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Comparative Evaluation of Quercetin, Isoquercetin and Rutin as Inhibitors of α-Glucosidase

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Three flavonoids from tartary buckwheat bran, namely, quercetin (Que), isoquercetin (Iso) and rutin (Rut), have been evaluated as α-glucosidase inhibitors by fluorescence spectroscopy and enzymatic kinetics and have also been compared with the market diabetes healer, acarbose. The results indicated that Que, Iso and Rut could bind α-glucosidase to form a new complex, which exhibited a strong static fluorescence quenching via nonradiation energy transfer, and an obvious blue shift of maximum fluorescence. The sequence of binding constants (K_A) was Que > Iso > Rut, and the number of binding sites was one for all of the three cases. The thermodynamic parameters were obtained by calculations based on data of binding constants. They revealed that the main driving force of the above-mentioned interaction was hydrophobic. Enzymatic kinetics measurements showed that all of the three compounds were effective inhibitors against α-glucosidase. Inhibitory modes all belonged to a mixed type of noncompetitive and anticompetitive. The sequence of affinity ($1/K_i$) was in accordance with the results of binding constants (K_A). The concentrations which gave 50% inhibition (IC_{50}) were 0.017 mmol·L^{-1}, 0.185 mmol·L^{-1} and 0.196 mmol·L^{-1}, compared with acarbose's IC_{50} (0.091 mmol·L^{-1}); the dose of acarbose was almost five times of that of Que and half of that of Iso and Rut. Our results explained why the inhibition on α-glucosidase of tartary buckwheat bran extractive substance (mainly Rut) was much weaker than that of its hydrolysis product (a mixture of Que, Iso and Rut). This work would be significant for the development of more powerful antidiabetes drugs and efficacious utilization of tartary buckwheat, which has been proved as an acknowledged food in the diet of diabetic patients.

KEYWORDS: Flavonoid compound; α-glucosidase; inhibitor; diabetes; tartary buckwheat

INTRODUCTION

For decades diabetes has loomed large as a serious problem in the world owing to the ever-enlarged number of patients that suffered from this consumptive disease. Controlling the blood glucose level is always an effective way to mitigate the illnesses and prevent hyperglycemic and diabetes exacerbation (*1*). It is well-known that all carbohydrates are hydrolyzed finally to monosaccharide in the intestine by enzymes. Among these enzymes, α-glucosidase which is located in the brush-border surface membrane of intestinal cells is the most important enzyme in carbohydrate digestion. Clinically acarbose and voglibose have been used as effective α-glucosidase inhibitors to delay glucose absorption (*2*,*3*). Research has shown that most type II diabetics' fasting and postprandial blood glucose, HbA, lipids and glycosylated hemoglobin were remarkably lowered and insulin sensitivity was improved after the patients had taken glucosidase inhibition drugs such as acarbose and voglibose (*1*−*3*). Seifarth et al. (*4*) reported that, after people with diabetes had taken glucosidase inhibition drugs, the chyme of carbohydrates, fats and proteins, etc. would get into the distal ileum where the glucagon-like peptide 1 (GLP-1) is the most abundant, and stimulate increased secretion of GLP-1 and released of insulin, thereby reducing the postprandial blood glucose concentration. It is also known that some glycosidase inhibitors might exhibit antiviral, antimetastatic and immunostimulatory activities through interference with the normal processing of glycoproteins and glycolipids (*5*).

Flavonoids, a class of natural drugs with high biological activity, are abundant in plants. They are reported to have protective effects against the development of diabetes (*6*, *7*) as well as a mitigation effect of diabetes consequences (*8*, *9*). Several papers have been published with regard to flavonoids as α-glucosidase inhibitors (*10*−*13*). They mainly dealt with the inhibitory activity of various flavonoids against α-glucosidases and their structure−activity relationships (*10*, *11*, *14*, *15*). However, studies on isoquercetin are scarce, and systematic investigations on the comparative evaluation of quercetin, its monoglycoside isoquercetin and its diglycoside rutin as inhibitors of α-glucosidase have not been reported.

