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Inhibitory Effects of *Citrus* Flavonoids on Starch Digestion and Antihyperglycemic Effects in HepG2 Cells

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Supporting Information

ABSTRACT: Flavonoids are a class of important bioactive natural products and are being extensively used in functional foods. In the present study, the effects of four *Citrus* flavonoids (i.e., hesperidin, naringin, neohesperidin, and nobiletin) on amylase-catalyzed starch digestion, major digestive enzyme activities (e.g., pancreatic α-amylase and α-glucosidase), and glucose use in HepG2 cells were investigated. The results showed that all of the tested *Citrus* flavonoids significantly inhibited amylase-catalyzed starch digestion. Moreover, naringin and neohesperidin mainly inhibited amylose digestion, whereas hesperidin and nobiletin inhibited both amylose and amylopectin digestion. However, these flavonoids showed weak inhibitory activities against digestive enzymes. Furthermore, glucose consumption, glycogen concentration, and glucokinase activity were significantly elevated, and glucose-6-phosphatase activity was markedly decreased by *Citrus* flavonoids. These results demonstrate that *Citrus* flavonoids play important roles in preventing the progression of hyperglycemia, partly by binding to starch, increasing hepatic glycolysis and the glycogen concentration, and lowering hepatic gluconeogenesis. This work suggests that *Citrus* flavonoids might be potentially used for the prevention of postprandial hyperglycemia.

KEYWORDS: *Functional foods, Citrus flavonoids, starch−Citrus flavonoid complexes, amylase-catalyzed digestion, HepG2 cell, glucose-regulating enzymes*

INTRODUCTION

Postprandial hyperglycemia has many serious hazards and can eventually cause type 2 diabetes mellitus, which some have called "postprandial diabetes", which has affected more than 170 million individuals in the world.[1−4] Postprandial hyperglycemia is being considered a main target for type 2 diabetes mellitus treatment.[2,3] The International Diabetes Federation and the American Diabetes Association have set stringent target values for postprandial blood glucose control.[5,6] Digestion of carbohydrates from food is the major source of postprandial hyperglycemia. Furthermore, hepatic glucose-reglulating enzymes, such as glucokinase and glucose-6-phosphatase, play critical roles in controlling postprandial hyperglycemia.[7] Therefore, delaying the digestion of carbohydrates in food and promoting glucose use in the liver could be an effective measure to prevent postprandial hyperglycemia.

In comparison to synthetic compounds, natural small molecules with special bioactivity have become the major resource of bioactive agents and played a key role in diabetes therapy.[8] Many studies have been conducted on the binding of lipids to starch, thus inhibiting starch digestion.[9−13] There are also a few studies suggesting that some flavonoids, such as epicatechin-dimethylgallate, rutin, and genistein, could bind to starch,[14,15] but only one study was concerned with the digestibility of starch.[14] However, whether it could time-dependently decrease starch digestion or inhibit digestive enzyme activities resulting in secondary delay starch digestion is unclear. In addition, epidemiological studies also show that dietary flavonoid intakes may reduce the risk of diabetes mellitus.[16,17] The above information inspires researchers to exploit new function of flavonoids as effective antidiabetic functional foods.

Citrus flavonoids have received much attention in recent years, for their potential therapeutic qualities and relatively low toxicity to animals.[18−20] *Citrus* flavonoids possess various pharmacological activities, such as antioxidant, anticancer, anti-inflammatory, chemopreventive, cardioprotective, and neuroprotective activities.[18−23] Hesperidin, naringin, and nobiletin also exhibit antidiabetic activities partly by lowering hepatic gluconeogenesis or improving insulin sensitivity in diabetic animals.[24−28] However, there is no information about the effects of *Citrus* flavonoids on carbohydrates digestion, which is the major source of postprandial hyperglycemia. The annual production of *Citrus* fruits is almost 1.02 hundred million tons, and they are consumed globally.[29] It is known that *Citrus* fruits are peeled, and *Citrus* peel contains a high content of flavonoids, including primarily hesperidin, nobiletin, neohesperidin, and naringin.[30] The abandoned peel will result in both environmental pollution and resource waste. Therefore, it is rational to exploit the *Citrus* peel resource and evaluate these flavonoids used as specific nutrients in functional foods.

In the present study, the effects of four *Citrus* flavonoids on starch digestion, digestive enzyme activities, hepatic glucose consumption, hepatic glycogen concentration, and hepatic glucose-regulating enzyme activities were investgated. On the basis of this research, we could make the best use of *Citrus* peel

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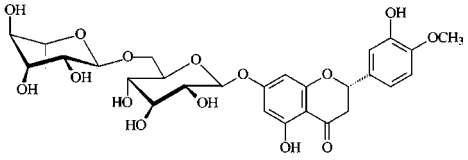
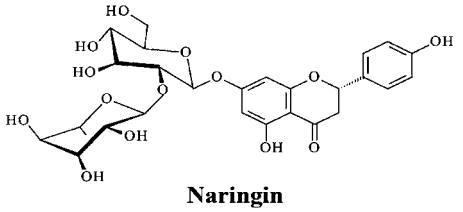
resources and offer a useful reference for future exploitation of new functional foods with potential prophylactic postprandial hyperglycemia properties.

MATERIALS AND METHODS

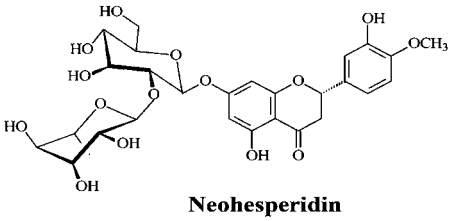
Reagents. *Citrus* flavonoids (hesperidin, neohesperidin, naringin, and nobiletin) were isolated from *Citrus* peel (flavedo and albedo) in our laboratory, as described by Lu et al.[30] Their structures are shown in Figure 1. Soluble starch was obtained from Sinopharm Chemical



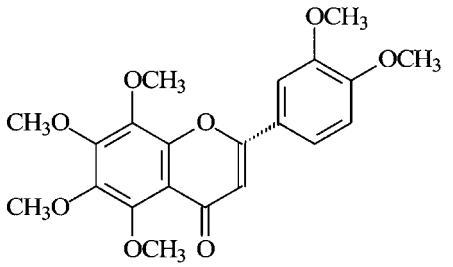
Hesperidin



Naringin



Neohesperidin



Nobiletin

Figure 1. Structures of *Citrus* flavonoids used in this study.

Reagent Co., Ltd. (Shanghai, China). α-(2-Chloro-4-nitrophenyl)-β-1,4-galactopyranosylmaltoside (Gal-G2-α-CNP) was purchased from Toyobo Co., Ltd. (Osaka, Japan). 2-Chloro-4-nitrophenol (CNP) was purchased from Energy Chemical (Shanghai, China). Porcine pancreas α-amylase, pancreatin from porcine pancreas, amylose, amylopectin, 4-hydroxybenzhydrazide, and acarbose were purchased from Sigma-Aldrich Co. (St. Louis, MO). Rat intestinal acetone powder (α-glucosidase) and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were kindly provided by Unilever Co., Ltd. (Shanghai, China). Metformin, glucose-6-phosphate dehydrogenase (G6PDH), ATP, nicotinamide adenine dinucleotide (NAD^+), $NADP^+$, dithiothreitol (DTT), mutarotase, glucose-6-phosphate (G6P), and imidazole−HCl were purchased from Moldepot Incorporation (Nanjing, China). Glucose dehydrogenase was obtained from American Amresco Company. All other chemicals were of analytical reagent (AR) grade.

