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First Identification of α-Glucosidase Inhibitors from Okra (*Abelmoschus esculentus*) Seeds

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Infusion of roasted okra seeds has long been consumed in Turkey for diabetes mellitus therapy. Previous reports of a hypoglycemic effect observed in rats administrated with okra seed extract indicated a possible connection with inhibition of intestinal α-glucosidase. An attempt to identify active components was first herein conducted using α-glucosidase-inhibition-guided isolation, yielding two major flavonol glucosides named isoquercetin (**2**) and quercetin-3-*O*-β-glucopyranosyl-(1‴→6″)-glucoside (**3**). They selectively inhibited rat intestinal maltase and sucrase, in which isoquercetin (**2**) was 6-10 times more potent than its related diglucoside **3**. This result suggested that an increase in hydrophilicity by the additional glucose residue in **3** led to a significant decline in the inhibitory effect and raised the possible involvement of the free 3-OH in exerting the inhibition. Our postulation was evaluated by examining α-glucosidase inhibition of quercetin (**1**), and the aglycone of **2** and **3**, whose 3-OH is free from any glucose moiety. Interestingly, **1** displayed a broad inhibitory effect toward rat intestinal and baker's yeast α-glucosidases, with improved potency. A kinetic study of **1** indicated that it inhibited maltase by two distinct mechanisms, in competitive (K_i 462 μM) and noncompetitive (K'_i 2153 μM) manners, whereas the mechanism underlying the inhibition of sucrase was verified as being of a competitive behavior (K_i 218 μM).

Keywords: Okra, *Abelmoschus esculentus*, Hypoglycemic, Diabetes mellitus, α-Glucosidase, Quercetin.

Maintaining good glycemic control is critical in diabetes therapy because it is associated with marked reduction in risk of developing complications such as cardiovascular diseases, neuropathy and retinopathy. Therefore, antihyperglycemic agents, particularly α-glucosidase inhibitors, are expected to provide such beneficial effects. Acarbose and voglibose are instances of potent α-glucosidase inhibitors currently used for diabetes therapy. They not only suppress postprandial glucose but also reduce the triglyceride level, body weight and systolic blood pressure. However, long-term intake of acarbose and voglibose resulted in undesired effects such as flatulence and diarrhea [1]. Therefore, new α-glucosidase inhibitors having minimized side effects are continuously required.

Edible plants are a prolific source of α-glucosidase inhibitors [2], in addition to their beneficial nutrition and safety on prolonged consumption. Prominent examples include corchoruside A, a flavonoid glycoside having a caffeoyl moiety isolated from *Corchorus olitorius* [3], which inhibited α-glucosidase three times more potently than acarbose. In the present study, we are intrigued by *Abelmoschus esculentus*, typically known as okra, because of its antidiabetic potential [4]. Okra fruit is high in mucilage [5,6], which was believed to delay glucose absorption into the bloodstream by its excellent water-holding and gel-forming capabilities. However, Palanuvej and coworkers [7] demonstrated that mucilage yield, glucose entrapping capability, and α-glucosidase inhibition of fruit aqueous extract were relatively low. Thus, the antidiabetic activity reported by Tomada [4] is not likely to be due to the mucilage.

Alternatively, roasted seeds, which are popularly consumed in Turkey as an herbal infusion, have long been recognized to attenuate blood glucose level. This ethnopharmacological claim was supported by *in vivo* antihyperglycemic activity. Oral administration of an aqueous extract in hyperglycemic rats prevented high blood glucose levels while a more pronounced and significant effect was observed in streptozotocin-induced diabetic rats [8]. However, there is no informative clue implied from early reports describing a possible mechanism underlying the hypoglycemic effect of okra seeds. Herein, we propose that α-glucosidase inhibition is one pathway in which okra seeds possibly exert their effect. An attempt to identify the active components in okra seeds resulted in the isolation of flavonol glucosides **2** and **3**. The mechanism underlying the inhibition is also reported.