Buckwheat is the only cultivated crop containing flavonoids and used as a staple food and folk medicine in some regions. It has

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been receiving more and more attention recently owing to its diverse utilities, such as in reducing blood cholesterol and regulating blood sugar, blood lipid and blood pressure in human body. Previously, we reported our study on the interaction between three flavonoid compounds (Que, Iso and Rut) and α-amylase (*16*). We also observed that α-glucosidase's activity was obviously affected by the extractive substance (mainly rutin) and its hydrolysis product (a mixture of quercetin, isoquercetin and rutin) from tartary buckwheat bran. The results showed that both the extractive substance and the hydrolytic product were able to inhibit the activity of α-glucosidase, and the hydrolytic product was much stronger than its precursor. Is such an increase in inhibiting ability related to the hydrolysis of rutin into quercetin and isoquercetin? And is the sequence of the three compounds' inhibiting ability toward α-glucosidase in accordance with that of α-amylase? It will be significant to answer these questions for the development of more powerful antidiabetic drugs and efficacious utilization of tartary buckwheat, which has been proved as an acknowledged food in the diet of diabetic patients.

In order to find out the binding behavior of flavonoids with α-glucosidase, their effect on enzymatic activity (inhibiting effect), and whether such an effect is related to the substitution of glycosyl on C3-OH from flavonoid molecules, we investigated the interaction between three similar flavonoids (i.e., Que, Iso and Rut) and α-glucosidase by fluorescence spectrum (FS) and ultraviolet (UV) spectrum methods. Based on these data we calculated and compared their binding constants (K_A) and the number of binding sites (n). We also compared their inhibiting degree toward the activity of α-glucosidase with that of acarbose by using enzymatic kinetics, and suggested a reasonable inhibiting mode.

MATERIALS AND METHODS

Materials. α-Glucosidase, generated by *Saccharomyces cerevisiae,* was a product of Sigma, USA. Biotech-grade pNP-α-Glu (4-nitrophenyl-α-D-glucopyranoside) was bought from Seebio, Shanghai, China, and L-glutathione (reduced) was obtained from Solarbio, Beijing, China. Que, Iso and Rut were from tartary buckwheat bran, supplied by Shanxi Academy of Agricultural Sciences. Their purity was 99.1%, 96.7% and 98.3% by HPLC, and the relative molecular masses were 302.23, 464.38 and 610.52 Da, respectively. Their structures are shown in **Figure 1**. Acarbose tablets were purchased from Bayer HealthCare Company Ltd. Beijing, China, with each tablet contains 50 mg of acarbose. Doubly distilled water, Hitachi F-2500 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan), Hitachi U-2010 UV spectrophotometer (Hitachi Instruments, Inc., Tokyo, Japan) and Inolab pH meter (WTW GmbH & Co. KG, Weilheim, Germany) were used in the experiments.

Fluorescence Spectra Measurements. The sample of α-glucosidase (5.0×10^{-7} mol·L^{-1}) was prepared by dissolving solid α-glucosidase into phosphate buffer (0.1 mol·L^{-1}, pH 6.8, with 0.1 mol·L^{-1} NaCl to sustain a consistent ion intensity), and flavonoids were in 60% ethanol. To do the FS measurement, a solution of 1.0 mL of α-glucosidase was added to a fluorescence cuvette at a given temperature and titrated with flavonoid. The later should be mingled with the former and kept for 5 min to let them to react before the measurements. Fluorescence spectra of α-glucosidase and α-glucosidase−flavonoid mixture were recorded in the range from 315 to 500 nm. Both slits of excitation and emission were 10 nm with an excitation wavelength at 295 nm and an optical path of 10 mm.

Analysis of Inhibitory Ability of Que, Iso, Rut and Acarbose. pNP-α-Glu would be hydrolyzed into pNP by α-glucosidase's catalysis. The absorptions of pNP at 410 nm were measured on an Hitachi U-2010 spectrophotometer, and they are consistent with the activity of α-glucosidase. To make a sample, α-glucosidase was dissolved in phosphate buffer (pH 6.8), and its concentration was 1.0×10^{-6} mol·L^{-1}. The changes of the absorption values of pNP after adding different quantities of Que, Iso, Rut and acarbose were investigated. The inhibitory degree (%) of flavonoids upon α-glucosidase is expressed as $(1 - \Delta A_{test}/\Delta A_{control}) \times 100$, where ΔA indicates the absorbance increase in 10 min. Acarbose was in doubly distilled water, filtered by filter membranes (0.22 μm), and its concentration was 7.7×10^{-3} mol·L^{-1}.