Preparation of Solution. Potassium iodide (1.5 g) was dissolved in 12.5 mL of distilled water. Iodine (0.635 g) was added to the solution, and the volume of the mixture of potassium iodide and iodine solution was adjusted to 50 mL by adding distilled water. The solution was stored in dark at 4 °C to be used as iodine solution. Sodium phosphate buffer (30 mM): 150 mL of 64 mM sodium phosphate solution (pH 7.1) was combined with 150 mL of 32 mM KCl−HCl (pH 2.0) in 20 mL of distilled water and stored at room temperature. Porcine pancreas α-amylase (5 mg/mL) and Gal-G2-α-CNP (5 mM) were dissolved in sodium phosphate buffer. Potassium phosphate buffer (0.1 M): 49.7 mL of 1 M potassium phosphate was combined with 50.3 mL of 1 M potassium dihydrogen phosphate. The combined solution was diluted to 1 L with distilled water. The pH of the diluted solution was adjusted to 6.8 and stored at room temperature. PNPG solution (8 mM): PNPG was dissolved in 0.1 M potassium phosphate buffer. α-Glucosidase solution: rat intestinal acetone powder (1 g) was suspended in 15 mL of potassium phosphate buffer; after ultrasonic treatment and centrifugation, the supernatant was used as α-glucosidase solution.

Preparation of Starch Suspensions. Soluble starch (10 mg) was suspended in 1 mL of gently boiling water. The suspensions were kept for 30 min at room temperature to make starch swell, and then 7.5 mL of 64 mM sodium phosphate (pH 7.1) and 7.5 mL of 32 mM KCl−HCl (pH 2.0) were added to the suspensions. The suspensions were homogenized using a homogenizer and then sonicated for 15 s to be used as soluble starch suspensions. The concentration of starch in the suspensions was 0.625 mg/mL. Amylose (0.625 mg/mL) and amylopectin (0.625 mg/mL) suspensions were prepared the same as soluble starch suspensions.

Formation of the Starch−Iodine Complex. Starch−iodine complex formation was studied using the spectrophotography described by Takahama and Hirota.[14,31] Namely, the effects of *Citrus* flavonoids on the formation of the starch−iodine complex was studied by recording absorption spectra from 900 to 500 nm using an UV-1800 double-beam spectrophotometer (Shimadzu, Kyoto, Japan). When soluble starch was used, the formation of the soluble starch−iodine complex was studied as follows: *Citrus* flavonoids (hesperidin, neohesperidin, naringin, and nobiletin) or reagent were added to 0.9 mL of soluble starch suspensions and incubated for 0.5 min, and then 0.1 mL of iodine solution was added to the suspensions. Immediately after the addition of iodine solution, measurements of absorption spectra were started. The formations of the amylose−iodine complex and the amylopectin−iodine complex were manipulated the same as the formation of the soluble starch−iodine complex.

Porcine Pancreas α-Amylase Catalyzed Starch Digestion. The digestion of starch, which was catalyzed by porcine pancreas α-amylase, was studied using an UV-1800 double-beam spectrophotometer. To digest starch, 2 μL of α-amylase suspension (5 mg/mL) was added to 0.9 mL of a starch suspension prepared as above. Immediately after the addition of α-amylase, the two solutions were mixed by top and bottom inversion of the cuvette. The suspension was incubated for defined periods at 37 °C, and then 0.1 mL of iodine solution was added. From the addition of iodine solution, amylase-catalyzed starch digestion was terminated. The degree of the digested and undigested starch species was estimated from the difference (ΔA) spectra of starch−iodine complexes after and before the digestion.

In addition, amylase-catalyzed starch digestion was also studied by quantifying reducing sugar using 4-hydroxybenzhydrazide.[14] Briefly, a starch suspension (0.5 mL) was digested by pancreas α-amylase (10 μg/mL) for different times at 37 °C. The reaction was terminated by adding 2.5 mL of the mixture of solutions I and II and then incubated in a boiling water bath for 6 min. After cooling to room temperature and removal of precipitates by centrifugation, the absorbance was detected at 410 nm.

Measurement of Digestive Enzyme Activities. The assay of porcine pancreas α-amylase activity was carried out using a modification method described previously.[32] Briefly, *Citrus* flavonoids at different concentrations were combined with 10 μL of 200 μg/mL α-amylase in 30 mM sodium phosphate buffer (pH 6.9). After shaking for intensive mixing, 100 μL of 5 mM Gal-G2-α-CNP solution was added to start the reaction at 37 °C. The total reaction volume was 200 μL. The final concentrations of *Citrus* flavonoids, α-amylase, and

Gal-G2-α-CNP were 0−160 μM, 10 μg/mL, and 2.5 mM, respectively. After incubation at 37 °C for 10 min, the released CNP was monitored at 405 nm by a Bio-Tek Power Wave XS2 micro well plate reader (Bio-Tek Instruments, Winooski, VT). Acarbose was used as a positive control.

The assay of α-glycosidase activity used PNPG as the substrate, which was hydrolyzed by α-glycosidase to release *p*-nitrophenol, a color agent that can be monitored at 405 nm.[33] The procedure was the same as that described for the measurement of amylase activity. The differences were that the concentrations of *Citrus* flavonoids were 0−100 μM and the reaction time was 15 min. Acarbose was also used as a positive control.

Cell Culture. The HepG2 cell line was obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in H-DMEM (25.27 mM glucose) containing 10% newborn calf serum at 37 °C in a humidified atmosphere incubator with 5% CO_2.

Glucose Consumption (GC). At 2 days before the experiments, the cells were plated into 96-well (4000 cells/well) tissue culture plates, with some wells left blank. The determination of GC was performed according to the modified method by Zheng et al.[34] Briefly, after the cells reached confluence, the medium was replaced by H-DMEM supplemented with 0.2% bovine serum albumin (BSA). After 12 h, the medium was removed and the same BSA H-DMEM containing different *Citrus* flavonoids or metformin was added to all wells, including the blank well. After 24 h, the medium was removed, and the glucose concentrations were determined by the glucose oxidase method. GC was calculated by measuring the glucose concentrations in the blank group and subtracting the remaining glucose concentration in the control or medicine group. To consider cell proliferation, glucose consumption was appraised by calculating the ratio of GC divided by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (GC/MTT).

MTT Method. A MTT assay was conducted after the GC experiment. A total of 20 μL of MTT (5 mg/mL) was added to each well. After 4 hours, the corresponding supernatants were discarded and then the formazan crystals were dissolved in 150 μL of dimethylsulfoxide (DMSO). After shaking, the absorption values were measured at 570 nm for the reading and at 630 nm for the reference wavelength on a Bio-Tek Power Wave XS2 micro well plate reader (Bio-Tek Instruments, Winooski, VT).

Hepatic Enzyme Activities. To determine hepatic enzyme activities and glycogen content, the cells were plated in 6-well (4×10^5 cells/well) tissue culture plates. After cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), the cells were lysed in a buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 150 mM sucrose, 2 mM sodium orthovanadate, 80 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.l% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonylfluoride (PMSF), and 1% protease inhibitor cocktail for mammalian cell culture for 10 min on an ice bath. The lysates were centrifugated at 1600*g* for 10 min at 4 °C. The pellet was discarded, and the supernatant was used for a hepatic enzyme activities assay.

Glucokinase (GK) activity was determined using a spectrophotometric continuous assay, as described by Slosberg et al.[35] Briefly, the assay buffer contained 100 mM Tris-HCl at pH 7.4, 100 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 1 mM $NADP^+$, 30 IU/mL G6PDH, and 0.5 or 100 mM glucose.

Glucose-6-phosphatase (G-6-Pase) activity was determined using a spectrophotometric continuous assay according to the method by Alegre et al.[36] Briefly, the reation mixture contained 26.5 mM G6P, 1.8 mM EDTA, both previously adjusted to pH 6.5, 2 mM NAD^+, 0.6 IU/mL mutarotase, 6 IU/mL glucose dehydrogenase, and 100 mM imidazole−HCl at pH 6.5.

