J Nutr Sci Vitaminol, **52**, 149–153, 2006

Inhibition of α-Glucosidase and α-Amylase by Flavonoids

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(Received June 30, 2005)

Summary The inhibitory activity of six groups of flavonoids against yeast and rat small intestinal α-glucosidases and porcine pancreatic α-amylase was compared, and chemical structures of flavonoids responsible for the inhibitory activity were evaluated. Yeast α-glucosidase was potently inhibited by the anthocyanidin, isoflavone and flavonol groups with the IC_{50} values less than 15 μM. The following structures enhanced the inhibitory activity: the unsaturated C ring, 3-OH, 4-CO, the linkage of the B ring at the 3 position, and the hydroxyl substitution on the B ring. Rat small intestinal α-glucosidase was weakly inhibited by many flavonoids, and slightly by the anthocyanidin and isoflavone groups. 3-OH and the hydroxyl substitution on the B ring increased the inhibitory activity. In porcine pancreatic α-amylase, luteolin, myricetin and quercetin were potent inhibitors with the IC_{50} values less than 500 μM. The 2,3-double bond, 5-OH, the linkage of the B ring at the 3 position, and the hydroxyl substitution on the B ring enhanced the inhibitory activity, while 3-OH reduced it.

Key Words α-glucosidase, α-amylase, inhibition, flavonoid, structure-activity relationship

Small intestinal α-glucosidase [EC 3.2.1.20] and pancreatic α-amylase [EC 3.2.1.1] are key enzymes of dietary carbohydrate digestion in humans. Inhibitors of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress postprandial hyperglycemia. Some of flavonoids are known to inhibit these enzymes, and the inhibitory activity of flavonoid glycosides is usually lower than that of the aglycones.

Flavonoids are widely distributed in plants such as vegetables and fruits. The primary structure of flavonoids consists of two moieties: benzopyran (A and C rings) and phenyl (B ring) groups. The variation in the C ring and the linkage between the benzopyran and phenyl groups are the basis for the classification of flavonoids into six groups: flavone, flavonol, flavanone, isoflavone, flavan-3-ol, and anthocyanidin groups (*1*).

The structure-activity relationship is elucidated in some flavonoid groups (*2–6*), but there is no paper to investigate the relationship by comparing the α-glucosidase inhibitory activity of six groups of flavonoids. Few papers report the inhibition of flavonoids against α-amylase (*7–9*). Since yeast α-glucosidase is distinct from the small intestinal enzyme, we here investigated the inhibitory activity of six groups of flavonoids against yeast and rat small intestinal α-glucosidases, and porcine pancreatic α-amylase; and the relationship between the structures of the A, B and C rings and the inhibitory activity. In this work, we selected luteolin, apigenin and baicalein (flavone group), myricetin, qurcetin, kaempferol and fisetin (flavonol group), naringenin and hesperetin (flavanone group), genistein and daidzein (isoflavone group), (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epigallocatechin gallate (flavan-3-ol group), and cyanidin (anthocyanidin group) on the basis of the structure of the C ring, the degree of hydroxylation, and the linkage between the benzopyran and phenyl groups.

MATERIALS AND METHODS

Materials. Luteolin, apigenin, quercetin dihydrate, kaempferol, fisetin, genistein, daidzein, (−)-epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin gallate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); myricetin, and (+)-catechin hydrate from Nacalai Tesque, Inc. (Kyoto, Japan); baicalein from Sigma Aldrich Japan Co. (Tokyo, Japan); naringenin from Tokyo Kasei Kogyo (Tokyo, Japan); hesperetin from Avocado Research Chemicals Ltd. (Lancashire, UK); cyanidin chloride from Extrasynthese (France). α-Glucosidase from yeast was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan); α-amylase from porcine pancreas (Type VI-B), and rat small intestine acetone powder from Sigma Aldrich Japan Co. *p*-Nitrophenyl α-D-glucopyranoside (PNPG) was from Nacalai Tesque, Inc.; Gluneo kit from Shinotest Co. (Tokyo, Japan); Amylase HR Reagent (freeze-dried non-reducing-end blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) plus a thermostable α-glucosidase) from Megazyme International Ireland (Wicklow, Ireland).

Assay of enzyme activity.

