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Evaluation of α-Glucosidase Inhibitory Assay using Different Sub-classes of Flavonoids

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Abstract

Diabetes mellitus is one of the metabolic disorders that cause high blood glucose levels with deficiency of released insulin from β cells in pancreas. Alternative medicinal treatment is to control blood glucose level through inhibition of α-glucosidase, leading to delayed starch degradation and slower rate of raising blood glucose level. However, the methods for α-glucosidase inhibitory assay were varied, leading to confusion on reliability to evaluate α-glucosidase inhibition of particular bioactive compounds. Interestingly, pervious researchers had reported anti-diabetic potential of flavonoids, which provided great inhibitory activities among phenolics. Therefore, the aim of this research was to evaluate different methodologies of α-glucosidase inhibitory assays using five selected flavonoids (epigallocathchin gallate (EGCG), genistein, myricetin, luteolin and naringenin) as the models. The results indicated that enzyme kinetic assay exhibited the advantages over other fundamental assay methods, including higher range of measurement and reliability of the enzyme reaction. All investigated flavonoids exhibited the half maximal inhibitory concentration (IC_{50}) in the range of 1-81 μM, which were corresponded to the previous literature. EGCG was the strongest inhibitor against α-glucosidase, while naringenin was the weakest. The outcome of our study might be useful for drug design and functional cure of diabetic patients in the future.

Keywords: diabetes, α-glucosidase, flavonoids, enzyme inhibition

1. Introduction

Type 2 diabetes mellitus (DM) is one of the metabolic disorders related to high blood glucose levels. The majority of diabetes patients, 90-95%, are inflicted with DM. Type 2 DM affects insulin resistance, since liver, muscles and fat cells cannot respond normally to insulin. The main target of diabetes treatments is controllingblood glucose levels to its set point. Currently, medicinal treatment of type 2 DM is focused on inhibition of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase. The former hydrolyzesα-1-4-glycosidic linkage on polysaccharides into smaller sugar units. The latter hydrolyzes polysaccharides at the terminal non-reducing α-1-4-glycosidic linkage. Inhibition of both enzymes can prevent starch digestion and delay rate of glucose absorption [1]. Commercial inhibitors such as acarbose, volibose and migiltol can reduce blood glucose levels in many patients. However, adverse effects including diarrhea, flatulence and abdominal pain are reported [2]. Therefore, green medicines from natural sources are currently of interest for diabetes management, due to less side effects and economical effectiveness.

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Many previous studies reported that plant extracts and biological active compounds from nature exhibited potency against diabetes, through α-glucosidase and α-amylase inhibitions. Among bioactive compounds, phenolics are mostly found in natural sources and can be divided into flavonoids, tannins, phenolic acids and coumarins. Interestingly, previous studies revealed that flavonoids are the most effective inhibitors [3]. Recent studies indicated that high consumption of flavonoids is correlated with lower risk of diabetes [4]. In addition, flavonoids seem to be greater effective inhibitorsagainst α-glucosidase than α-amylase [3]. Therefore, the present study was focused on α-glucosidase inhibition.

Research methodology of previous studies was focused on the effect of flavonoids against α-glucosidase based on the end point assay. However, several disadvantages including sensitivity to noises, turbidity and interferences were reported [5]. On the other hand, enzyme kinetics assay possesses higher precision, higher reproducibility, higher measurement range and lower interfering substances [5, 6]. Even though the α-glucosidase assay was previously investigated, proper concentrations of enzyme and substrate are unknown in the inhibitory reaction. Therefore, the purpose of this study was to optimize assay conditions (enzyme and substrate concentrations) and to evaluate assay methods utilizing enzyme kinetics and selected flavonoids as the model inhibitors.

2. Materials and Methods

2.1 Materials and chemicals

The enzyme, *Saccharomyces cerevisiae* α-glucosidase (Type 1, ≥10 unit/mg), and substrate, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), were received from Sigma–Aldrich (*St. Louis, MO*, USA).Flavonoids including myricetin (purity ≥ 98%), luteolin (purity ≥ 97%), naringenin (purity ≥ 95%), genistein (purity ≥ 97%) and epigallocatechingallate (EGCG, purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents including hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate trihydrate ($K_2HPO_4•3H_2O$) were received from Merck (New Jersey, USA). Solvents including dimethyl sulfoxide (DMSO), hexane, ethyl acetate, ethanol, acetonitrile (CH_3CN), dichloromethane (CH_2Cl_2) and methanol were received from RCI Labscan (Bangkok, Thailand).