Protein concentrations in the supernatant were determined using the Bradford method, with BSA as the standard. Enzyme activities were indicated as nanomoles per minute per milligram of protein.

Hepatic Glycogen Assay. The glycogen content was determined with a modified method as previously described by Lee et al.[26] Briefly, after cells were washed 3 times with ice-cold PBS, the cells were collected in Eppendorf tubes and centrifuged at 10000*g* for 5 min at 4 °C. The pellet was hydrolyzed in 0.3 mL of 30% (w/v) KOH solution in a boiling water bath for 30 min. The glycogen was precipitated by adding 0.8 mL of ethanol and then centrifuged at 10000*g* for 5 min at room temperature. The glycogen pellet was dissolved in 0.3 mL of distilled water, and the glycogen concentration was determined by treatment with anthrone reagent (2 g of anthrone/L of 98% H_2SO_4) and measuring the absorbance at 620 nm.

Data Presentation and Statistical Analysis. All experiments were repeated at least 3 times. Data were presented as the mean ± standard deviation (SD). The results were evaluated by one-way analysis of variance (ANOVA), and Duncan's test was used to determine significant differences of multiple comparisons. Calculations were performed using SPSS, version 17.0 (SPSS, Chicago, IL). A value of $p < 0.05$ was taken to be statistically significant.

RESULTS AND DISCUSSION

Inhibitory Effects of *Citrus* Flavonoids on Starch−Iodine Complex Formation and Starch Digestion. Figures 2−5 show the interactions among *Citrus* flavonoids, iodine, and soluble starch. Panels A of Figures 2−5 illustrate that *Citrus* flavonoids affected the absorption spectrum of iodine solution. ΔA spectra, which were observed between the presence and absence of *Citrus* flavonoids, indicate that naringin, hesperidin, and neohesperidin suppressed the absorption spectrum of iodine solution (panels A of Figures 2−4). However, nobiletin increased the absorption spectrum of iodine solution (Figure 5A). These results suggest that *Citrus* flavonoid−iodine complexes could form after the addition of *Citrus* flavonoids to iodine solution. The formations of the rutin−iodine complex and the quercetin−iodine complex have been reported.[14] Some flavonoids have hydroxyl groups and may result in the formation of anion in the solution.[37] Nobiletin has no hydroxyl group, while hesperidin, naringin, and neohesperidin have hydroxyl groups (Figure 1). Therefore, it is possible that the presence of a hydroxyl group leads to the formation of anion in the solution, and the anion may be attributed to the difference between nobiletin and other flavonoids on the absorption spectrum of iodine solution.

It is known that the starch−iodine complex can be used to estimate the concentration of starch and that the color of the amylose−iodine complex (blue; λ_{max} = 540−660 nm) is different from that of the amylopectin−iodine complex (purple; λ_{max} = 500−540 nm).[14,31,38−41] Panels B of Figures 2−5 illustrate that four *Citrus* flavonoids also suppressed the formation of the starch−iodine complex and the suppression effects increased with increasing concentrations. If *Citrus* flavonoids did not suppress the formation of the starch−iodine complex, the ΔA spectra observed in the presence of *Citrus* flavonoids should be similar to those observed in the absence of *Citrus* flavonoids. The starch−iodine ΔA spectra suppressed by hesperidin and neohesperidin had a broad peak at around 562 nm (panels B of Figures 3 and 4). The starch−iodine ΔA spectrum suppressed by 1 mM naringin had a peak at 549 nm (trace 4 in Figure 2B). These results suggest that hesperidin, neohesperidin, and naringin could combine with starch, especially amylose, thus suppressing starch−iodine complex formation. Starch−iodine ΔA spectra suppressed by nobiletin had a broad peak at 570 nm and a low peak at 535 nm. This result suggests that nobiletin could combine with both amylose and amylopectin.

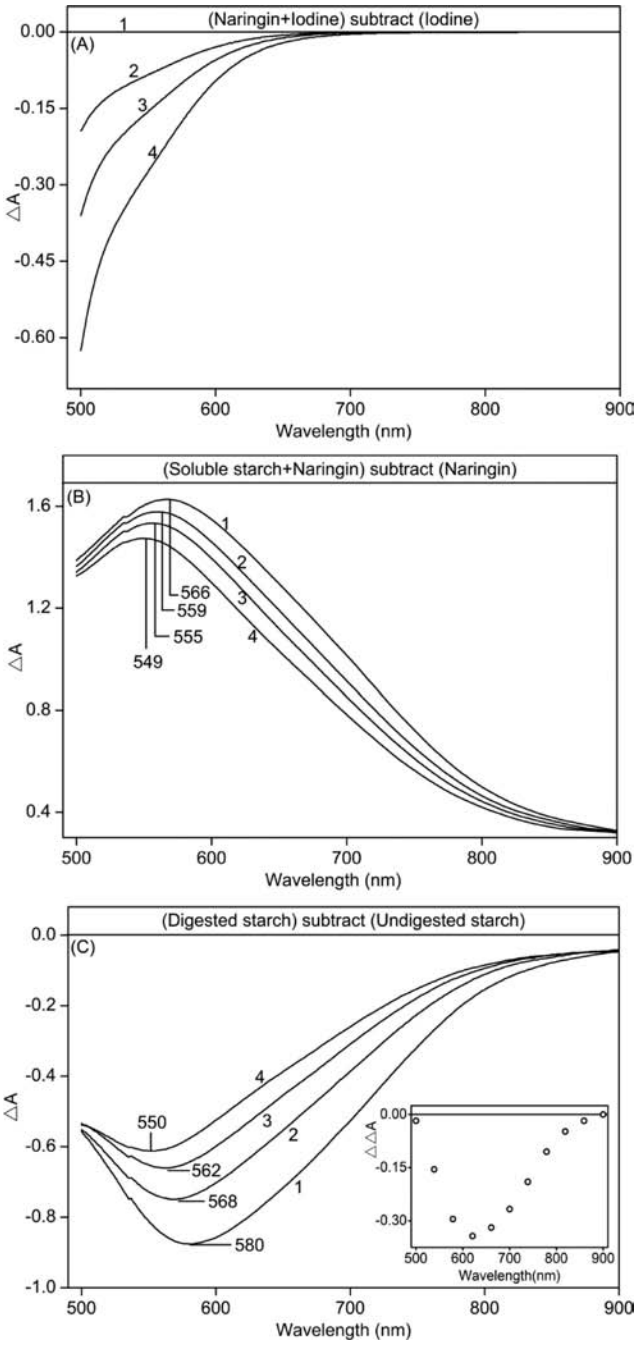


Figure 2. Interactions among naringin, iodine, and soluble starch. (A) ΔA spectra between the presence and absence of naringin in iodine solution. (B) Suppression of soluble starch−iodine complex formation by naringin. (C) Inhibition of pancreas α-amylase-catalyzed soluble starch digestion by naringin. Digestion time = 1 min. (Inset) $\Delta\Delta A$ spectrum of trace 1 minus trace 4. Traces 1, 2, 3, and 4 represent 0, 0.25, 0.5, and 1 mM naringin, respectively.