Measurements of Nonradiation Energy Transfer. The fluorescence emission spectrum of α-glucosidase and UV absorbance spectrum of the three flavonoids were obtained under the condition of the same concentration, 5.0×10^{-7} mol·L^{-1}, for all the samples. The overlapping area of the two spectra and the distance (r) between the donor and the acceptor were calculated.

All glassware were routinely washed in 1.0 mol·L^{-1} HNO_3 and then were rinsed with doubly distilled water.

RESULTS AND DISCUSSION

Fluorescence Spectra. In a protein molecule, tryptophan (Trp) has the most powerful activity to emit fluorescence. Thus we can take its fluorescent change as a probe to follow the protein's conformation variation (*17*). Under our measurement condition (i.e., from 315 to 500 nm), all fluorescence emissions from the flavonoids, buffer and other reagents are so weak that their impact can be ignored safely. At room temperature, with the addition of flavonoid into the cuvette, fluorescence intensity of α-glucosidase was quenched gradually and the absorption peaks (near 345 nm) were hypsochromic shifted (**Figure 2**). The sequence of quenching ability was Que > Iso ≈ Rut, and the emission peaks were shifted from 346 to 340 nm (with the addition of Que), 345 to 333 nm (Iso) and 345 to 342 nm (Rut), respectively. These changes suggested that the interaction between the three flavonoids and α-glucosidase had resulted in a polarity variation for Trp (*18*) which belongs to the molecule of enzyme. In another words, the reaction had made the microenvironment of Trp residues change from hydrophilic to hydrophobic. The fluorescence peak is near 345 nm, indicating it is from interior Trp residues of α-glucosidase (*19*).

Mechanism of Fluorescence Quenching. There are two types of fluorescence quenching, namely, dynamic quenching and static quenching (*20*). Dynamic quenching stems from the collisions

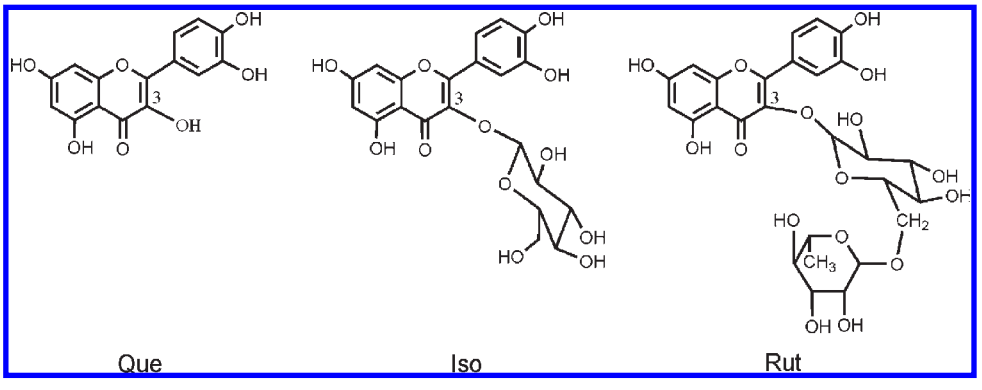

Figure 1. Molecular structures of Que, Iso and Rut.