2.2 Optimization of assay reaction

To optimize enzyme concentration, the α-glucosidase assay consisted of *Saccharomyces cerevisiae* α-glucosidase (0.006 to 0.10 U/mL) and *p*NPG (1.25 mM) in 50 mM KPB (pH 7.0). The reaction was monitored at a wavelength of 405 nm at 37°C for 30 minutes using a 96-well microplate reader (SynergyTM HT 96-well UV-visible spectrophotometer) and a Gen5 data analysis software (BioTek Instruments, Inc., Winooski, VT).

To optimize substrate concentration, the enzyme kinetic was performed as follow. The *Michaelis–Menten* constant, K_m, and maximum velocity, V_{max}, were determined using *Saccharomyces cerevisiae* α-glucosidase (0.01- 0.05 U/mL) with ten substrate concentrations (0.1–1.2 mM) in 50 mM KPB (pH 7.0). The assay was performed using the microplate reader. The results were calculated as specific activity using the molar extinction coefficient of *p*-nitrophenol product (18,000 M^{-1} cm^{-1}) at wavelength of 405 nm [7]. The initial rate was fitted by the Michaelis–Menten equation with least squares fit parameter using a GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

2.3 Assay of α-glucosidase inhibitory activity

The assay consisted of *Saccharomyces cerevisiae* α-glucosidase (0.01 U/mL), *p*NPG (1.25 mM) and flavonoids in 50 mM KPB (pH 7.0). All flavonoids reactions were monitored at a wavelength of 405 nm at 37°C for 30 minutes using a 96-well microplate reader. The results were calculated as a percentage of inhibitory activity using the following equation;

$$\% \text{ inhibition} = \left(1 - \frac{B-b}{A-a}\right) \text{x } 100,$$

where A is an initial velocity of the control reaction with enzyme (control), a is an initial velocity of the control reaction without enzyme (control blank), B is an initial velocity of the enzyme reaction with sample and b is an initial velocity of the reaction with sample but without enzyme (sample blank).

2.4 Statistical analysis

All of the experiments were carried out in triplicate. The data were expressed as mean ± standard deviation (SD). All statistical analyses were evaluated using a GraphPad Prism version 5. One way analysis of variance (ANOVA) and Duncan's test were performed to determine the significant differences between values. Significance of difference was defined at $p < 0.05$.

3. Results and Discussion

3.1 Optimization of enzyme concentrations

In order to determine a range of optimal enzyme concentrations for α-glucosidase using colorimetric microplate assay method, *p*NPG was fixed at the concentration of 1.25 mM, while the enzyme concentration was varied (0.006 to 0.10 U/mL) (Figure 1). The enzyme reaction was demonstrated as the colorless *p*NPG converted to glucose and yellow *p*-nitrophenol, which can be monitored at the wavelength of 405 nm. The rate of the reaction using high enzyme concentration increased faster than the reaction using low enzyme concentration. This result is expected since more enzymes can react with the substrate, causing rapidly increased reaction rate. The rate of the reaction with high enzyme concentration began to slowly increase and eventually plateaued as the reaction continued, indicating limitation of the available enzyme that can interact with the substrate.

In enzyme assay using the enzyme concentrations of 0.06-0.10 U/mL, rapid reaction rates that became plateau quickly were observed (Figure 1). The study found that the reaction rate was optimized and reaching its V_{max} (as being observed as plateau curve) over the first time period of 15 minutes after initiating the reaction using 0.10 U/mL of the enzyme. Other enzyme reactions using lower enzyme concentrations subsequently showed longer time periods of linear initial velocity. Therefore, the time period of linear initial velocity became a significant factor to determine suitable enzyme concentration for inhibitory assay. However, in order to collect kinetics data such as initial velocity, a long linear phase on the enzyme reaction is required [8, 9]. Therefore, to establish optimal inhibitory assay conditions using kinetics analysis, the appropriated enzyme concentrationsare in the range of 0.006-0.05 U/mL, which provide the longest time period of linear initial velocity.