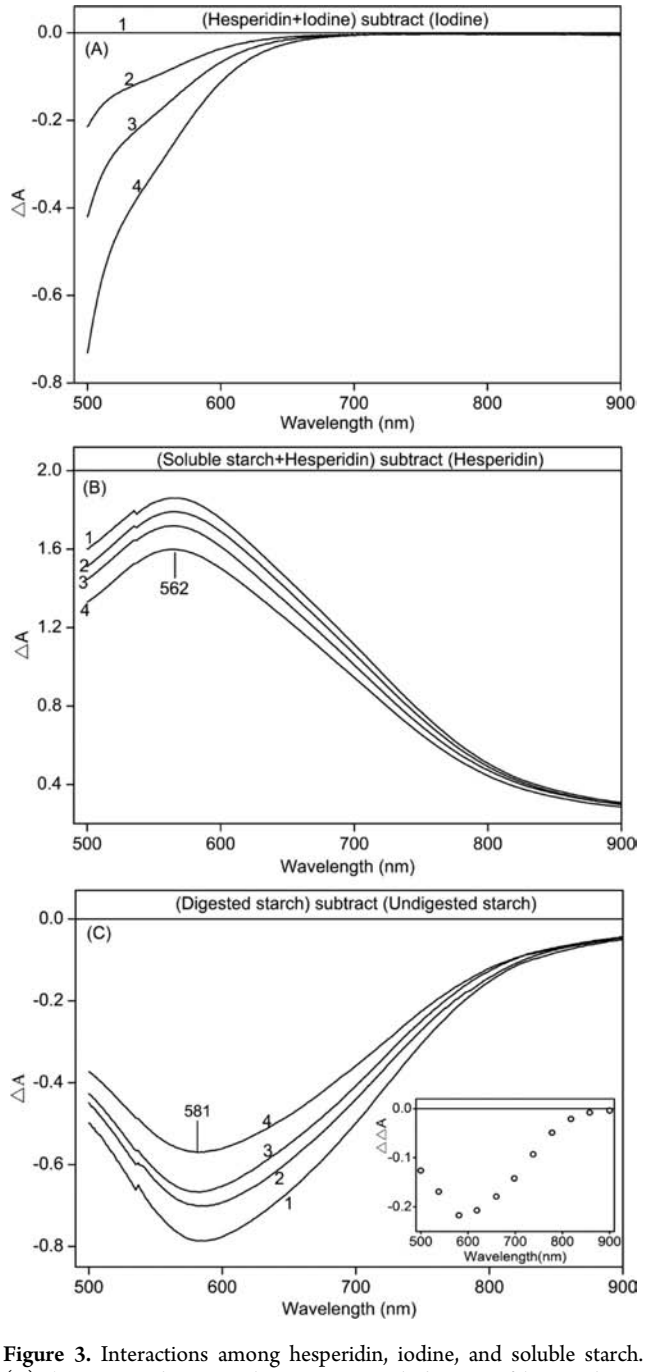


Figure 3. Interactions among hesperidin, iodine, and soluble starch. (A) ΔA spectra between the presence and absence of hesperidin in iodine solution. (B) Suppression of soluble starch−iodine complex formation by hesperidin. (C) Inhibition of pancreas α-amylase-catalyzed soluble starch digestion by hesperidin. Digestion time = 1 min. (Inset) $\Delta\Delta A$ spectrum of trace 1 minus trace 4. Traces 1, 2, 3, and 4 represent 0, 0.6, 1.2, and 2.4 mM hesperidin, respectively.

Panels C of Figures 2−5 illustrate that *Citrus* flavonoids suppressed pancreas α-amylase catalyzing soluble starch digestion and the inhibition increased with increasing concentrations of *Citrus* flavonoids. The peak of the ΔA spectra also suggest that the four *Citrus* flavonoids may mainly inhibit amylose digestion. The $\Delta\Delta A$ spectrum (inset of Figure 2C), which was obtained by subtracting trace 4 from trace 1, had a peak at about 620 nm but no significant difference at around 500 nm. This result also indicates that naringin mainly inhibited amylose digestion. The $\Delta\Delta A$ spectrum in the inset of Figure 4C was similar to the $\Delta\Delta A$ spectrum in the inset of Figure 2C, indicating that neohesperidin mainly inhibited amylose digestion. However, the $\Delta\Delta A$ spectra in the inset of panels C of Figures 3 and 5 showed significant difference at around 500 nm, which were different from the $\Delta\Delta A$ spectra in the inset of Figure 2C. These results suggest that, under the conditions of this study, the largest dose of hesperidin and nobiletin resulted in the inhibition of both amylose and amylopectin digestion.

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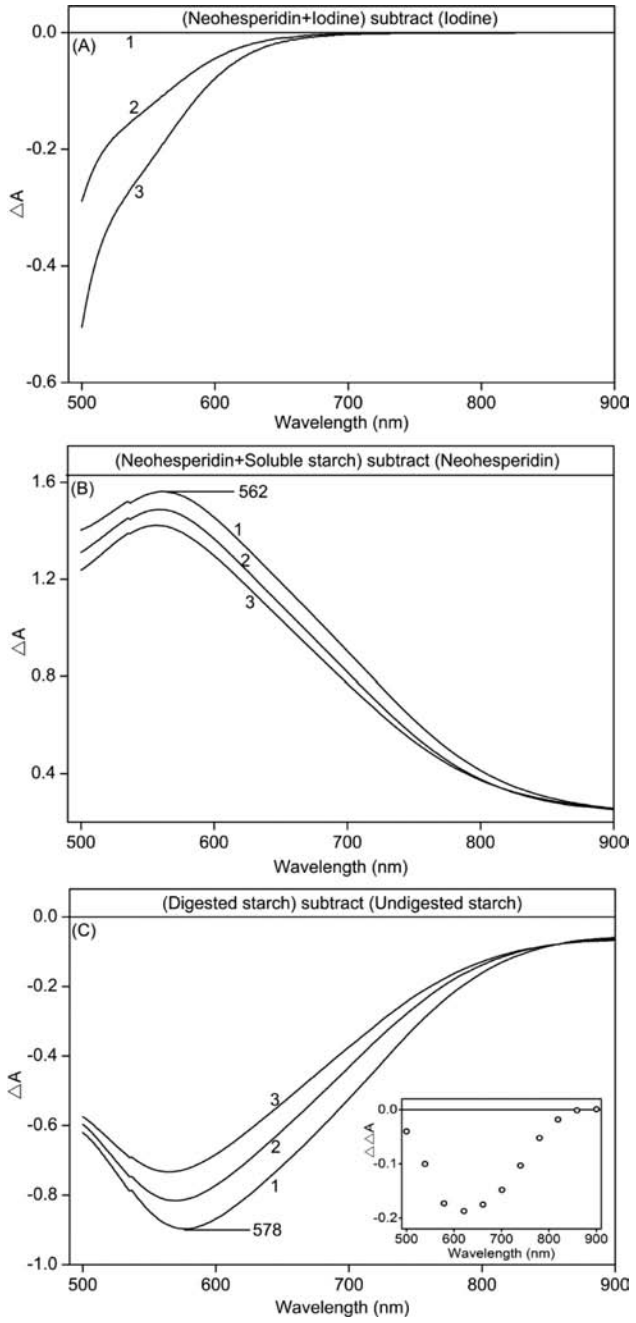

Figure 4. Interactions among neohesperidin, iodine, and soluble starch. (A) Δ*A* spectra between the presence and absence of neohesperidin in iodine solution. (B) Suppression of soluble starch–iodine complex formation by neohesperidin. (C) Inhibition of pancreas α-amylase-catalyzed soluble starch digestion by neohesperidin. Digestion time = 1 min. (Inset) ΔΔ*A* spectrum of trace 1 minus trace 3. Traces 1, 2, and 3 represent 0, 1, and 2 mM neohesperidin, respectively.