An attempt to identify the active compounds responsible for suppressing blood glucose level was performed using an α-glucosidase inhibitory assay as guidance, coupled with chromatographic separation. The methanolic extract of the seeds when fractionated by vacuum column chromatography, afforded four major fractions. The combined second fraction (Fr2) displayed the highest inhibitory activity against rat intestinal maltase (76.3%) and sucrose (69.8%) (Figure 1). Further purification of the active fraction afforded the two principal components, which based on NMR and MS data, were identified as isoquercetin (**2**) and quercetin-3-*O*-β-glucopyranosyl-(1‴→6″)-glucoside (**3**) [9,10].

Flavonol glucosides **2** and **3** were subjected to evaluation for their inhibitory effects against α-glucosidases from two different sources, baker's yeast and rat intestine. Apparently, they selectively inhibited rat intestinal maltase and sucrose, whereas inhibitory activity against baker's yeast α-glucosidase was not observed (Table 1). Isoquercetin (**2**) revealed inhibition toward maltase and sucrase with IC_{50} values of 64.1 and 42.5 μM, respectively, which were 10 and 5 times, respectively, more potent than its corresponding diglucoside **3**. This observation suggested that the increase in hydrophilicity by the extra glucose moiety in **3** dramatically reduced the inhibitory effect. We subsequently

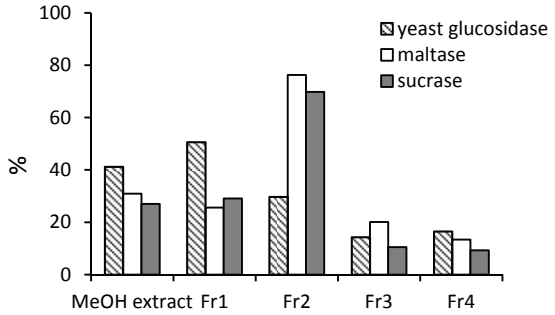

Figure 1: α-Glucosidase inhibitory effect of methanol extract and fractions after first-step purification, examined at concentration of 1.0 mg/mL.

investigated the inhibitory activity of quercetin, the aglycone of **2** and **3**, in order to gain insight into the effect of 3-OH in exerting inhibition. Quercetin (**1**) used in our experiment was prepared from commercially available rutin by HCl-catalyzed hydrolysis.

Table 1: α-Glucosidase inhibitory effect of flavonoids **1**-**3**.

Compds	IC_{50} (μM)		
	Baker's yeast	Maltase	Sucrase
1	62.8 ± 2.1	12.3 ± 0.6	31.7 ± 1.5
2	NI[a]	64.1 ± 3.3	42.5 ± 1.2
3	NI	646 ± 5.8	265.7 ± 3.1
Acarbose	403.9 ± 0.4	1.50 ± 0.14	2.38 ± 0.02

[a]No inhibition, inhibitory effect less than 30% at 10 mg/mL.

Interestingly, **1** displayed a broad inhibitory effect against baker's yeast α-glucosidase (IC_{50} 62.8 μM), rat intestinal maltase (IC_{50} 12.3 μM) and sucrase (IC_{50} 31.7 μM), with improved potency (Table 1). Therefore, the presence of the free 3-OH in quercetin is required for α-glucosidase inhibition. To gain insight into the mechanism underlying this inhibitory effect, a kinetic study of **1** toward maltase and sucrase, instead of baker's yeast α-glucosidase, was carried out.

We designed such an experiment based on the fact that maltase and sucrase are more closely relevant to the α-glucosidases present in the human small intestine [11]. The Lineweaver–Burk plot of 1/v and 1/[maltose] (Figure 2) demonstrated a series of straight lines; all of which had different Y intercepts but intersected in the second quadrant. Kinetic analysis showed that V_{max} decreased with elevated K_m in the presence of increasing concentrations of **1**, therefore suggesting that maltase was inhibited by quercetin (**1**) in two distinct manners, competitive and noncompetitive (Table 2). This phenomenon could be rationalized by simultaneous formation of an enzyme-inhibitor (EI) complex in a competitive manner and an enzyme-substrate-inhibitor (ESI) complex in a noncompetitive manner.