Nevertheless, previous literatures had reported the enzyme concentration of 0.05-0.13 U/mL, which was employed for determination of α-glucosidase inhibitory activity [10-12]. Higher range of enzyme concentration is chosen for inhibitory assay may be due to these previous studies used the end point type of the enzyme assay to determine α-glucosidase inhibition. Thus, the completion of the enzyme reaction is required before terminating the reaction and measuring the product. In order to avoid time consuming during incubation of the enzyme mixture and to speed

up the assay reaction, high concentration of enzyme is normally appreciated. However, the enzyme concentration of 0.01 U/mL was used in the inhibitory assay in this present study. Lower enzyme concentration can be chosen in the kinetics type of the inhibitory assay, since initial rate of the enzyme reaction is used to determine inhibitory activity, while the completion of the enzyme assay is not required.

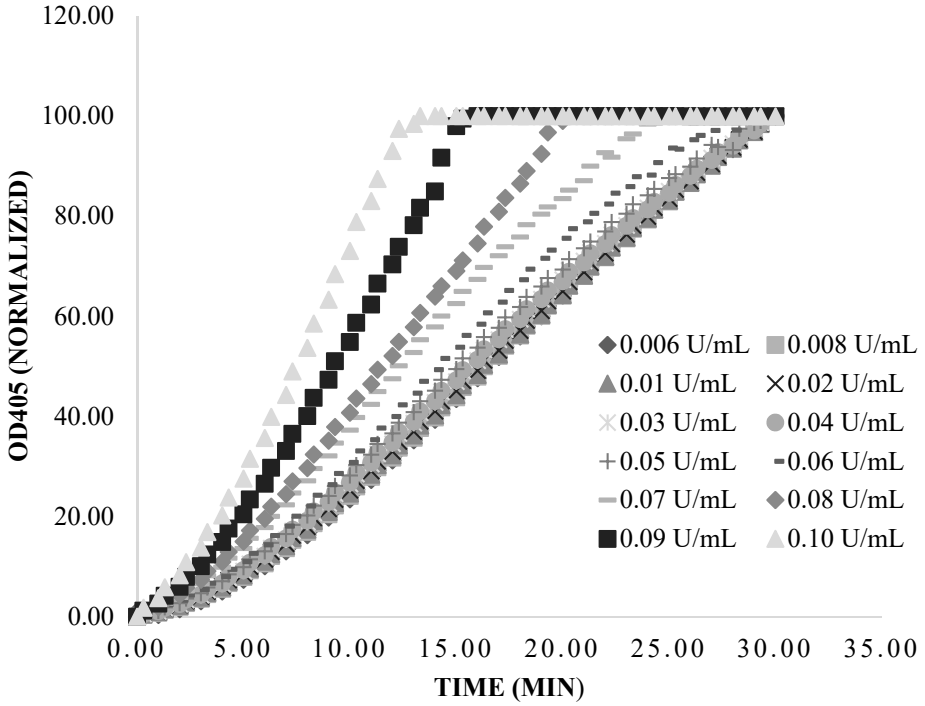

Figure 1. Time-dependent reaction on α-glucosidase from *Saccharomyces cerevisiae* (0.006 to 0.10 U/mL) through running the enzyme assay.

3.2 Optimization of substrate concentrations

To define a suitable substrate concentration of α-glucosidase reaction, enzyme kinetics was investigated (Figure 2). Based on Michaelis-Menten kinetics model, low substrate concentration (0.1 to 0.8 mM) showed rapid increase in reaction rate. At this point, the binding site of enzyme is available for the substrate. However, continuingly increased substrate concentration caused a slow increase in the reaction rate, indicating that all the binding sites on enzyme are filled up by the substrate. At this point, addition of more substrates did not affect the reaction rate, and the reaction rate is finally optimized (V_{max}) [13]. For enzyme kinetics, K_m and V_{max} play an important role in the reaction rate. The K_m indicated the binding affinity between enzyme and substrate. Lower K_m presentsgreater binding affinity of the substrate to the enzyme. In addition, V_{max} is expressed as all active sites on the enzyme are bound to the substrate, and no free enzyme is available. In this study, K_m value of 0.30±0.01 mM and V_{max} value of 6,136±410.82 μmol•min^{-1}•mg^{-1}were reported. Therefore, the optimal substrate concentration for the inhibitory assay should be more than 1.2 mM, where the enzyme reaction reaches its V_{max}.