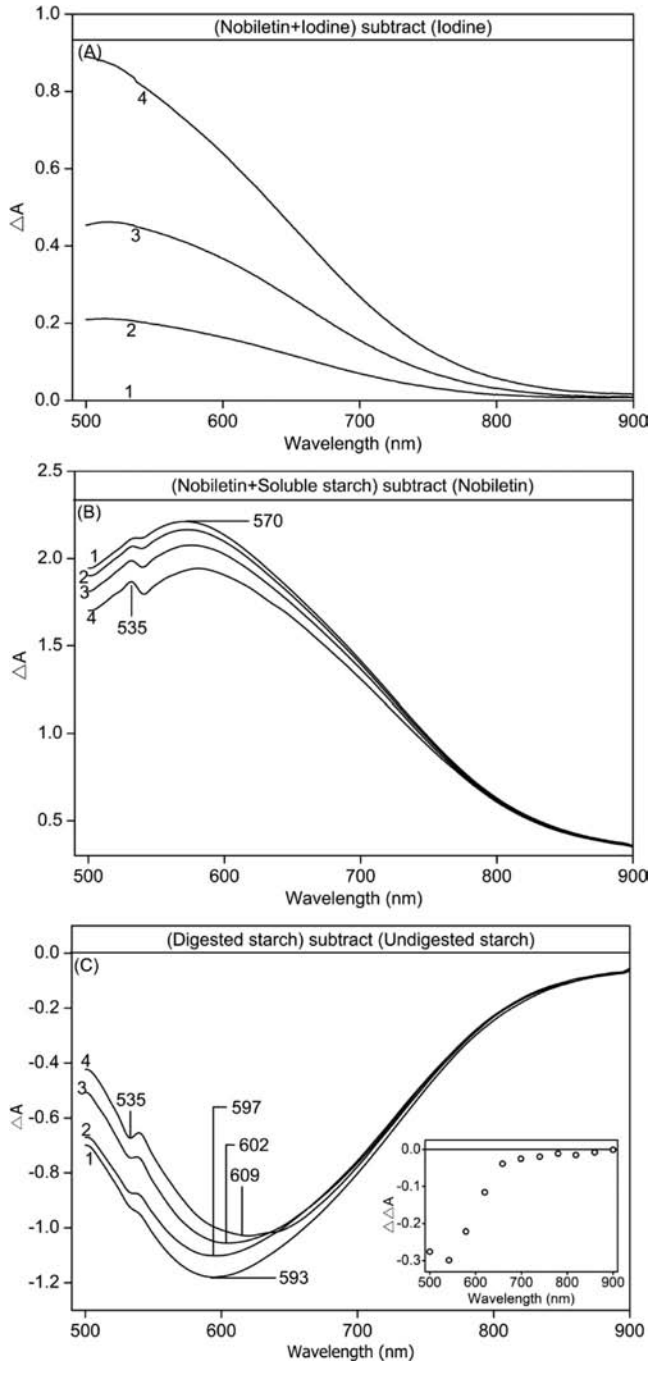

Figure 5. Interactions among nobiletin, iodine, and soluble starch. (A) Δ*A* spectra between the presence and absence of nobiletin in iodine solution. (B) Suppression of soluble starch–iodine complex formation by nobiletin. (C) Inhibition of pancreas α-amylase-catalyzed soluble starch digestion by nobiletin. Digestion time = 1 min. (Inset) ΔΔ*A* spectrum of trace 1 minus trace 4. Traces 1, 2, 3, and 4 represent 0, 20, 40, and 80 μM nobiletin, respectively.

The results obtained above suggest that naringin and neohesperidin could bind to amylose and inhibit amylose digestion, whereas hesperidin and nobiletin could bind to both amylose and amylopectin and inhibit their digestion. Confirming experiments were performed in the next study.

Inhibitory Effects of *Citrus* Flavonoids on Amylose–Iodine Complex and Amylopectin–Iodine Complex Formation and Digestion. Panels A of Figures 6 and 7 show the suppression effects of hesperidin and naringin on amylose–iodine complex formation, and the suppression effects increased with increasing concentrations. The amylose–iodine complex had a peak at 540–660 nm, and Wang et al. finally chose 615 nm as the detection wavelength of the amylose content.[38] Therefore, the peak at 612 nm of the Δ*A* spectra (panels A of Figures 6 and 7) was due to the formation of the amylose–iodine complex. These results suggest that hesperidin and naringin could bind to amylose and suppress the formation

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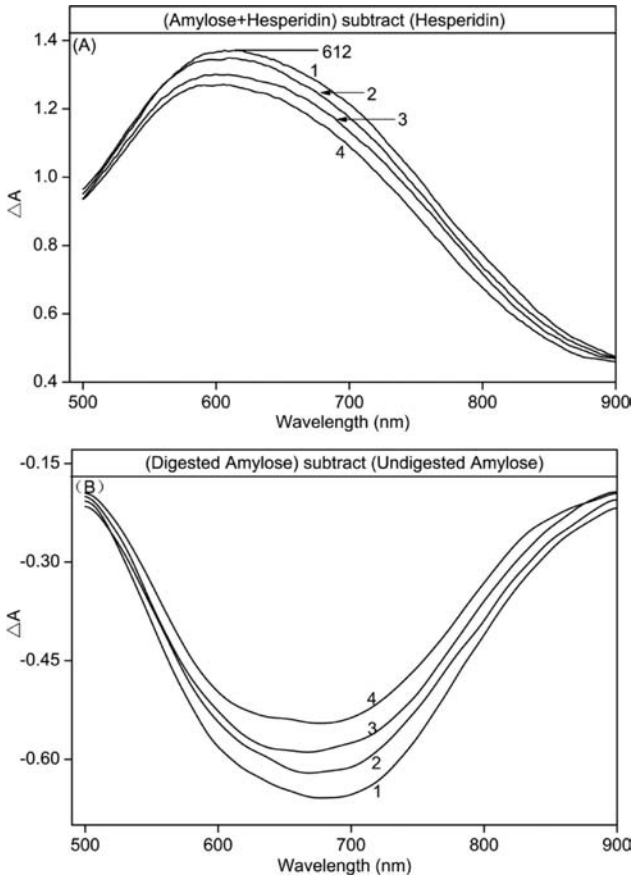

Figure 6. Interactions between hesperidin and amylose. (A) Suppression of amylose–iodine complex formation by hesperidin. (B) Inhibition of pancreas α-amylase-catalyzed amylose digestion by hesperidin. Digestion time = 1 min. Traces 1, 2, 3, and 4 represent 0, 0.6, 1.2, and 2.4 mM hesperidin, respectively.

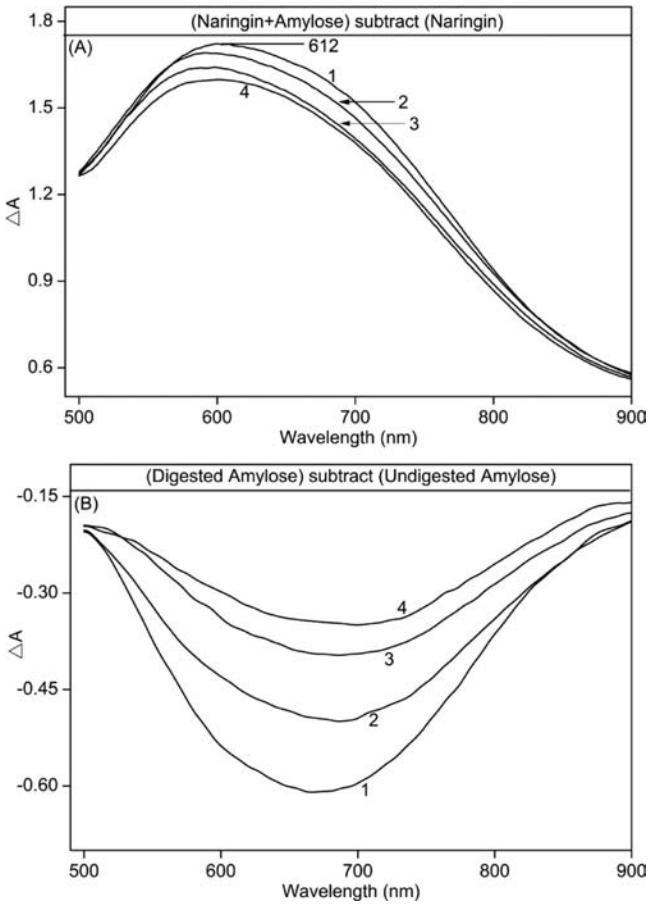

Figure 7. Interactions between naringin and amylose. (A) Suppression of amylose–iodine complex formation by naringin. (B) Inhibition of pancreas α amylase-catalyzed amylose digestion by naringin. Digestion time = 1 min. Traces 1, 2, 3, and 4 represent 0, 0.25, 0.5, and 1 mM naringin, respectively.

