THE Society for Food Science & Technology
Journal of Food Science
A Publication of the Institute of Food Technologists

Study on the Interaction between 3 Flavonoid Compounds and α-Amylase by Fluorescence Spectroscopy and Enzymatic Kinetics

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ABSTRACT: The interaction between α-amylase and 3 flavonoid compounds from tartary buckwheat bran, namely, quercetin (Que), its monoglycoside isoquercetin (Iso), and its diglycoside rutinb (Rut), has been studied by fluorescence spectroscopy and enzymatic kinetics. The results indicate that Que, Iso, and Rut could bind with α-amylase to form a new complex, which exhibits an obvious fluorescence quenching. We deduce that such a quenching is a static quenching via nonradiation energy transfer. Results from plots and calculations show that the sequence of binding constants (K_A) is Iso > Que > Rut. Calculation on thermodynamic parameters reveals that the main driving force of above-mentioned interaction is hydrophobic. Enzyme activity measurements show that all of the 3 flavonoid compounds are effective inhibitors toward α-amylase, and the inhibitory mode belongs to a competitive type. The sequence of affinity ($1/K_i$) is in accordance with the results of binding constants (K_A) from fluorescence experiments.

Keywords: α-amylase, fluorescence spectroscopy, isoquercetin, quercetin, rutin

Introduction

Flavonoid compounds, a class of natural drugs with high biological activity, are abundant in plants in nature. Flavonoid is reported to exert antihyperglycemic effect (Wang and others 1992; Kamalakkannan and Stanely 2006), protective effect against the development of diabetes (Odetti and others 1990; Srinivasan and others 2005) as well as a mitigation effect of diabetes consequences (Je and others 2002; Nagasawa and others 2003). However, the acting mechanism is not well known. Diabetes is a common consumptive disease in the today's world and effective medicines that can completely cure diabetes are still not available. Control of the sugar level in patients' blood is an effective way to mitigate and prevent the illnesses of hyperglycemic and diabetes from exacerbation. After starch is taken into human body it is hydrolyzed to oligosaccharides by amylase at first, then decomposed into disaccharides and further into monosaccharides by glocosidase. Inhibitors of amylase can effectively defer or partly retard the digestion and assimilation at the early stage of starch digestion, and thus lead to a lower level of blood sugar for diabetic patients after meals. So amylase inhibitors can be served as good auxiliary medicines for treatment of diabetes.

In the paper titled "Binding of Selected Phenolic Compounds to Proteins" (Rawel and others 2005), the noncovalent binding of selected phenolic compounds (quercetin, rutin, and isoquercetin) to different proteins (human serum albumin, bovine serum albumin, soy glycinin, and lysozyme) was studied. The results showed that there are noncovalent binding between quercetin, rutin, isoquercetin, and lysozyme, amylase, but the work was not concerned with inhibitory function. Yang Juan and others (2006) researched the interaction between drug rutin and pancreatic α-amylase and came to the conclusion that rutin inhibits pancreatic α-amylase in a noncompetitive manner. Another paper, "Inhibitory Effect of Tartary Buckwheat Extract on Trypsin and Amylase Activity," authored by Hu and others (2003), proved that rutin could inhibit the activity of α-amylase and β-amylase and that leaching liquor of tartary buckwheat could inhibit the activity of α-amylase and β-amylase as well. They suggested that the inhibition might be due to the flavonoid in tartary buckwheat.

Previously, we obtained the extractive (mainly rutin) from tartary buckwheat bran and its hydrolysis product (a mixture of quercetin, isoquercetin and rutin). Based on this, we started to investigate the interaction between α-amylase and the extractive or its hydrolysis product. Our studies showed that both the extractive and its hydrolysis product were able to inhibit the activity of α-amylase, but the hydrolytic product was much stronger than its precursor. Is such an increase in inhibitory ability related to the hydrolysis of rutin into quercetin and isoquercetin? It should be significant to answer this question for the development of more powerful antidiabetes drugs and efficacious utilization of tartary buckwheat, which have been proved as an acknowledged food in the diet of diabetic patients. Reports on previous disquisitions about isoquercetin are scarce, and the systematic studies on the interaction between α-amylase and quercetin, its monoglycoside isoquercetin and its diglycoside rutin have not been reported up to now.

