the food matrix in which they are contained. Thus, if contained in a liquid form, they will obviously have greater access to the enzymes than if contained in a solid form requiring extensive chewing. Even so, it should be clear that the hydrolytic activity will start in the oral cavity and continue through the passage of the food throughout the aerodigestive tract.

The mechanism of hydrolysis was presumably through β-glucosidases, but the source of these enzymes was difficult to pinpoint. Bacterial flora in the oral cavity play a role. This can be deduced from the effectiveness of the antibacterial agents chlorhexidine and Listerine in inhibiting the salivary hydrolysis and is also strongly supported by the experiments using cell culture techniques, either for the whole saliva or for...
subcultured oral bacterial colonies. However, cytosols from cultured oral epithelial cells also displayed a high rate of hydrolysis and the saliva from all subjects, in particular after brushing the cheeks, gums, and tongue, contained high numbers of epithelial cells. It is interesting to note that the cytosolic hydrolysis of quercetin 4′-glucoside to quercetin proceeded with an apparent $K_m$ of 34 $\mu$mol/L and a $V_{max}$ of 64 nmol/(h $\cdot$ mg protein), very similar to the hydrolysis by cytosol from human liver and small intestine (14), implying the involvement of the same $\beta$-glucosidase enzyme. Clearly, more studies will be needed to better determine the contribution of bacterial and human epithelial $\beta$-glucosidases.

Finally, it was of great interest to note that both quercetin and genistein were able to inhibit proliferation of the oral squamous carcinoma SCC-9 cells. This occurred with an MEC of 5–10 $\mu$mol/L for these flavonoids, which should be achievable in the oral cavity after consumption of a diet containing these flavonoids. The mechanisms of these effects are likely different. Quercetin inhibits PI3-kinase (26), whereas genistein inhibits tyrosine kinases (26). A mixture of flavonoids with different mechanistic properties may be an advantage in cancer prevention.

In conclusion, flavonoid glucosides were hydrolyzed to their aglycones in the oral cavity. The $\beta$-glucosidase enzymes responsible were derived both from bacteria and shedded oral epithelial cells. Two of the flavonoid aglycones studied, quercetin and genistein, showed potent inhibition of oral cancer cell proliferation. The ability of some individuals but not others to hydrolyze these protective dietary flavonoids in the oral cavity should be an important consideration in future studies.

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LITERATURE CITED