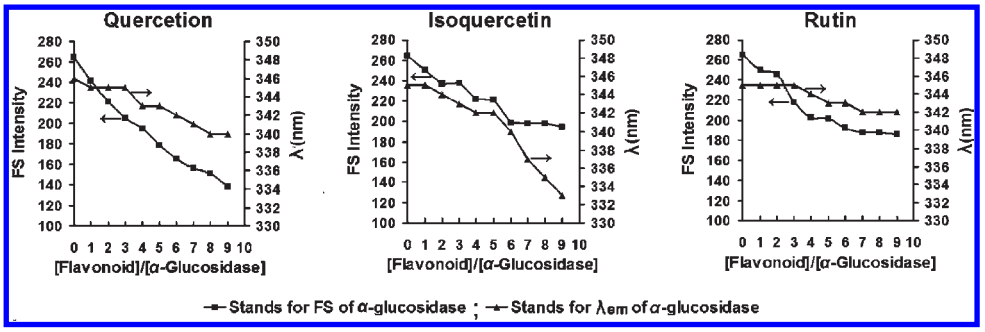

Figure 2. The effect of Que, Iso and Rut on fluorescence spectrum of α-glucosidase after they were added into the enzyme solution. Measurement conditions: [α-glucosidase] = 5.0×10^{-7} mol·L^{-1}, pH 6.8, at room temperature, λ_{ex} = 295 nm, λ_{em} = 345 nm.

between two fluorescent luminophors, while static quenching results from the formation of a new nonfluorescent complex. According to the fluorescence quenching theory (*20*), at a certain range of temperature, dynamic quenching constant (K_{sv}) rises along with the increase of temperature, while with static quenching, the case is the opposite. The rate constant (K_q) in the process of dynamic quenching usually is up-limited to be not greater than 2.0×10^{10} L·mol^{-1}·s^{-1}, which is the maximal value for macromolecule-participating quenching rate constant in dynamic quenching (*21*). So if $K_q \gg 2.0 \times 10^{10}$ L·mol^{-1}·s^{-1}, we can rule out the quenching to be static quenching. K_{sv} and K_q can be calculated from the Stern−Volmer equation (*22*):

$$F_0/F = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

where F_0 and F are fluorescence intensity of fore-and-aft interaction between α-glucosidase and flavonoid, [Q] is the concentration of quencher and here flavonoid, and τ_0 is the average life of fluorescent substance without quencher, valued about 10^{-8} s (*21*). K_{sv} and K_q are the dynamic quenching constant and the rate constant in the process of double molecule quenching.

The quenching fluorescence spectra of α-glucosidase by flavonoids were recorded at three temperatures (25, 30 and 37 °C). The values of K_{sv} and K_q were obtained with the Stern−Volmer equation from plots of linear equations obtained by F_0/F vs [Q]. As seen from **Table 1**, the values of K_{sv} decreased with the increase of temperature and K_q are much more than 2.0×10^{10} L·mol^{-1}·s^{-1}. Therefore, the process of quenching is a static quenching by forming a complex.

Binding Constants (K_A) and Binding Sites (n). Static quenching follows the equations (*23*)

$$\lg\frac{F_0 - F}{F} = \lg K_A + n \lg [Q]_f \quad (2)$$

$$[\text{flavonoid}]_f = [\text{flavonoid}] - n[\alpha\text{-glucosidase-flavonoid}_n] \quad (3)$$

$$[\alpha\text{-glucosidase-flavonoid}_n] = \frac{F_0 - F}{F_0 - F_\infty}[\alpha\text{-glucosidase}] \quad (4)$$

Here $[Q]_f$ is the concentration of free flavonoid, $[\text{flavonoid}]_f$; $[\alpha\text{-glucosidase-flavonoid}_n]$ is the concentration of α-glucosidase bound with the flavonoid.

From the plots of linear eq 2 obtained by $\lg[(F_0 - F)/F]$ vs $\lg [Q]_f$, one can calculate the values of K_A and n. The results (**Table 2**) demonstrated that the sequence of binding constants (K_A) is Que > Iso > Rut, namely, with the increase of their volumes and polarity the flavonoids would be more difficult to bind with a protein. The number of binding sites (n) was close to one at 37 °C, but smaller with the reaction temperature lowered. This means

Table 1. Constants of K_{sv} and K_q of the Interaction between α-Glucosidase and Que, Iso, Rut

flavonoid	T (°C)	$K_{sv}/10^5$ (L·mol^{-1})	$K_q/10^{13}$ (L·mol^{-1}·s^{-1})	R^2
quercetin	25	1.94	1.94	0.9985
	30	1.87	1.87	0.9849
	37	1.40	1.40	0.9804
isoquercetin	25	1.02	1.02	0.9818
	30	0.91	0.91	0.9819
	37	0.88	0.88	0.9819
rutin	25	1.48	1.48	0.9798
	30	1.42	1.42	0.9864
	37	1.37	1.37	0.9841