For a drug there is a strong relation between the activity and its structure. Yang Ran and others (2006) studied the interaction between lysozyme and 3 flavonoids, their results showed that the existence of C3–OH on flavonoid molecule was disadvantageous to the interaction between flavones and enzymes. Hodnick and others (1986) thought that the C3–OH on flavonoid molecule would deteriorate their antioxidization property. To find out whether the change in binding and inhibiting ability of α-amylase is resulted from the substitution of indicans on C3–OH, if there is any difference with the increase of substituent group on indicans, and what is the varying law within them, we abstracted rutin from tartary buckwheat bran, obtained quercetin and isoquercetin via

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doi: 10.1111/j.1750-3841.2009.01080.x

hydrolysis, and applied fluorescence spectrum (FS) and ultraviolet absorption (UV) spectrum methods to investigate the interaction between the aforesaid 3 similar flavonoid compounds and α-amylase, compared their binding constants (K_A) and binding sites (n). We also studied their inhibition toward α-amylase and proposed a reasonable inhibiting mode.

Materials and Methods

Materials

α-Amylase was purchased from HeFei BoMei Biotechnology Co. Ltd., made in Japan, biochemistry grade. Que, Iso, and Rut (supplied by Shanxi Academy of Agricultural Sciences) are products from tartary buckwheat bran and their structures are shown in Figure 1. The purity of these flavonoid compounds is 99.1%, 96.7%, and 98.3%, respectively, determined by HPLC. They were dissolved in 60% grain alcohol and their relative molecular mass were 302.23, 464.38, and 610.52 Da. Dissolubility starch solution, standard maltose solution, 0.4 mol/L NaOH, DNS, and other reagents were all analytical grade. The water used in all experiments was double distilled water.

Instruments

Fluorescence spectra were recorded on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan), UV spectra were obtained on a Hitachi U-2010 UV spectrophotometer (Hitachi Instruments, Inc., Tokyo, Japan), and visible spectra on an Unico UV-2000 spectrophotometer (UNICO Instruments Co., Ltd., Shanghai, China). Measurements of pH values were carried out on an Inolab pH meter (WTW GmbH & Co. KG, Weilheim, Germany). All glassware were routinely washed in 1.0 mol/L HNO_3 and then rinsed with doubly distilled water.

Methods

Fluorescence spectrum. The fluorescence spectrum of α-amylase was recorded on a Hitachi F-2500 fluorescence spectrophotometer. A certain amount of α-amylase (with a concentration and volume of 8.0×10^{-6} mol/L and 1.0 mL, respectively) was dissolved in Tris-HCl buffer (0.1 mol/L, pH 7.4, with 0.1 mol/L NaCl to keep the same ion intensity). After addition of different concentrations of Que, Iso, and Rut to 3 samples of α-amylase solution, they were slowly dissolved and mixed within 5 min. The emission spectrum was recorded until the solution temperature reached to that of room. Both slit widths of excitation and emission were 10 nm, excitation wavelength was 295 nm. All the fluorescence data were recorded in the range of 300 to 600 nm. As the volumes of Que, Iso, and Rut were much less than that of α-amylase, dilution effect was neglected.

Measurements of α-amylase activity and specific activity. Applying the method of Bernfeld (1955), the absorption of reducing sugar at 520 nm was measured on an Unico UV-2000 spectrophotometer. α-Amylase was dissolved in phosphate buffer (pH 6.8), and its concentration was 2.0×10^{-5} mol/L.

Inhibitory kinetics of α-amylase. Under the same experimental condition as above, the changes of enzyme activity after adding different concentrations of Que, Iso, and Rut were investigated. Finally, the inhibitory types of Que, Iso, Rut, and the values of K_i, $1/K_i$ were obtained by the methods of Lineweaver–Burk (Zhang and others 2007) and Dixon (Wang and others 2004).