of the amylose–iodine complex, which is familiar to some flavonoids, such as rutin, quercetin, and isoflavone genistein in early studies.[14,15]

The inhibitory effects of hesperidin and naringin on amylose digestion were shown in panels B of Figures 6 and 7, and the inhibition increased with increasing concentrations. Neohesperidin and nobiletin also suppressed the formation of the amylose–iodine complex and inhibited the amylase-catalyzed amylose digestion (data not shown). These results are in general accordance with a previous study that suggests some flavonoids, such as rutin and quercetin, could bind to amylose by occupying its helical structures, thus inhibiting amylose digestion.[14]

The suppression effects of hesperidin and nobiletin on the formation of the amylopectin–iodine complex were shown in panels A and B of Figure 8. The peak at 535 nm of ΔA spectra can be taken as the formation of the amylopectin–iodine complex. This is deduced from the report that the amylopectin–iodine complex had a peak at 500–540 nm, and Wang et al. finally chose 535 nm as the detection wavelength of the amylopectin content.[38] We did not obtain significant ΔA spectra of amylopectin digestion, which can be explained by the production of reducing sugars. When soluble starch and amylose were used, after 1 min of amylase treatment, the absorbance of ΔA_{410} was about 0.44 and 0.4, respectively (panels A and B of Figure 9). However, for amylopectin, under the same conditions, the absorbance of ΔA_{410} was about 0.125. Even after 8 min of amylase treatment, the absorbance of ΔA_{410} was about 0.295 (Figure 9C). The slight degradation of amylopectin by amylase may explain why we did not obtain significant ΔA spectra of amylopectin digestion.

The results in Figures 6–8 confirm the conclusion induced from Figures 2–5 that naringin and neohesperidin could bind to amylose, whereas hesperidin and nobiletin could bind to both amylose and amylopectin and inhibited their digestion.

Fatty acids, such as myristic, palmitic, stearic, oleic, linoleic, and stearic acids, could occupy the helical structures of amylose, thus inhibiting amylose digesiton.[9−14] The digestion of starch was inhibited by *Citrus* flavonoids, suggesting that flavonoids could also occupy the hydrophobic helical structures of amylose, thus inhibiting starch digestion. It has been reported that flavonoids, such as rutin, quercetin, and genistein, could occupy the helical structures of amylose.[14,15] Amylopectin also has a hydrophobic helical structure in the molecule, but the length of the helical structure of amylopectin is shorter than that of amylose. Hesperidin and nobiletin could bind to the hydrophobic helical structure of amylopectin because of their smaller spatial conformation and size of hydrophobic regions compared to neohesperidin and naringin. This ideal is supported by the report that octenyl succinic anhydride, which has a smaller spatial conformation and size of hydrophobic regions, decreased wax maize starch enzyme accessibility because of the cross-linking of amylopectin and the

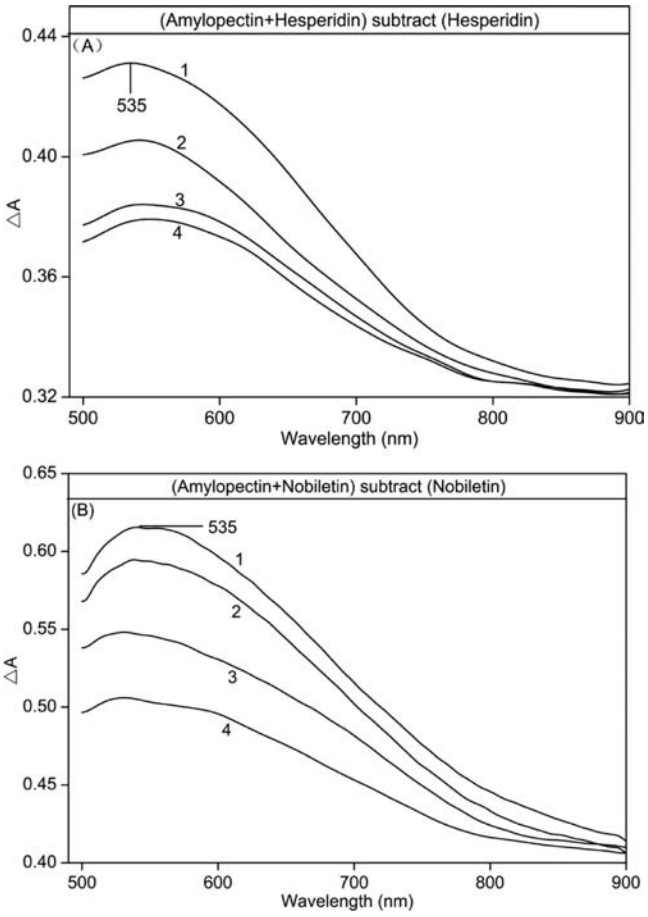

Figure 8. Interactions between hesperidin or nobiletin and amylopectin. (A) Suppression of amylopectin−iodine complex formation by hesperidin. Traces 1, 2, 3, and 4 represent 0, 0.6, 1.2, and 2.4 mM hesperidin, respectively. (B) Suppression of amylopectin−iodine complex formation by nobiletin. Traces 1, 2, 3, and 4 represent 0, 20, 40, and 80 μM nobiletin, respectively.

hydrophobic interaction between octenyl succinic anhydride and amylopectin.[42] Figure 8 demonstrates that both hesperidin and nobiletin could bind to amylopectin. Interestingly, the ΔA spectrum of the soluble starch−iodine complex suppressed by nobiletin had a peak at 535 nm (Figure 5B), but hesperidin did not have a peak at 535 nm (Figure 3B). It has been reported that iodine could bind to the amylose−lipid complex and the amylose−fatty acid−protein ternary complex, thus resulting in the different absorption spectra.[41] The absorbance of iodine was increased by the formation of the nobiletin−iodine complex but decreased by the formation of the hesperidin−iodine complex. Therefore, it is possible that the absorption spectrum of iodine binding to the soluble starch−nobiletin complex has a peak at 535 nm, whereas the absorption spectrum of iodine binding to the soluble starch−hesperidin complex has no peak at 535 nm. According to the above discussion, it is important to take the presence and absence of free helical structures in starch into consideration to discuss the interactions between starch and *Citrus* flavonoids in relation to starch digestion.

