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Inhibitory Activity of Naturally Occurring Compounds Towards Rat Intestinal α-glucosidase using p-nitrophenyl-α-d-glucopyranoside (PNP-G) as a Substrate

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ABSTRACT

The purpose of this study was to examine potential antidiabetic effects of plant polyphenols commonly found in foods. Inhibitors of the enzyme α-glucosidase have been shown to be useful as antidiabetic agents. Eleven naturally occurring compounds were tested for inhibitory activity with the enzyme α-glucosidase obtained from rat intestine using the substrate p-nitrophenyl-α-D-glucopyranoside (PNP-G). These compounds included 10 polyphenols, 4 of which are anthocyanidins and trans-cinnamic acid. Acarbose was used as the standard. Both total inhibition and kinetic measurements were made in this study. All of the anthocyanidins tested showed inhibitory activity. All polyphenols tested were active except for caffeic acid. trans-Cinnamic acid was also weakly active. Ferulic acid, rutin, cyanidin, malvidin and delphinidin showed competitive inhibition behavior. However, tannic acid and pelargonidin showed noncompetitive behavior. Quercetin and trans-cinnamic acid showed mixed inhibition behavior. All compounds containing at least one ether or glycosidic link show competitive behavior. Cyanidin, delphinidin and gallic acid do not have any ether or glycosidic links but still displayed competitive kinetic behavior. The relation between chemical structure and inhibitory activity of the compounds are discussed. In summary, the majority of the compounds tested showed significant α-glucosidase inhibitory activity. The type of inhibition varied between compounds. The mechanism of inhibition needs to be further explored.

Key words: α-Glucosidase, inhibition, non-insulin-dependent diabetes mellitus, anthocyanidins, polyphenols, kinetics

INTRODUCTION

Non-insulin-dependent diabetes mellitus is a chronic disease that affects many adults. Long-term side effects of the disease include neuropathy, retinopathy and cataracts (Israili, 2011). In recent years, the enzyme α-glucosidase (AGH) has been chosen as a target for the development of new antidiabetic agents (Baron, 1998). Inhibition of this enzyme would limit the release of glucose from ingested starch or sucrose. Therefore, substances which inhibit AGH are believed to be good candidates for antidiabetic drugs. Three such substances, acarbose (Van de Laar *et al*., 2005), miglitol (Van de Laar *et al*., 2005) and voglibose (Matsumoto *et al*., 1998) are currently being used as antidiabetic drugs.

Many plant and natural extracts and natural compounds have been shown to have AGH inhibitory activity (McDougall *et al*., 2005; Li *et al*., 2009; Kolawole *et al*., 2011; Priya *et al*., 2012; Khan *et al*., 2013; Saha *et al*., 2011). Compounds which have shown AGH inhibitory activity include many polyphenols including anthocyanins (Benalla *et al*., 2010). Polyphenol derivatives having either ether, glycosidic, or acyl moieties appear to show the most inhibition (Matsui *et al*., 2001, 2007). This is believed to be due to the structural resemblance between these moieties and the glycosidic links found in natural substrates. Inhibition type has been shown to be either competitive, noncompetitive, or mixed dependent upon the substance tested (Lianzhu *et al*., 2011).

These studies have been complicated by a lack of consistency of the methods being used. For example, AGH is obtained from mammalian intestines or *Saccharomyces cerevisiae*. Mammalian sources include rat, rabbit and pig. Studies have shown the enzyme from different sources have yielded widely conflicting results in some cases. Oki *et al*. (1999) demonstrated significant differences in inhibitory activity of many compounds towards AGH obtained from mammalian and yeast sources.

Studies have also varied with regard to the AGH substrates used. The most common method uses *p*-nitrophenyl-α-D-glucopyranoside (PNP-G) as a substrate. Release of p-nitrophenol is monitored spectrophotometrically by increase in absorbance at 400 nm (Matsui *et al*., 1996). A second method uses either maltose or sucrose as substrates and release of glucose is measured using the glucose oxidase assay. This method has the advantage of measuring both maltase and sucrase inhibition of crude AGH extracts (Pluempanuput *et al*., 2007). Differences in reactivity towards chemical compounds have also been shown to exist between this method and PNP-G method (Oki *et al*., 1999).

Due to the differing methodologies used, it is currently difficult to ascertain a profile of reactivity of various compounds towards AGH. In this study, the inhibitory activity of several naturally occurring compounds was investigated using AGH obtained from rat intestine and PNP-G as the substrate. These natural substances are polyphenols including anthocyanidins. Both percent inhibition and kinetic parameters were measured. The aim of this study is to serve as a beginning of a compilation of AGH inhibitory data of various compounds by each method reported. Such a compilation of data would be very useful to investigators.

MATERIALS AND METHODS

Materials: The experiment was conducted during 2011 to 2012 at the Department of Agriculture of University of Arkansas, USA. Glucosidase (AGH) from intestinal acetone powder from rat was purchased from Sigma-Aldrich Co. (St. Louis, MO). Quercetin dihydrate, rutin, caffeic acid, ferulic acid, gallic acid, trans-cinnamic acid, sodium chloride and the synthetic substrate p-nitrophenyl α-D-glucopyranoside (PNP-G) were purchased from ACROS Organics (New Jersey). Tannic acid was obtained from Fisher Scientific (Fair Lawn, NJ). Delphinidin chloride was purchased from INDOFINE Chemical Co. (Hillsborough, NJ). Malvidin and cyanidin chloride were purchased from Alexis Biochemicals (San Diego, CA). Pelargonidin chloride was purchased from MP Biomedicals, LLC (Solon, OH). The synthetic inhibitor acarbose was obtained from LKT Laboratories, Inc. (St. Paul, MN). Phosphate buffer (pH 7.4) was purchased from RICCA Chemical Co. (Arlington, TX). Other reagents were of analytical grade and used without further purification.