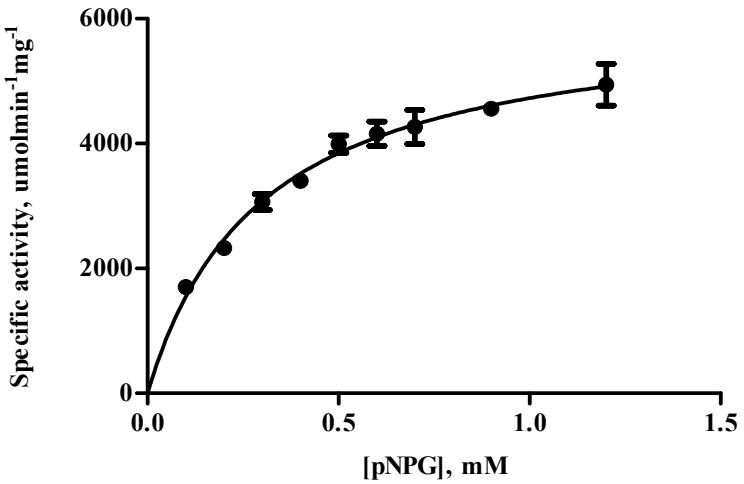

Figure 2. Determination of enzyme kinetics of *Saccharomyces cerevisiae* α-glucosidase activity at various *p*NPG concentrations (0.1–1.2 mM). The values are mean ± SD (n=3). Curves were fitted to the *Michaelis–Menten* equation on the data.

Previous studies, however, suggested different K_m and V_{max} values, in which K_m of 4 mM and V_{max} of 12,062.50 μmol•min^{-1}•mg^{-1}were reported using the same enzyme and substrate system [10]. The substrate concentration of 1.25 mM was chosen for inhibitory assay in this study [10]. Another research had found that K_m and V_{max} values of yeast α-glucosidase and *p*NPG were 3.5 mM and 35,740.74 μmol•min^{-1}•mg^{-1}, respectively [11]. The selected substrate concentration for inhibitory assay in this study was 5.56 mM [11]. The other study showed that the substrate concentration in the inhibitory assay was 1 mM, where the K_m and V_{max} values were 0.431 mM and 7,768.25 μmol•min^{-1}•mg^{-1}, respectively [12]. Various K_m, V_{max} and substrate concentrations as being detected in these previous literatures might be corresponded to assay factors being employed in each assay methods. These factors are temperature of a reaction, pH of solutions, types of buffer solutions, concentrations of buffer, wavelength of absorbance, sensitivity of spectrophotometer, reaction time and solvent.

3.3 α-Glucosidase inhibitory profile of flavonoids

To evaluate the potency of flavonoids on α-glucosidase inhibition, each sub-classes of flavonoids including EGCG (flavan-3-ol), genistein (isoflavone), myricetin (flavonol), luteolin (flavone) and naringenin (flavanone) were selected. However, anthocyanidins were not examined in this study due to their low stability in assay conditions. Many factors affect anthocyanidin stability including temperature, pH, light and storage time [14]. Especially pH, α-glucosidase becomes inactive at acidic pH (pH 2-5, data not shown), while anthocyanidins are likely stable in a low pH (1 to 3) [15]. Additionally, the optimal assay for the study of anthocyanidins against α-glucosidase was also unproven.