Results and Discussion

Fluorescence spectra

In a protein molecule, tryptophan is the principal residue to be responsible for the fluorescence intensity of the macromolecule.

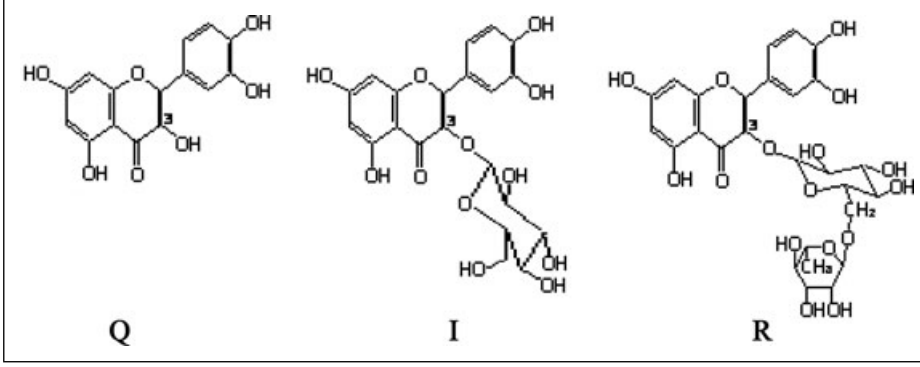

Figure 1 — The scheme of molecular structures for Que, Iso, and Rut.

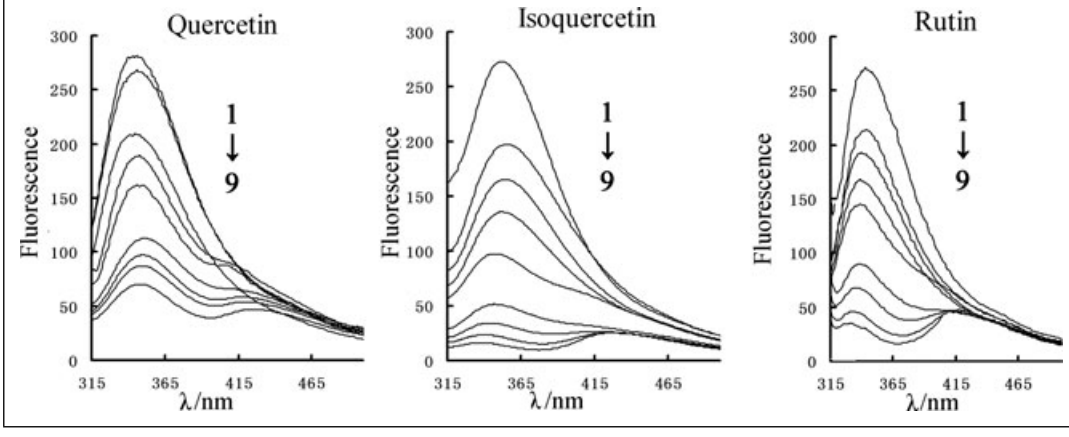

Figure 2—The effect of Que, Iso, and Rut on fluorescence spectrum of α-amylase λ_{ex} = 295 nm, pH 7.4 at 18 °C; in all cases [α-amylase] = 8.0 × 10^{-6} mol/L, the volume of α-amylase is 1 mL, curves 1 to 9 ([flavonoid]/[α-amylase]): 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0.