Table 2. Values of K_A and n of the Interaction between α-Glucosidase and Que, Iso, Rut

flavonoid	T (°C)	$K_A/10^5$ (L·mol^{-1})	n	R^2
quercetin	25	1.4564	0.8021	0.9950
	30	1.6772	0.8680	0.9884
	37	2.0450	0.9904	0.9923
isoquercetin	25	1.0363	0.8039	0.9945
	30	1.3394	0.8366	0.9549
	37	1.8147	0.8656	0.9803
rutin	25	1.0346	0.8733	0.9731
	30	1.1749	0.8085	0.9840
	37	1.4289	0.9075	0.9893

that 37 °C is the most suitable temperature for the three flavonoid molecules to bind with α-glucosidase, and such a temperature is just the human body's temperature.

Thermodynamics of Que, Iso and Rut Acting with α-Glucosidase. The interaction between a quencher and a protein molecule belongs to weak intermolecular forces, including hydrogen bonding, van der Waals force, electrostatic attraction, hydrophobic interaction and so on. The type of the main interaction between a flavonoid and a protein molecule could be judged or assessed according to the relative values' change in enthalpy and entropy (ΔH and ΔS) (*24*). More specifically, if $\Delta H > 0$ and $\Delta S > 0$, the main force would be hydrophobic force; if $\Delta H < 0$ and $\Delta S < 0$, it would be hydrogen bonding; if $\Delta H < 0$ and $\Delta S > 0$, it would be electrostatic force. With the following thermodynamic equations, we obtained the thermodynamic parameters, and they have been listed in **Table 3**.

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

$$\Delta G = -RT \ln K \quad (6)$$

$$\ln(k_2/k_1) = (1/T_1 - 1/T_2)\Delta H/R \quad (7)$$

Under our experimental conditions, the values of ΔG for the three flavonoids were all negative, while their ΔH and ΔS were all

positive. This means that the interaction between α-glucosidase and the three flavonoids is a spontaneous process of entropy increasing and free energy decreasing and driven mainly by hydrophobic force. However, hydrogen binding should not be ruled out (although not as the main contributor) because the experimental system is in aqueous solution and there exist many hydroxyl groups on the molecules of flavonoids and protein.

Nonradiation Energy Transfer. According to Förster's dipole−dipole nonradiation energy transfer theory (*25*), energy transfer will happen when a donor can emit fluorescence, and at the same time there is an overlapping area between the donor's fluorescence emission spectrum and the acceptor's UV absorption spectrum, and the distance (r) between the donor and the acceptor should approach and be lower than 7 nm (*26*). The results (**Figure 3**) revealed that there was overlapping between α-glucosidase's fluorescence emission spectrum and the UV absorption spectra of Que, Iso and Rut. The distances (r) between them were calculated, and they are 3.73, 3.50 and 3.47 nm, respectively, for Que, Iso and Rut. As we noted, the three distances are all less than 7 nm; this further proved that the fluorescence quenching between Que, Iso, Rut and α-glucosidase was caused by nonradiation energy transfer and it also meant that flavonoid molecule can partly insert into α-glucosidase.

Table 3. Thermodynamic Parameters of the Interaction between α-Glucosidase and Que, Iso, Rut

flavonoid	T (°C)	ΔG (kJ·mol^{-1})	ΔH (kJ·mol^{-1})	ΔS (J·mol^{-1}K^{-1})
quercetin	25	−23.0854	130.43	516.3
	30	−24.5969		
	37	−30.6271		
isoquercetin	25	−22.0969	38.69	206.7
	30	−24.1447		
	37	−25.2619		
rutin	25	−28.9369	113.76	478.6
	30	−32.1781		
	37	−35.337		

Inhibitory Kinetics of α-Glucosidase. The experimental results (**Figure 4**) revealed that Que, Iso and Rut were all powerful inhibitors toward α-glucosidase. The inhibitory sequence was consistent with the binding order, Que > Iso ≈ Rut, and the values of IC_{50} were 0.017, 0.185 and 0.196 mmol·L^{-1} for Que, Iso and Rut, respectively. To compare with acarbose's IC_{50} (0.091 mmol·L^{-1}), the dose of acarbose was almost five times that of Que and half that of Iso and Rut. Applying double reciprocal or Lineweaver−Burk plot of $1/v$ against 1/[S] (**Figure 5**), we deduced that the inhibitory mode of Que, Iso and Rut toward α-glucosidase was a mixture type of noncompetitive and anticompetitive (*27*). According to the Michaelis equation (*28*),