Inhibition of Soluble Starch, Amylose, and Amylopectin Production of Reducing Sugars by *Citrus* Flavonoids. Panels A and B of Figure 9 show that naringin and neohesperidin time-dependently inhibited amylase-catalyzed soluble starch and amylose digestion. Naringin and

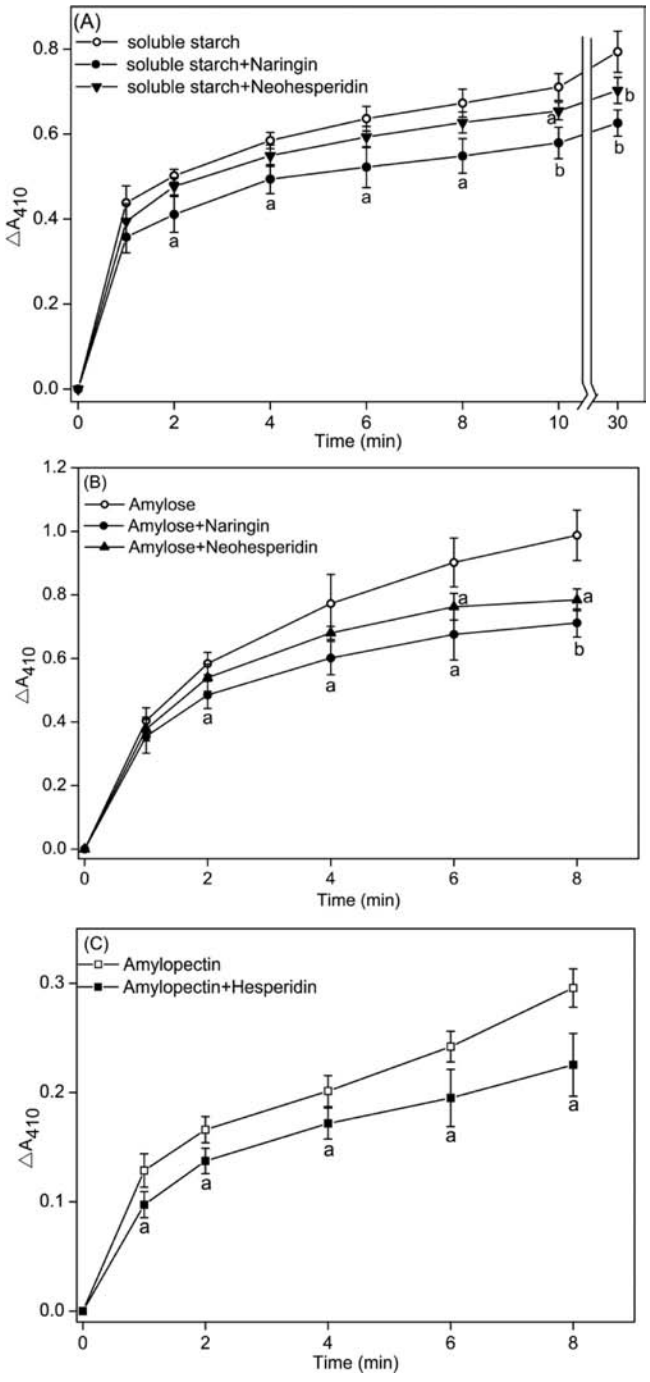

Figure 9. Inhibition of pancreas α-amylase-catalyzed digestion of (A) soluble starch, (B) amylose, and (C) amylopectin by *Citrus* flavonoids. Starch digestion was estimated by the production of reducing sugars. Each data point represents the average of three independent experiments. The concentrations of neohesperidin, naringin, and hesperidin were 0.8, 1, and 1.2 mM, respectively. (a) $p < 0.05$ and (b) $p < 0.01$ compared to soluble starch, amylose, or amylopectin.

neohesperidin significantly ($p < 0.01$) decreased the soluble starch digestion at 30 min (Figure 9A). The inhibition ratio of naringin and neohesperidin were about 22 and 12%, respectively. Naringin also significantly ($p < 0.01$) decreased the amylose digestion at 8 min, and neohesperidin significantly ($p < 0.05$) decreased the amylose digestion after 6 min of amylase treatment (Figure 9B). Naringin and neohesperidin inhibited amylose digestion by about 28 and 21%, respectively,

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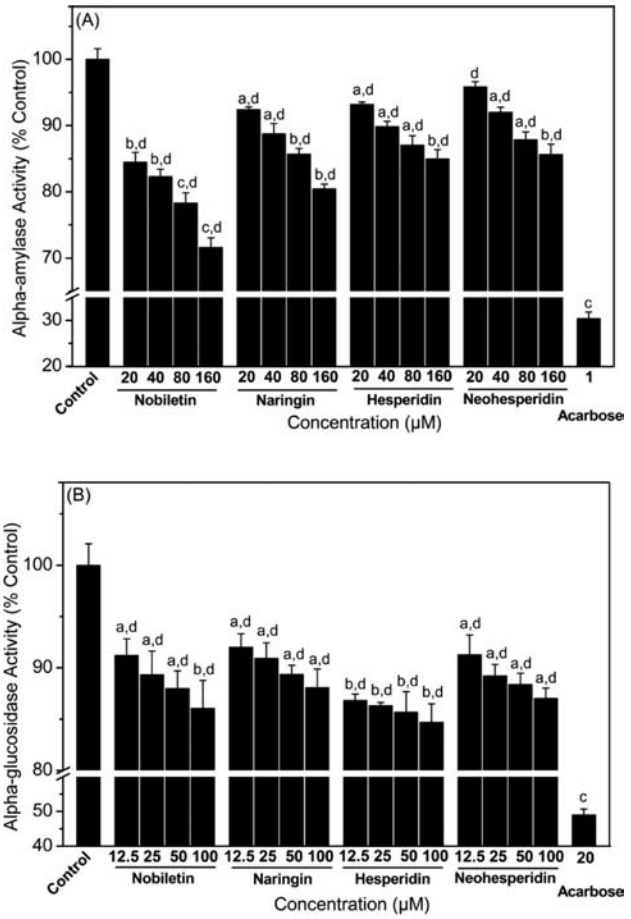

Figure 10. Inhibition of (A) α-amylase activity and (B) α-glucosidase activity by *Citrus* flavonoids. Values are normalized to vehicle-treated controls and expressed as the mean ± SD of four experiments. (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ compared to the control group. (d) $p < 0.001$ compared to the acarbose group.

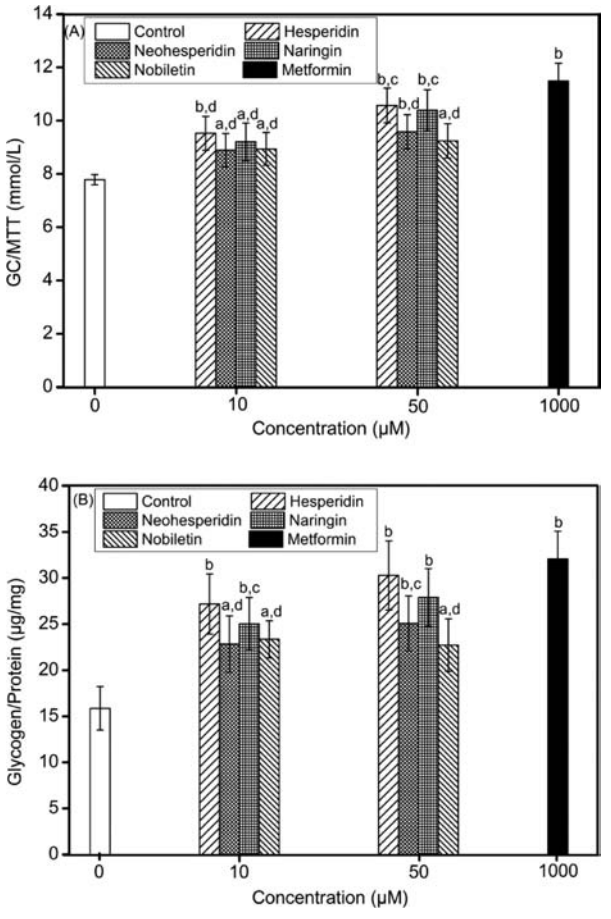

Figure 11. Effects of *Citrus* flavonoids on the GC and glycogen content in HepG2 cells. GC/MTT was the ratio of the GC divided by the optical density (OD) value of MTT. Each value represents the mean ± SD of three experiments. (a) $p < 0.05$ and (b) $p < 0.01$ compared to the control group. (c) $p < 0.05$ and (d) $p < 0.01$ compared to the metformin group.

at the terminal time. Hesperidin also significantly ($p < 0.05$) decreased the amylopectin production of reducing sugars after 1 min of amylase treatment (Figure 9C). Hesperidin and nobiletin also significantly ($p < 0.01$) decreased the soluble starch and amylose production of reducing sugars (data not shown).