For α-amylase molecule variety of relative molecular weight, sequence of amino acid (a.a.) and the number of Try may occur for different sources of the enzyme. The α-amylase used in this study is from the species of *Bacillus Subtilis*. It contains about 650 a.a. (from Gene Bank), among them there are 14 Try residues, located at the positions of 56, 58, 99, 101, 161, 171, 175, 188, 235, 305, 321, 331, 574 (or 573), and 596 (or 595), respectively. Since Try has the major fluorescence intensity in α-amylase molecule, so we can track the change of α-amylase's conformation by taking Try as the intrinsic fluorescence probe. As can be seen in Figure 2, at pH 7.4 and room temperature, there is a fluorescence peak at near 345 nm when excitation wavelength is 295 nm. Because of Que, Iso, and Rut have no obvious fluorescence emission information at 345 nm their interference to α-amylase can be neglected. With the addition of flavonoid, the fluorescence intensity was quenched gradually. The curves appeared as a slight red shift with the addition of Que, whereas with the addition of Iso and Rut, it appeared as a blue shift (see Figure 2). These changes suggest that the interaction between α-amylase and the 3 flavonoid compounds have resulted in a polarity variation for Try (Yang and others 2006). The fluorescence peak is at near 345 nm, indicating the peak belongs to Try residues of α-amylase, locates at protein interior (Burstein and others 1973).

Fluorescence quenching can be divided up into 2 types, namely, dynamic quenching and static quenching. Dynamic quenching stems from the collisions between 2 fluorescent luminophors by which a fluorescent body at excited state loses its excited energy and goes back to its ground state and thus leads to the quenching of fluorescence. So dynamic quenching is also called colliding quenching. Static quenching arises from the formation of a new nonfluorescent complex that formed between fluorescent luminophors and quencher.

Dynamic quenching follows the Stern–Volmer equation (Zhang and others 1999):

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

where F_0 and F are fluorescence intensity of fore-and-aft interaction between α-amylase and flavonoid, $[Q]$ is the concentration of quencher and here flavonoid, τ_0 is the average life of fluorescent substance without quencher, valued about 10^{-8} s (Liu and others 2004). K_q is the rate constant in the process of double molecules quenching, K_{sv} is the dynamic quenching constant.

How can we distinguish fluorescence dynamic quenching from static quenching? Since dynamic quenching mainly depend on the collision, its K_q usually is up-limited to be not greater than 2.0×10^{10} per mol/s which is the maximal value for macromolecule-participating quenching rate constant in dynamic quenching (Liu and others 2004). So if $K_q >> 2.0 \times 10^{10}$ per mol/s, we can rule out the quenching to be dynamic one. Another method to make a distinction between dynamic quenching and static quenching is to inspect their relationship with the temperature change. For dynamic quenching, since it heavily depends on the collision, rising of the temperature will be advantageous, thus enlarge its K_{sv} value. While for static quenching, rising of the temperature will be unfavorable to the stability of the complex, thus reduce the value of the formation constant (K_{sv}) of the complex. Applying Stern–Volmer equation we can obtain the values of K_{sv} and K_q by the plots of linear equations got by F_0/F compared with $[Q]$. As shown in Table 1, the values of K_q are much more than 2.0×10^{10} per mol/s and K_{sv} (18 °C) > K_{sv} (37 °C). Therefore, the process of quenching is not the dynamic quenching induced by the collision of molecules, but the static quenching by forming a complex.

Binding constants (K_A) and binding sites (n)

By applying correlative static quenching equations (Xie and others 2004) we can obtain the plots of linear equations calculated by $\lg(F_0 - F)/F$ compared with $\lg[Q]_f$. Through these plots, we calculated the values of K_A and n furthermore. In the following equations, $[Q]_f$ is the concentration of free flavonoid, [α-Amylase–Flavonoid$_n$] is the concentration of α-amylase bound the flavonoid.

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

$$[\text{Flavonoid}]_f = [\text{Flavonid}] - n[\alpha\text{-Amylase} - \text{Flavonoid}_n] \quad (3)$$

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

In Table 2, the sequence of binding constants (K_A) of Que, Iso, and Rut is Iso > Que > Rut, the binding sites (n) are all one. Usually, with the increase of the volume and polarity, a molecule would be harder to bind with a protein. But in our case, with replacement of C3–OH by 1 indican, Que turns into Iso, and its volume and polarity is larger than Que's. However, from our data (Table 2), Iso's K_A increases evidently. So we think that replacement of Que's C3–OH by 1 indican is advantageous to its binding with α-amylase than Que itself. This result also shows that C3–OH on flavonoid molecules is not advantageous to their binding with proteins. It is consistent with the results of Yang Ran and others (2006) and Hodnick and others (1986). However, steric hindrance is unfavorable to the

Table 1—Constant values of K_{sv} and K_q of interaction between α-amylase and Que, Iso, Rut.