To envisage the pathway in which quercetin (**1**) preferentially proceeded, the dissociation constants of EI (K_i) and ESI (K'_i) complexes were determined. The secondary plot of slope of lines in the Lineweaver–Burk relation versus the inhibitor concentration produced a K_i value of 462 μM, whereas the secondary plot of intercept versus inhibitor concentration generated a K'_i value of 2153 μM (Table 2). An approximately 5-fold lower dissociation constant of the EI complex suggested a closer binding between quercetin (**1**) and maltase and pointed out that competitive inhibition was predominant over noncompetitive behavior (Scheme 1). In addition, we also investigated the mechanism underlying the inhibitory effect of quercetin (**1**) against sucrase using the aforementioned approach. The Lineweaver–Burk plot of **1** against

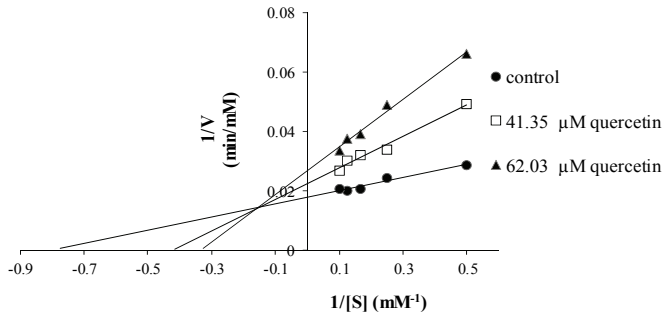

Figure 2: Lineweaver-Burk plot for inhibitory activity of quercetin (**1**) against rat intestinal maltase.

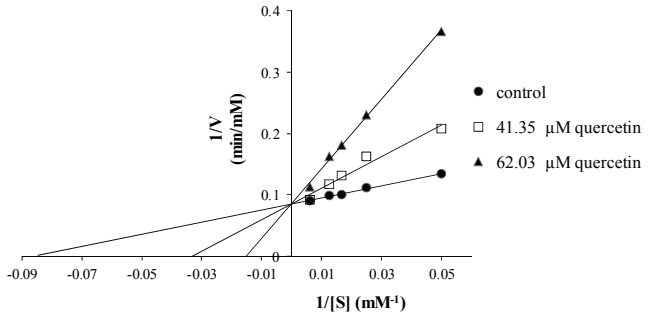

Figure 3: Lineweaver-Burk plot for inhibitory activity of quercetin (**1**) against rat intestinal sucrase.

Scheme 1: Proposed inhibition mechanisms of quercetin (**1**, I) against maltase and sucrase; where E, S and P are maltase (or sucrase), maltose (or sucrose) and glucose, respectively.

Table 2: Kinetic data of intestinal α-glucosidase inhibition by quercetin (**1**).

Flavonoid	Maltase		Sucrase	
	K_i (μM)	Inhibition type	K_i (μM)	Inhibition type
1	462 2153[a]	Mixed	218	Competitive

[a] K'_i value for noncompetitive manner.

sucrase (Figure 3) revealed a series of straight lines, all of which intersected at the Y axis. Kinetic analysis revealed that K_m increased while V_{max} remained constant in the presence of increasing concentrations of **1**, therefore indicating competitive inhibition (Scheme 1) with a K_i value of 218 μM (Table 2).

Quercetin (**1**) was previously reported as a potent inhibitor against baker's yeast α-glucosidase. However, the mechanism underlying the inhibitory effect reported by two groups is different; it was first reported as a noncompetitive inhibitor by Lee [12] and subsequently as a mixed-type of noncompetitive and anticompetitive inhibitor by Li [13]. Although a significant amount of **1** could not be detected in our experiment, its corresponding glucosides **2** and **3** could be transformed into aglycone **1** by lactase phlorizin hydrolase [14,15], an extracellular enzyme located at the brush border membrane of intestinal cells. In addition, Turkish ethnopharmacological use of roasted okra seeds as an infusion is likely to enhance liberation of quercetin from its glycosides by thermal-assisted hydrolysis.

In summary, we are the first to identify flavonol glucosides **2** and **3**, present in okra seeds, as the major inhibitors against α-glucosidase. The glucosides **2** and **3** possibly exert a beneficial effect on diabetes mellitus therapy by being transformed into the active quercetin (**1**), which can attenuate blood glucose levels through inhibiting maltase and sucrase functions. The highly improved inhibitory effect of **1** over **2** and **3** indicated that free 3-OH is required for retarding the α-glucosidase function. The mechanism underlying the inhibition of quercetin discovered in our investigation suggested a possible guidance for the use of okra seeds as a single alternative drug or in combination with currently used antidiabetic drugs.