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (8)$$

The calculated value of K_m for α-glucosidase was 1.48×10^{-4} mol·L^{-1} under the conditions of this work. The values of inhibition constants (K_i) can be obtained by the Dixon method. As is seen in **Table 4**, we deduced the sequence of affinity ($1/K_i$) between the three flavonoids and α-glucosidase to be Que > Iso > Rut, consistent with K_A.

On the structure−activity relation of flavonoids, Chen et al. thought that the glycosylation of C3-OH had little effect on antioxidant activities (*29*), while other scholars deduced that change of hydroxide radical into glycosyl would decline or even exterminate the antioxidant activity of flavonoid drugs (*30*). Tadera et al. (*10*) suggested that 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 enhanced the α-glucosidase inhibitory activity, and Gao et al. (*14*, *15*) studied the importance of the A ring and B ring and their hydroxyl substitution on the α-glucosidase inhibitory activity. They deemed that the

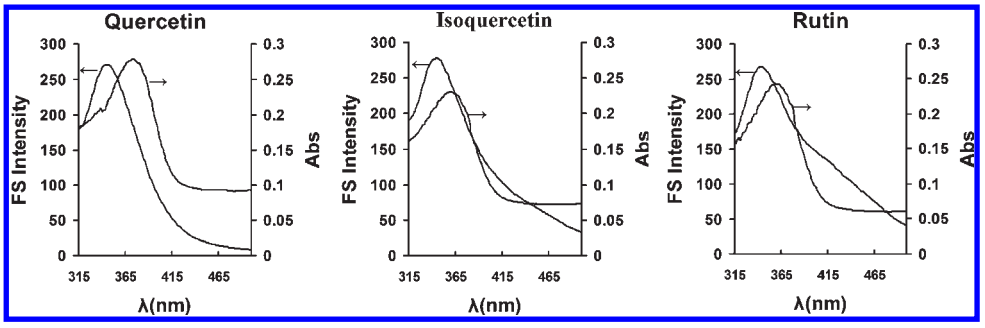

Figure 3. Overlap of α-glucosidase fluorescence spectra with three flavonoids UV absorption spectra. [Flavonoid] = [α-glucosidase] = 5.0×10^{-7} mol·L^{-1}, pH 6.8, at room temperature.

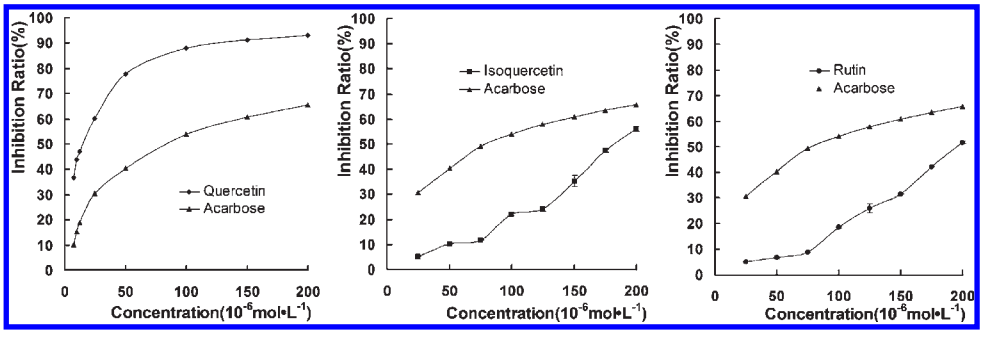

Figure 4. Inhibitory percentage of α-glucosidase by flavonoids and acarbose. [α-Glucosidase] = 1.0×10^{-6} mol·L^{-1}, pH 6.8, at room temperature, SD < 0.1.