Flavonoid	T (°C)	$K_{sv}/10^5$ (L/mol)	$K_q/10^{13}$ (L/mol/s)	R^2
Quercetin	18	0.310	0.310	0.9999
	37	0.188	0.188	0.9859
Isoquercetin	18	1.161	1.161	0.9831
	37	1.102	1.102	0.9873
Rutin	18	0.712	0.712	0.9815
	37	0.660	0.660	0.9861

Table 2—Values of K_A and n of interaction between α-amylase and Que, Iso, Rut.

Flavonoid	T (°C)	$K_A/10^5$ (L/mol)	n	R^2
Quercetin	18	1.338	1.152	0.9999
	37	1.370	1.195	0.9859
Isoquercetin	18	3.529	1.125	0.9783
	37	8.656	1.226	0.9708
Rutin	18	1.044	1.066	0.9882
	37	1.149	1.390	0.9695

Table 3—Thermodynamic parameters of interaction between α-amylase and Que, Iso, Rut.

Flavonoid	T (°C)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol/K)
Quercetin	18	−28.524	61.424	307.9
	25	−29.330		
	30	−32.574		
	37	−33.925		
Isoquercitrin	18	−31.096	32.565	219.3
	25	−32.973		
	30	−33.962		
	37	−35.291		
Rutin	18	−28.011	10.984	134.1
	25	−28.958		
	30	−29.767		
	37	−30.525		

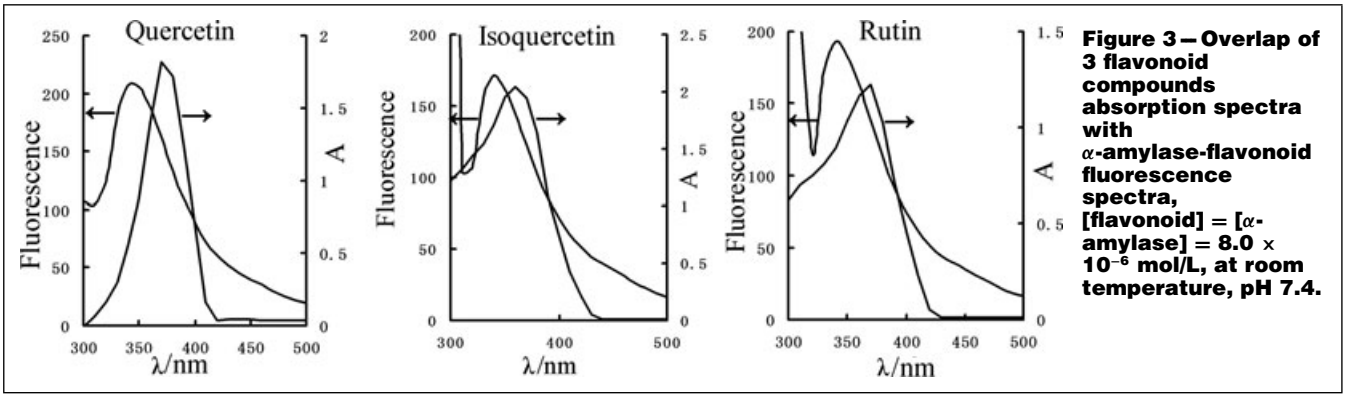

Figure 3—Overlap of 3 flavonoid compounds absorption spectra with α-amylase-flavonoid fluorescence spectra, [flavonoid] = [α-amylase] = 8.0 × 10^{-6} mol/L, at room temperature, pH 7.4.