Experimental

Plant material: Okra (*Abelmoschus esculentus*) seeds were collected at Nakornpatom, Thailand, in November 2010. The plant material was identified by the botanist, Mrs Parinyanutch Klinrat, and the voucher specimens (BCU013433) were deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Thailand.

Extraction and isolation: The air-dried seeds (62 g) were ground and extracted with methanol (3 × 250 mL) at room temperature. The extract was evaporated under reduced pressure to afford a brown syrup (12 g). The methanolic extract was absorbed on silica gel (20 g) and subjected to chromatography on silica gel, eluting (500 mL each fraction) with dichloromethane, methanol-dichloromethane (1:9), methanol-dichloromethane (2:8) and methanol-dichloromethane (1:1). The active fraction (Fr2), which was mainly eluted with methanol-dichloromethane (2:8), was dissolved in ethyl acetate and centrifuged. The precipitates, which are soluble in methanol, were further purified on a Sephadex LH-20 column (methanol), yielding quercetin-3-*O*-β-glucopyranoside or isoquercetin (**2**, 490 mg). In addition, the other active fractions, which were mainly eluted with methanol-dichloromethane (1:1) were subsequently purified by Sephadex LH-20 CC (methanol), affording quercetin-3-*O*-β-glucopyranosyl-(1‴→6″)-glucoside (**3**, 189 mg).

Preparation of quercetin: Rutin (100 mg) was dissolved in 3M methanolic HCl (10 mL) and refluxed at 60-70°C for 6 h. The reaction mixture was diluted with water (2 mL) and centrifuged. The precipitate obtained after centrifugation was washed with water until the supernatant was neutral to universal indicator paper, and then oven dried (80-85°C) to afford quercetin (**1**, 47 mg).

α-Glucosidase inhibition assay: The inhibitory effect against α-glucosidase from baker's yeast was tested using our previously reported protocol [16,17]. Briefly, the α-glucosidase (0.1 U/mL) and substrate (1 mM *p*-nitrophenyl-α-D-glucopyranoside) were dissolved in 0.1 M phosphate buffer, pH 6.9. Ten μL of test compound (1 mg/mL in DMSO) was incubated with 40 μL of α-glucosidase at 37ºC for 10 min. Fifty μL substrate solution was then added to the reaction mixture and incubated at 37ºC for an additional 20 min. After the reaction had been terminated by adding 100 μL of 1 M Na_2CO_3, enzymatic activity was quantified by measuring the absorbance at 405 nm (Bio-Red microplate reader model 3550 UV). The percentage inhibition was calculated by $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance without the sample, and A_1 is the absorbance with it. The IC_{50} value was determined from a plot of the percentage inhibition versus the sample concentration. Acarbose was used as the standard control and the experiment was performed in triplicate.

In addition, inhibitory activity against α-glucosidase from rat intestine was determined using our developed method [16,17]. The crude enzyme solution prepared from rat intestinal acetone powder was used as a source of maltase and sucrase. Rat intestinal acetone powder (1 g) was homogenized in 30 mL of 0.9% NaCl solution. After centrifugation (12,000 *g* × 30 min), the aliquot was subjected to assay. Ten μL of test compound was added to a substrate solution (10 mM maltose and 100 mM sucrose, each 20 μL), 0.1 M phosphate buffer (pH 6.9, 30 μL), glucose oxidase solution (80 μL) and enzyme solution (20 μL). The reaction mixture was incubated at 37°C (10 min for maltase and 40 min for sucrose) and its absorbance determined at 500 nm. Percentage inhibition and IC_{50} values were obtained using the aforementioned methodology.

Kinetic study of α-glucosidase inhibitory effect: The mechanism underlying the inhibitory effect was investigated by analyzing the enzyme kinetic data using the above reaction. Maltase activity was maintained at 0.45 U/mg protein in the presence of quercetin (from 0.1–1.0 mg/mL) at various concentrations of maltose (2, 6, 10, 16, 20 mM). A series of V_{max} and K_m values were obtained from the Y intercepts and calculated by slope × V_{max}, respectively. A similar method was also applied for sucrase, in which its activity was kept at 0.07 U/mg protein**.**

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