(1) Yeast α-glucosidase: Basal conditions for the assay of the yeast α-glucosidase inhibitory activity were as follows. A reaction mixture containing 320 μL of 100 mM phosphate buffer (pH 6.8), 50 μL of 10 mM PNPG in the buffer, and 10 μL of flavonoid in dimethylsulfoxide (DMSO) was incubated at 30°C for 5 min, and then 20 μL of the buffer containing 0.01 mg/mL of the

enzyme was added to the mixture. After further incubation at 30°C for 5 min, 3.0 mL of 50 mM sodium hydroxide was added to the mixture, and the absorbance at 410 nm of the liberated *p*-nitrophenol was measured. The reaction mixture showed the absorbance of about 0.45 in the absence of flavonoid.

(2) Rat small intestinal α-glucosidase: A crude rat intestinal α-glucosidase solution was prepared by the method of Deguchi et al. (*10*). A reaction mixture consisting of 300 μL of 56 mM maleate buffer (pH 6.0), 600 μL of 2% maltose in the buffer, and 300 μL of flavonoid in DMSO was incubated at 37°C for 5 min, and then 600 μL of the crude enzyme solution was added to the mixture. After further incubation at 37°C for 30 min, the reaction was terminated by heating for 10 min in a boiling water bath. To 900 μL of the reaction mixture was added 50 mg of polyvinyl polypyrolidone to remove flavonoid which interfered with glucose measurement. The liberated glucose was determined with a Gluneo kit.

(3) Porcine pancreatic α-amylase: A synthetic substrate, BPNPG7, was used as substrate in the assay of the porcine pancreatic α-amylase inhibitory activity. A reaction mixture containing 100 μL of Amylase HR Reagent and 40 μL of flavonoid in methanol was incubated at 37°C for 5 min, and then 60 μL of 0.1 mg/mL enzyme in 0.1 M HEPES buffer (pH 6.9) was added to the reaction mixture. After further incubation at 37°C for 10 min, the liberated *p*-nitrophenol was determined as described in the assay of the yeast α-glucosidase activity.

Estimation of inhibition of flavonoids. Inhibition (%) was calculated as $(A-B)/A\times100$, where A was the quantity of the reaction product in the absence of flavonoid, and B was that in the presence of flavonoid. IC_{50} was the concentration of flavonoid required for 50% inhibition of the enzyme activity under the assay conditions, and obtained graphically by an inhibition curve.

The inhibitory activity of all the flavonoids tested against rat small intestinal α-glucosidase and porcine pancreatic α-amylase was not determined at the concentrations over 500 μM where some flavonoids were insoluble.

RESULTS AND DISCUSSION

Inhibition of yeast α-glucosidase by flavonoids

Six groups of flavonoids in Fig. 1 were evaluated for the inhibitory activity against yeast α-glucosidase, rat small intestinal α-glucosidase, and porcine pancreatic α-amylase. Table 1 shows the inhibitory activity of flavonoids against yeast α-glucosidase in the inhibition (%) at 200 μM and the IC_{50} values. All flavonoids except for apigenin, of which the inhibition did not increase at the concentrations over 25 μM, inhibited the enzyme in a dose-dependent manner. The IC_{50} values in this work were compatible with the reported ones (*2*, *11*, *12*). The anthocyanidin, isoflavone and flavonol groups, and epigallocatechin gallate in the flavan-3-ol group were potent inhibitors with the IC_{50} values less than 15 μM.

First, the effect of the hydroxyl substitution on the B ring on the inhibitory activity was evaluated (Table 1). Baicalein lacking 4¢OH little inhibited the enzyme, and the methoxyl substitution of 4¢OH was unfavorable to the inhibitory activity (the inhibitory activity of hesperetin was lower than that of naringenin, that is, hesperetin<naringenin), showing that 4¢OH was important to the inhibitory activity. A comparison of the inhibitory activity of 4¢hydroxylated, 4¢5¢dihydroxylated, and 3¢4¢5¢trihydroxylated flavonoids in the same flavonoid groups indicated that the inhibitory activity increased considerably with an increase in the number of the hydroxyl group on the B ring (Flavonol: myricetin>quercetin>kaempferol. Flavone: luteolin>apigenin. Flavan-3-ol: epigallocatechin>epicatechin).