In this study, flavonoids exhibited IC_{50} values in the range of 1-82 μM (Figure 3, Table 1). The IC_{50} values of flavonoids in this study corresponded to the previous reportusing end point assay analysis [16], in which the IC_{50} values were ranged 2-75 μM (Table 1). Even though the results of the enzyme inhibition were similar, enzyme kinetics method has advantage over other methodologies [5]. In kinetics analysis, the reaction can be continuously monitored in real time, thus any errors on enzyme reaction can be visualized immediately. The shape of enzyme kinetics

can also reveal impurities and artifacts on the reaction. Besides, the percentage of inhibition is analyzed using initial velocity collectedfrom several data points (corresponding to signal slope or initial rate of reaction), thus increasing reliability of the method. Moreover, since kinetics assay relies on the set of data collected over a period of time, the range of measurable activity of kinetics method is wider than the end point method, which is limited on a single measurement after stopping the reaction [5, 6]. Additionally, in the end point assay, the percentage of inhibition is normally calculated from one single data point at the stopped time. Thus, any errors in the reaction can be easily missed. If the end point assay cannot be avoided, detection (or measurement) at several time points must be taken into consideration to ensure the existence of the reaction.

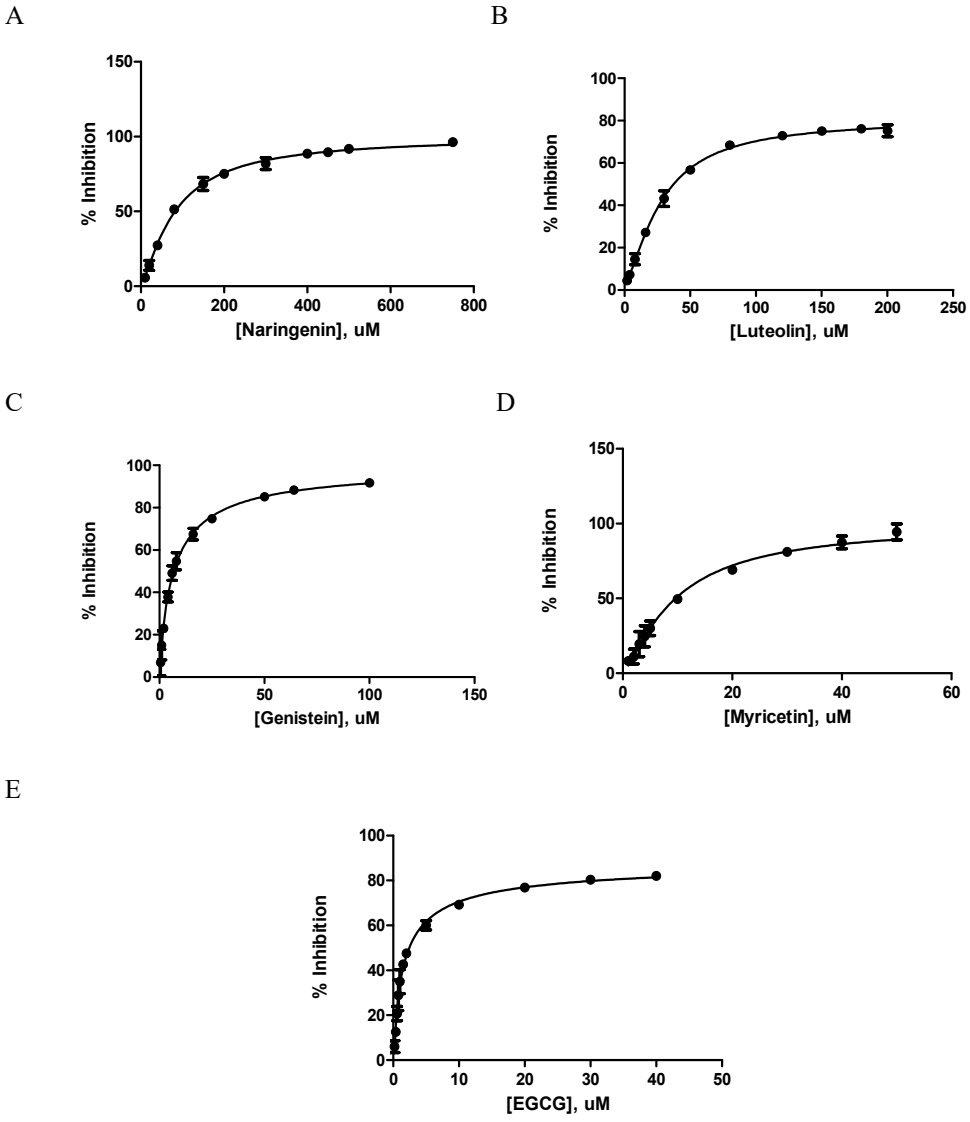

Figure 3. Inhibitory activities of selected representative of sub-classes of flavonoids on α-glucosidase from *Saccharomyces cerevisiae.* Naringenin (A), Luteolin (B), Genistein (C), Myricetin (D) and EGCG (E). The values are expressed as mean ± SD (n=3).

Table 1. The IC_{50} values of representative of sub-classes of flavonoids against α-glucosidase activity

Flavonoids	IC_{50} (μM)	
	Present study	**Previous study** [11]
Epigallocathchin gallate (EGCG)	0.99 ± 0.07^{a}	2
Genistein	6.85 ± 0.39^{cd}	7
Luteolin	27.99 ± 2.15^{b}	21
Myricetin	9.65 ± 0.85^{c}	5
Naringenin	82.26 ± 1.98^{e}	75

All flavonoids were dissolved in 50% DMSO except luteolin was dissolved in 75% DMSO.
IC50 values were determined by nonlinear regression. The values are presented as mean±SD of three replications. $^{a-e}$Different superscripts letters were analyzed with one way analysis of variance (ANOVA) and Duncan's test at $p< 0.05$.

The results also revealed that EGCG was the most effective inhibitor, while naringenin was the weakest against α-glucosidase. Previous research had suggested that due to the relationship between chemical structures of flavonoids and α-glucosidase inhibition, EGCG with galloyl moietymight play an important role on increasing binding affinity with the enzyme [17]. Furthermore, increasing of hydroxylation on the ring structure also elevated inhibitory activity. On the other hand, naringenin with fewer hydroxyl substitutions on ring structure exhibited lower enzyme inhibition [16].