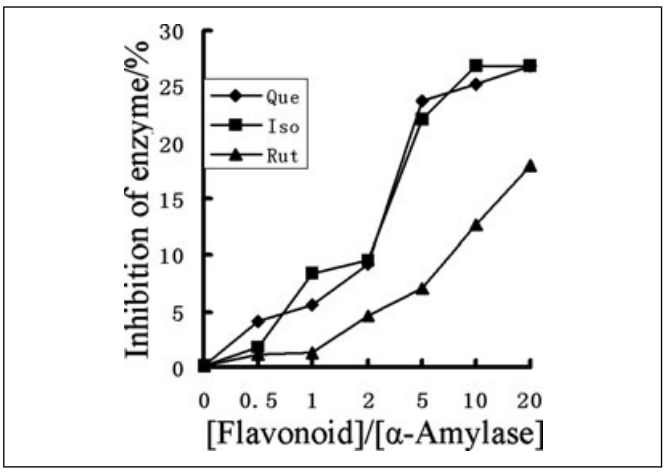

Figure 4—The inhibition of flavonoids on α-amylase under various concentrations, [α-amylase] = 2.0 × 10^{-5} mol/L, pH 6.8, at room temperature, SD < 0.1.

binding. Thus for Rut, it is derived from Que with replacement of C3–OH by 2 indicans and now the steric hindrance effect is more obvious. These 2 opposite interactions lead to a final result that the K_A of Rut is equal to or a little bit less than that of Que.

Thermodynamic parameters and types of main acting force

Ross and Subramanian (1981) once suggested that the types of main acting force between pharmaceutical molecule and protein could be judged or assessed according to the relative values' change in enthalpy and entropy (ΔH and ΔS). 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 listed them in Table 3.

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

$$\Delta G = -RT\ln K \tag{6}$$

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

As shown in Table 3, under our experimental conditions, the values of ΔG for our 3 flavonoids are all negative, while their ΔH and ΔS are all positive. This means that the interaction between α-amylase and the 3 flavonoids is a spontaneous process of entropy increasing, free energy decreasing and driven mainly by hydrophobic force. From the structural point, the catalytic center of α-amylase is a hydrophobic tubby composed of $(\beta/\alpha)_8$, so the interaction between α-amylase and the 3 flavonoids should be driven mainly by hydrophobic force. However, hydrogen binding should not be neglected because the experimental system is in aqueous solution and there exist many hydroxyl groups on the molecules of drugs and macromolecules, here a protein.

Nonradiative energy transferring

As can be seen in Figure 3, flavonoids absorption spectra and α-amylase-flavonoid (with the molar ratio 1:1) fluorescence spectra overlap at 300 to 500 nm. According to Förster's theory on nonradiative energy transferring (Yang and Gao 2002), this means a nonradiative energy transferring between Que, Iso, Rut, and Try residues on α-amylase, thus results in fluorescence quenching.

Inhibitory kinetics of α-amylase

The activity and specific activity of α-amylase are 83.25 ± 0.23 U and 5789.0 ± 2.75 U/mg, respectively. Figure 4 shows that all the 3 flavonoids inhibit the activity of α-amylase with the addition of Que, Iso, and Rut gradually. The ability of inhibition is Iso ≈ Que > Rut (SD < 0.1). Applying double reciprocal or Lineweaver–Burk plot of $1/v$ against $1/[S]$ (Figure 5), we deduce that the inhibitory mode of Que, Iso, and Rut toward α-amylase is competitive type (Hames and Hooper 2000). According to Michealis equation:

$$v = \frac{V_{\max}[S]}{K_m + [S]} \tag{8}$$

the K_m of α-amylase is about 0.089 ± 0.0001 g/mL under the condition of this study. In addition, the values of inhibition constants (K_i) can be obtained by Dixon method. As shown in Table 4, we can deduce the sequence of affinity ($1/K_i$) between 3 flavonoids and α-amylase as Iso > Que > Rut, being consistent with K_A.