Then, the effect of the structures of the A and C rings on the inhibitory activity was evaluated by comparing the inhibitory activity of six groups of flavonoids containing the same B ring at 200 μM (Table 1). In 4¢hydroxylated flavonoids, the decreasing order of the inhibitory activity was genistein>daidzein>kaempferol>naringenin>apigenin. But the inhibitory activity of apigenin (43%) was higher than that of naringenin (25%) at 25 μM where the apigenin showed the maximum acitivity (Table 1). In 3¢4¢dihydroxylated flavonoids, the decreasing order of the inhibitory activity was cyanidin>quercetin>luteolin>catechin>epicatechin, and in 3¢4¢5¢trihydroxylate flavonoids, it

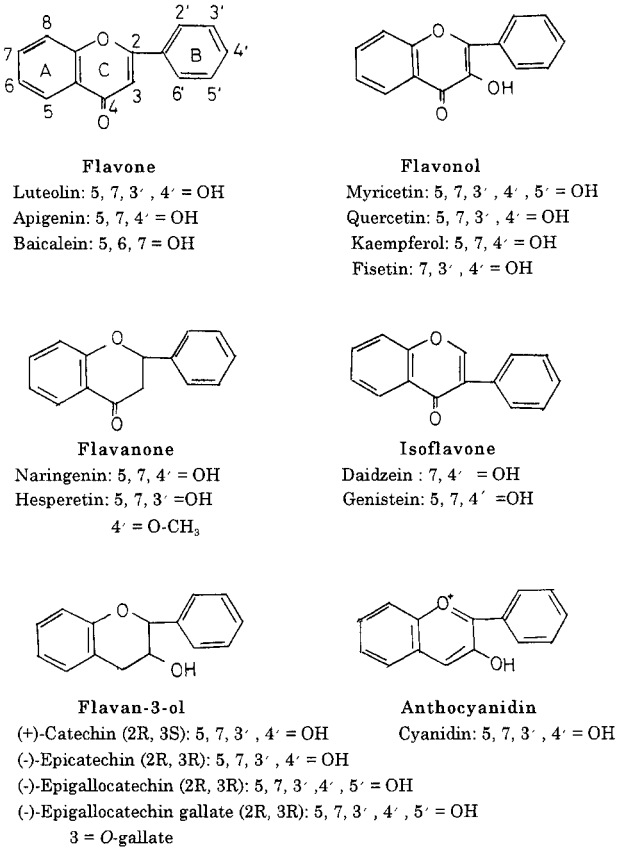

Fig. 1. Structures of flavonoids. Flavonoids are classified into six groups on the basis of the variation in the C ring and the linkage between the benzopyran and phenyl groups.

Table 1. Inhibitory activity of flavonoid against yeast α-glucosidase.

Flavonoid	Inhibition[a,b] (%)	IC_{50} (μM)[a,c]		Flavonoid	Inhibition[a,b] (%)	IC_{50} (μM)[a,c]	
		This work[a]	Reported value			This work[a]	Reported value
Flavonol				Isoflavone			
Myricetin	94	5	4[e]	Daidzein	89 (67)[d]	14	
Quercetin	91	7	8[e], <24[f]	Genistein	93 (78)[d]	7	8[g]
Kaempferol	82 (64)[d]	12	20[e]	Flavan-3-ol			
Fisetin	88	13	8[e]	Catechin	45	>200	
Flavone				Epicatechin	24	>200	
Luteolin	92	21		Epigallocatechin	71	75	
Apigenin	43 (43)[d]	>200		Epigallocatechin gallate	89	2	
Baicalein	5	>200		Anthocyanidin			
Flavanone				Cyanidin	99	4	
Naringenin	73 (25)[d]	75	50[e]				
Hesperetin	61	150					

The enzyme activity was estimated by measuring *p*-nitrophenol liberated. Experimental details are described in "Materials and Methods."

[a] The result was an average of three determinations.

[b] Inhibition by 200 μM flavonoid.

[c] Concentration required for 50% inhibition of the enzyme activity under the assay conditions.

[d] Inhibition by 25 μM flavonoid.

[e] Iio et al. (*2*).

[f] Watanabe et al. (*11*).

[g] Lee and Lee (*12*).

was myricetin>epigallocatechin.