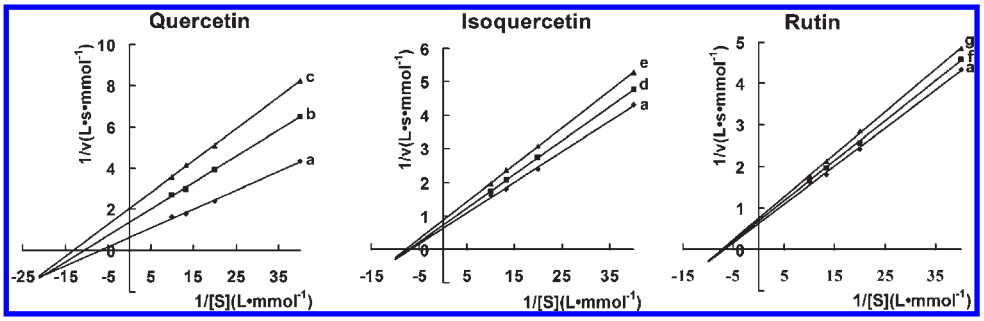

Figure 5. Plots of $1/v$ versus 1/[S]. [α-Glucosidase] = 1.0×10^{-6} mol·L^{-1}; [flavonoid]: in a, 0 mol·L^{-1}; in b, 1.0×10^{-5} mol·L^{-1}; in c, 2.0×10^{-5} mol·L^{-1}; in d and f, 5.0×10^{-5} mol·L^{-1}; in e and g, 1.0×10^{-4} mol·L^{-1}. pH 6.8, at room temperature.

Table 4. K_i and $1/K_i$ Values of Interaction between α-Glucosidase and Que, Iso, Rut

	$K_i/10^{-6}$ (mol·L^{-1})	$1/K_i/10^4$ (L·mol^{-1})
quercetin	6.68	15.0
isoquercetin	25.0	4.00
rutin	28.3	3.53

5,6,7-trihydroxyflavone structure was concluded to be crucial for the potent inhibitory activity, and hydroxyl substitution on the B ring of 5,6,7-trihydroxyflavones was favorable to the activity. Yang et al. (*18*) compared the interaction between quercetin (with C3-OH), luteocin (without C3-OH) and lysozyme, and their results showed that C3-OH was disadvantageous for the hydrophobic interaction and led to a reduction of the binding between quercetin and lysozyme. In our previous work (*16*), we found that the inhibitory effects of Que, Iso and Rut on α-amylase showed an inhibitory sequence as Iso ≈ Que > Rut, which was in accordance with the results of binding constants from fluorescence experiments. So far, no publications have reported investigation of the relation between the α-glucosidase inhibitory activity and the replacement of C3−OH on flavonoids by glycosyl.

As the main driving force of the interaction between Que, Iso, Rut and α-glucosidase was hydrophobic, with the replacement of C3−OH by one glycosyl, Que turns into Iso, and its volume and polarity become larger than that of Que's, and the inhibitory ability of Que was stronger than that of Iso. However, such an impact was not in direct proportion with the increase of glycosyl (such as the inhibitory ability of Iso ≈ that of Rut). This suggested that the effect of steric hindrance is far less than that of the substitution of glycosyl, and also showed that the mechanism of interaction between molecules is very complex, affected by a variety of factors.

In summary, we have compared the inhibition ability of three flavonoids (with similar structures) and acarbose toward α-glucosidase's activity and studied the structure−activity relationships of flavonoids. Our objective is to search for more effective and safer α-glucosidase inhibitors from natural materials and develop some intensified functional or health care food for diabetic patients. Our results have explained with satisfaction why the inhibition ability of tartary buckwheat bran extractive substance (mainly Rut) against α-glucosidase was much weaker than that of its hydrolysis product (a mixture of Que, Iso and Rut). And we found that quercetin is a much more potent natural product than acarbose in controlling blood glucose level. We want to stress here that it will not be an effective method in treating hyperglycemia and obesity by merely taking raw tartary buckwheat or using Rut in food. It is necessary for us to develop some new technologies for deep-processing of tartary buckwheat, say, to transform Rut into Iso and Que by special hydrolytic methods, thus making them possess remarkable effects in curing diseases as hyperglycemia and obesity. It is even possible to commercialize such a process to benefit society.

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