It is known that different kinds of α-amylases all contain 4 conservative domains (named A, B, C, D) and have the same secondary structure. Domain A in a tubby shape is the center of catalysis and composed of $(\beta/\alpha)_8$. Domains B, C, and D are relevant to specificity and stability of substrate, while the carboxyl terminal of α-amylase (E-SBD) can bind with starch (Macregor and Janecek 2001; Birte Sevensson 2003). According to Hames and Hooper (2000), the 3 flavonoid compounds all bind with domain A of α-amylase in our experiments in a competitive inhibitory mode (Figure 5). Further investigations or more information about the inhibition can be explored with NMR and other techniques.

Conclusions

Using the methods of spectroscopy, analytical chemistry, and enzymology, we have studied the interaction between α-amylase and 3 flavonoid compounds from tartary buckwheat on both macro and micro levels. The results revealed that Que, Iso, and Rut could bind with α-amylase to form a new steady complex,

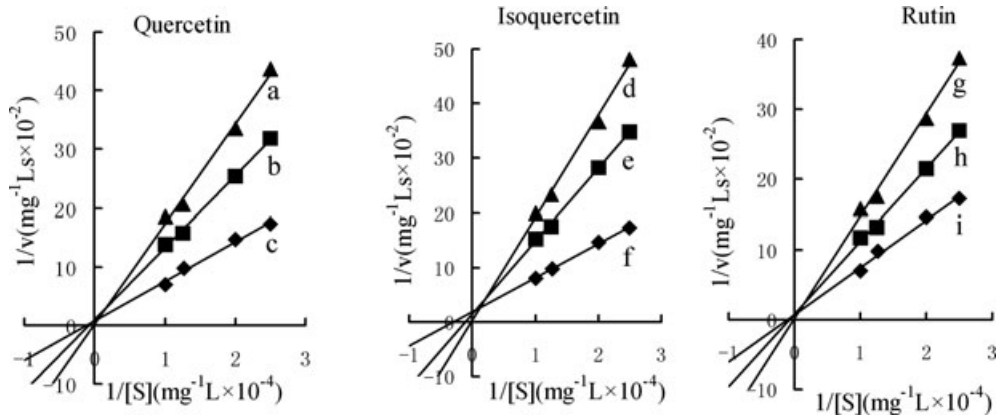

Figure 5—Plots of 1/*v* compared with 1/[*S*] for judging the type of inhibition pH 6.8, at room temperature, [α-amylase] = 2.0 × 10^{-5} mol/L, c, f, i: [flavonoid]/[starch] = 0; b, e, h: [flavonoid]/[starch] = 0.5; a, d, g: [flavonoid]/[starch] = 1.0.

Table 4—K_i and $1/K_i$ values of interaction between α-amylase and Que, Iso, Rut.

Flavonoid	$K_i/10^{-5}$ (mol/L)	$1/K_i/10^5$ (L/mol)	SD
Quercetin	0.636	1.572	0.02
Isoquercetin	0.535	1.896	0.02
Rutin	0.896	1.116	0.05

which led to fluorescence of α-amylase static quenching and nonradiation energy transferring, and went further to inhibit enzyme's activity. At the same time, we deduced that the inhibitory type belongs to a competitive manner.

In summary, the inhibitory sequence of Que, Iso, and Rut is consistent with the binding order. They are all Iso > Que > Rut. As Rut constitutes 90% of flavonoid in tartary buckwheat (only a few Que and nearly no Iso), so taking original tartary buckwheat or using Rut in food to decrease blood sugar may not have an obvious effect on curing high blood sugar and fat. Applying some special hydrolytic methods to transform Rut into Iso and Que may be a good idea. The present study will increase some useful knowledge to the fields of green medicine on decreasing the levels of sugar and fat in human blood.

Acknowledgment

This study was supported by the Natl. Natural Science Foundation of China (nr 30771310 and nr 30740038) and Natl. Sci-Tech Support Program of Eleventh Five-Year Plan (nr 2006BAD02B06), Ministry of Sci-Tech, P.R.China.

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