From the above results, the decreasing order of the inhibitory activity of six flavonoid groups was concluded to be anthocyanidin≧isoflavone≧flavonol>flavone>flavanone>flavan-3-ol. The structures of the A, B and C rings were closely related to the inhibitory activity. As to the A and C rings, hydroxylations at the 3 and 5 positions of flavone enhanced the inhibitory activity (Hydroxylation at the 3 position: quercetin>luteolin; kaempferol>apigenin. Hydroxylation at the 5 position: quercetin>fisetin; genistein>daidzein). A great difference in inhibition between the isoflavone and flavone groups (genistein>apigenin) indicated that the linkage of the B ring at the 3 position enhanced the inhibitory activity. Saturation of the 2,3-double bond in the C ring seemed to decrease the inhibitory activity (naringenin<apigenin). Flavan-3-ol lacking both the 2,3-double bond and 4-CO showed the lowest inhibitory activity. However, the bonding of the galloyl group at the 3 position of epigallocatechin resulted in a high inhibitory activity, especially at the concentrations under 10 μM. Cyanidin, which contains the 1,2- and 3,4-double bonds and lacks 4-CO, was a potent inhibitor. Recently, its 3-rutinoside has been found as a new inhibitor against yeast α-glucosidase (*13*). As to the B ring, 4¢OH was important, and 3¢ and 5¢OH were favorable to the inhibitory activity as described above.

The type of inhibition and the inhibitor constant were estimated by kinetic analysis for some flavonoids in five flavonoid groups (Table 2). Ki and Ki¢ which are dissociation constants of the enzyme-inhibitor complex and the enzyme-substrate-inhibitor complex, respectively, were determined from the slope and intercept on the vertical axis in a Lineweaver-Burk plot (*2*). The inhibitor constant of quercetin was similar to that reported by Iio et al. (*2*). The ratio of Ki/Ki¢tended to be high in the isoflavone group showing the mixed type inhibition.

Inhibition of rat small intestinal α-glucosidase by flavonoids

Sucrose is used as substrate in most of studies on the inhibition of flavonoids against rat small intestinal α-glucosidase. In this work, maltose was used, because maltose is the main digestive product of starch in the small intestine.

Rat small intestinal α-glucosidase was weakly inhibited by many flavonoids, and slightly by the isoflavone and anthocyanidin groups (Table 3). The inhibitory effect of the flavan-3-ol group was almost the same as that of tea polyphenols on the PNPG-hydrolytic activity (*14*). Acylated anthocyanins and anthocyanins inhibit rat small intestinal α-glucosidase with an IC_{50} of 60 μM and 4.6 mM, respectively (*15*). In this work, a slight inhibitory activity of cyanidin was observed. Bicalein (5,6,7-trihydroxyflavone), which is known as a potent inhibitor of rat small intestinal α-glucosidase with sucrose as substrate (IC_{50} of 32 μM (*4*) or 45 μM (5)), weakly inhibited the maltose-hydrolytic action. The IC_{50} value was over 500 μM (Table 3).

The following structures enhanced the inhibitory activity: hydroxylation at the 3 position of flavone (quercetin>luteolin; kaempferol>apigenin), and the hydroxyl substitution on the B ring (Flavonol: myricetin≒quercetin>kaempferol. Flavone: luteolin> apigenin). 5-OH did not affect the inhibitory activity

Table 2. Type and kinetic constant of yeast α-glucosidase inhibition by flavonoid.

Flavonoid	Type of inhibition	Ki[a] (μM)	Ki¢[a] (μM)
Myricetin	Mixed type, close to non-competitive	3.0	7.6
Quercetin	Mixed type, close to non-competitive	3.4	14
Luteolin	Mixed type, close to non-competitive	29	38
Naringenin	Mixed type, close to non-competitive	120	60
Daidzein	Mixed type	36	9.3
Genistein	Mixed type	52	4.2
Epigallocatechi gallate	Mixed type, close to non-competitive	3.0	7.6

The reaction conditions are described in "Materials and Methods." Ki and Ki¢values were determined from the slope and intercept on the vertical axis in a double-reciprocal plot (*2*).
[a] The result was an average of three determinations.

Table 3. Inhibitory activity of flavonoid against rat small intestinal α-glucosidase and porcine pancreatic α-amylase.