It is well-known that the time required for gastric digestion cannot be stated with absolute accuracy. In general, the period for the complete digestion of food is over 3 h.[43] Although naringin and neohesperidin did not significantly decrease the soluble starch digestion in the first few minutes, they all significantly ($p < 0.01$) decreased the soluble starch production of reducing sugars at 30 min. The inhibition increased with the prolongation of time. The above information suggests that *Citrus* flavonoids might be potentially used as specific nutrients in functional foods and may significantly delay starch digestion.

Inhibition of Digestive Enzyme Activities by *Citrus* Flavonoids. It is known that flavonoids can inhibit digestive enzyme activities.[33,44−47] However, there are few studies about the inhibitory effects of *Citrus* flavonoids on digestive enzyme activities. If *Citrus* flavonoids were used in functional foods, these flavonoids could be released when food were digested in the intestinal tract. However, whether *Citrus* flavonoids could inhibit digestive enzyme activities, thus causing a secondary hypoglycemic effect, is unclear. Pancreas α-amylase and α-glucosidase are the major digestive enzymes involved in the hydrolysis of dietary starch and food.[46,47] Therefore, we studied the effects of *Citrus* flavonoids on pancreas α-amylase and α-glucosidase activities.

Figure 10A shows the inhibitory activity of *Citrus* flavonoids against pancreas α-amylase. Although nobiletin showed a better inhibition effect compared to the other three *Citrus* flavonoids, the largest dose (160 μM) inhibition ratio of nobiletin was below 30%. Acarbose was used as a positive control and inhibited pancreas α-amylase by 70% at 1 μM. Figure 10B shows the effect of *Citrus* flavonoids on α-glucosidase activity. Four flavonoids all showed weak inhibitory activity on α-glucosidase; the inhibition ratios were all bellow 20%, even at the largest dose (100 μM). Acarbose was also used as a positive control and inhibited α-glucosidase by 52% at 20 μM. These results indicate that *Citrus* flavonoids exhibited weak digestive enzyme inhibition activities and may not be effective digestive enzyme inhibitors.

According to the report by Kim and his co-workers,[48] *Dangyuja* extract (main compositions are neohesperidin, naringin, and hesperidin) showed slight or no inhibitory activities on rat intestinal α-glucosidase and porcine pancreatic α-amylase. Converting these aglycones into their hydroxylated flavanones by *Aspergillus saitoi* had significantly increased their inhibitory activities on rat intestinal α-glucosidase and porcine

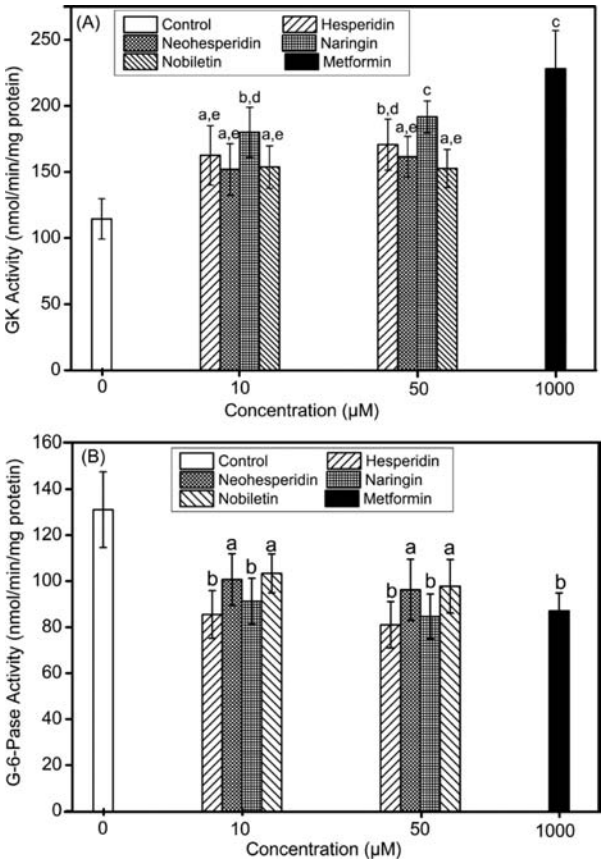

Figure 12. Effects of *Citrus* flavonoids on (A) GK activity and (B) G-6-Pase activity in HepG2 cells. Each value represents the mean ± SD of three experiment. (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ compared to the control group. (d) $p < 0.05$ and (e) $p < 0.01$ compared to the metformin group.

pancreatic α-amylase compared to their glycosides and aglycones. These results indicate that more hydroxyls in the aglycone may contribute to better inhibitory activities against digestive enzymes.

Effects of *Citrus* Flavonoids on the GC and Glycogen Content in HepG2 Cells. The results in Figure 11 show that the GC/MTT and glycogen content were significantly increased by 10 and 50 μM *Citrus* flavonoids. These results were similar to the report that hesperidin and naringin increased diabetic mice hepatic glucose use and hepatic glycogen concentration.[26] In the same condition, 1 mM metformin showed a stronger effect of promoting the GC rate and glycogen content.

Effects of *Citrus* Flavonoids on Hepatic Enzyme Activities in HepG2 Cells. Hepatic GK activity was significantly elevated, and G-6-Pase activity was markedly decreased in *Citrus* flavonoid groups when compared to the control group (Figure 12). Hesperidin and naringin showed better effects on increasing glycolysis and lowering gluconeogenesis. It has been reported that hesperidin and naringin significantly increased diabetic animal hepatic GK activity and GK mRNA level and markedly decreased hepatic G-6-Pase activity and G-6-Pase mRNA level.[25−28] Nobiletin also significantly decreased diabetic mice hepatic G-6-Pase mRNA level.[24] Our results along with the reported results indicate that *Citrus* flavonoids play important roles in preventing the progression of hyperglycemia, partly by increasing hepatic glycolysis and glycogen content and lowering hepatic gluconeogenesis.

In conclusion, the present study provides the evidence that *Citrus* flavonoids exhibited weak digestive enzyme inhibition activities, therefore, may not be effective inhibitors of digestive enzymes, but could bind to starch, thus significantly delaying starch digestion. Moreover, *Citrus* flavonoids also increased glucose use, which seemingly was mediated via elevated glycolysis, increased hepatic glycogen concentration, and decreased gluconeogenesis, resulting from the effect on GK and G-6-Pase. This work suggests that *Citrus* flavonoids could control the postprandial rises in blood glucose and prevent postprandial hyperglycemia. Further *in vivo* studies are needed to investigate the metabolism of *Citrus* flavonoids used in functional foods and the blood glucose regulation mechanism by *Citrus* flavonoids.

■ ASSOCIATED CONTENT

Supporting Information

Amylase-catalyzed digestion of soluble starch (Figure S1), inhibition of amylose digestion by neohesperidin (Figure S2) and nobiletin (Figure S3), and inhibition of amylase-catalyzed digestion of soluble starch and amylose by hesperidin (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

Gal-G2-α-CNP, α-(2-chloro-4-nitrophenyl)-β-1,4-galactopyranosylmaltoside; CNP, 2-chloro-4-nitrophenol; PNPG, *p*-nitrophenyl-α-D-glucopyranoside; GK, glucokinase; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; G-6-Pase, glucose-6-phosphatase; DTT, dithiothreitol; NAD^+, nicotinamide adenine dinucleotide; GC, glucose consumption; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

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