EGCG can be mostly found in green tea and is accounted for 50-80% of total green tea content. Green tea extract exhibited the IC_{50} value of 0.5-4.4 μg/mL against α-glucosidase [18, 19]. Moreover, the synergistic effect between green tea extract and acarbose (1:1 ratio) was reported to be an effective combination against α-glucosidase [18]. However, higher concentration (1:1 ratio) showed antagonized mode of action [18]. Other components of green tea extract might, as well, contribute to inhibitory activity such as catechin, epicatechin, epigallocatechin, epigallocatechin gallate, gallocatechin gallate and epicatechin gallate [20]. Several controlled trials indicate that green tea administration could decrease blood glucose level and control diabetic status in patients with type 2 DM [21]. Intake of catechins rich plant extracts could lead to lower blood glucose levels and improve insulin resistance [22].

High concentration of naringenin is found in citrus fruit such as grapefruit, orange and pummel [23]. It was previously reported that grapefruit extract exhibited the IC_{50} value of 0.41 mg/mL against α-glucosidase [24]. Other compositions including kaempferol-3-O-glucoside and chlorogenic acid-3-O-glucoside in grapefruit extract also showed inhibitory potential against α-glucosidase [24, 25]. These may imply inhibitory activity depending on the bioactive compounds and constituent of plant sources.

4. Conclusions

Several advantages of the enzyme inhibitory assay using kinetics analysis are high sensitivity, high reliable detection and reproducibility due to elimination of interferences. On the other hand, other methodologies such as end point assay are sensitive to noises, turbidity and interfering substances. Enzyme kinetics analysis of α-glucosidase assay possesses advantages over other methodologies, including high reproducibility, high sensitivity, less interferences, reliable results and shorter analysis time. Comparing to previous literatures, all flavonoids exhibited great α-glucosidase inhibition with EGCG being the strongest α-glucosidase inhibitor. This finding can lead to effective methodology for prediction of flavonoids rich food and natural sources in diabetes

management. Furthermore, this study may be the useful information for drug development for diabetes prevention and reducing adverse effects in patients.

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