Flavonoid	α-Glucosidase	α-Amylase		Flavonoid	α-Glucosidase	α-Amylase	
	Inhibition[a,b] (%)	Inhibition[a,b] (%)	IC_{50}[a,c] (mM)		Inhibition[a,b] (%)	Inhibition[a,b] (%)	IC_{50}[a,c] (mM)
Flavonol				Isoflavone			
Myricetin	29	64	0.38	Daidzein	0	19	>0.50
Quercetin	28	50	0.50	Genistein	2	33	>0.50
Kaempferol	8	18	>0.50	Flavan-3-ol			
Fisetin	26	33	>0.50	Catechin	1	4	>0.50
Flavone				Epicatechin	5	14	>0.50
Luteolin	19	61	0.36	Epigallocatechin	7	5	>0.50
Apigenin	3	21	>0.50	Epigallocatechin gallate	32	21	>0.50
Baicalein	16	31	>0.50	Anthocyanidin			
Flavanone				Cyanidin	6	37	>0.50
Naringenin	1	5	>0.50				
Hesperetin	2	16	>0.50				

The activity of rat small intestinal α-glucosidase was estimated by measuring glucose liberated from maltose, and that of porcine pancreatic α-amylase by measuring *p*-nitrophenol from BPNPG7. Experimental details are described in "Materials and Methods."
[a] The result was an average of three determinations.
[b] Inhibition by 0.50 mM flavonoid.
[c] Concentration of flavonoid required for 50% inhibition of the enzyme activity under the assay conditions.

(fisetin≒quercetin).

Inhibition of porcine pancreatic α-amylase by flavonoids

The inhibitory activity of six groups of flavonoids against porcine pancreatic α-amylase is shown in Table 3. Luteolin, myricetin and quercetin were potent inhibitors with IC_{50} of 0.36, 0.38 and 0.50 mM, respectively. The IC_{50} value for luteolin was compatible with the result of Kim et al. (0.17–1.7 mM) (*7*).

A comparison of the inhibitory activity of 4¢hydroxylated, 4¢5¢dihydroxylated, and 3¢4¢5¢trihydroxylated flavonoids in the same flavonoid groups showed that the inhibitory activity increased appreciably with an increase in the number of the hydroxyl group on the B ring (Flavonol: myricetin>quercetin>kaempferol. Flavone: luteolin>apigenin). The inhibitory activity of six groups of flavonoids containing the same B ring was compared. In 4¢hydroxylated flavonoids, the decreasing order of the inhibitory activity was genistein>apigenin>daidzein>kaempferol>naringenin; in 3¢4¢dihydroxylated flavonoids, it was luteolin>quercetin>cyanidin>epicatechin>catechin; in 3¢4¢5¢trihydroxylated flavonoids, it was myricetin>epigallocatechin. From the above results, the decreasing order of the inhibitory activity of six flavonoid groups was concluded to be isoflavone>flavone>flavonol>anthocyanidine>flavanone≒flavan-3-ol.

The structures of the A, B and C rings were related to the activity. Linkage of the B ring at the 3 position (genistein>apigenin), the 2,3-double bond (isoflavone, flavone, and flavonol>flavanone and flavan-3-ol), and hydroxylation at the 5 position of flavonol or isoflavone (quercetin>fisetin; genistein>daidzein) enhanced the inhibitory activity. Since the inhibitory activity appreciably increased with an increase in the number of the hydroxyl group on the B ring as described above, 3¢ hydroxylation of genistein was inferred to result in a

higher inhibitory activity than luteolin. Hydroxylation at the 3-position of flavone was unfavorable to the inhibitory activity (quercetin<luteolin; kaempferol< apigenin) in contrast to the two α-glucosidases.

Tea polyphenols inhibit human salivary α-amylase potently, and the IC_{50} for epigallocatechin gallate is 260 μM (*9*). Some anthocyanin extracts from plants inhibit human salivary α-amylase (*8*). In both works, starch is used as substrate. In this work using a synthetic substrate, BPNPG7, the inhibitory activity of epigallocatechin gallate and cyanidin was weak (Table 3). Then, we made a preliminary experiment to evaluate the inhibitory effect of epigallocatechin gallate and cyanidin on starch-hydrolytic activity of porcine pancreatic α-amylase, in which digestion of starch was measured by the method of Matsui et al. (*8*). It was found that epigallocatechin gallate and cyanidin potently inhibited starch-hydrolytic action of porcine pancreatic α-amylase too, and the IC_{50} values were 400 and 80 μM, respectively. The different assay methods gave the different results.

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