AGH solution preparation from intestinal acetone powder from rat: AGH solution was prepared by a slight modified procedure reported by Oki *et al.* (1999). Specifically, 200 mg of intestinal acetone powder was added to 3 mL of pH 7.4 phosphate buffer (100 mM NaCl) and vortexed for 2 min. After centrifugation at 3000 g for 20 min at 4°C, the supernatant was removed and used in the endpoint and kinetic inhibitory assays.

Endpoint AGH assay: The inhibition (%) calculated in the endpoint assay was determined by a slight modification of a procedure reported by Matsui *et al.* (1996). A 10 mM working solution was prepared for every compound tested for inhibition. Acarbose and pelargonidin were both dissolved in water. All other compounds were dissolved in methanol. For each experiment, 10 μL of inhibitor was added to 40 μL of AGH solution and pre-incubated at 37°C for 5 min. After pre-incubation, the reaction was initiated by adding 950 μL of PNP-G (1.4 mM) in pH 7.4 phosphate buffer and incubating at 37°C for 40 minutes. The reaction was stopped by adding 1.0 mL of 0.5 M Tris base. The AGH activity was determined by measuring the release of p-nitrophenol from PNP-G at 400 nm. Absorbances for endpoint assay were measured with a BIOCHROM ASYS 340 microplate spectrometer (Eugendorf, Austria). Inhibition (%) for all compounds was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Ac-As}}{\text{Ac-AB}} \times 100$$

where, A_c, A_s and A_B represent the absorbances measured for the control, sample and blank. Anthocyanidins and certain other polyphenols absorb at 400 nm. To correct for this absorbance, the blank included 10 μL of the inhibitor.

Kinetic AGH assay: Kinetic behavior was determined for each compound by slight modifying a procedure described by Oki *et al.* (1999) Absorbance was measured at 400 nm at one minute intervals for 50 min with a Synergy HT multi-mode microplate reader from BioTek Instruments, Inc. (Winooski, VT). Five PNP-G concentrations were used and ranged from 0.7- 4.0 mM. A 10 mM solution of each compound tested was prepared. Again, certain compounds absorb light at 400 nm. To prevent absorbance due to the compounds tested, the 10 mM solutions were diluted. For quercetin, rutin, cyanidin and pelargonidin, 1:100 dilutions were made. For delphinidin and malvidin, 1:1000 dilutions were prepared. A 1:5 dilution was made for tannic acid. These dilutions were based on relative absorbance of each compound at 400 nm. Since the object of the kinetic study was to determine only inhibition type and not degree of inhibition these dilutions are justified on the basis that different inhibitor concentrations do not change inhibition type. For each experiment, 10 μL of inhibitor was added to 40 μL of AGH solution and pre-incubated at 37°C for 5 min. After pre-incubation, the reaction was initiated by adding 75 μL of PNP-G in pH 7.4 phosphate buffer.

The types of inhibition, Km and Vmax values were determined graphically from double-reciprocal Lineweaver-Burk plots.

Statistical analysis of kinetic data: Km and Vmax replicate values of AGH runs containing test compounds with varying concentrations of PNP-G were compared to Km and Vmax replicate values of AGH runs containing varying concentrations of PNP-G but with no test compounds using a

2-tailed student t-test (Zar, 1984). Compounds displaying a significant difference ($p<0.05$) in Km values from control, but not with Vmax were considered competitive inhibitors. Compounds with a significant difference in Vmax, but not Km, were considered noncompetitive inhibitors. Compounds which displayed a significant difference from control in both Km and Vmax values are considered mixed inhibitors. Compounds displaying no significant difference between Vmax and Km values were considered inactive at the inhibitor concentration used.

RESULTS AND DISCUSSION

The results of the percent inhibition data for the compounds tested are shown in Table 1. The data in Table 1 indicated that all compounds displayed significant inhibitory activity except for caffeic acid. Of the polyphenols, tannic acid was the most active (76.24% inhibition), having more activity than acarbose (50.0% inhibition). Of the anthocyanidins, malvidin (33.5%) and pelargonidin (36.9%) were the most active.

These data disagree with those of Adisakwattana *et al.* (2009) concerning the activity of caffeic acid. This group found caffeic acid to have significant AGH inhibitory activity. However, AGH inhibitory activity was determined by measuring concentrations of glucose released from sucrose and maltose substrates. In our study, using PNP-G as a substrate, caffeic acid was found to have no significant AGH inhibitory activity.

Intestinal rat AGH is composed of a variety of carbohydrases on the surface of small intestines (Oki *et al.*, 1999). These enzymes accept a variety of carbohydrate substrates. However, maltase and sucrase accept only maltose and sucrose, respectively as substrates. Therefore, one might expect some differences in inhibitory activity between the PNP-G model and the maltose/sucrose model. D-xylose and (+)-catechin showed inhibition against rat AGH using maltose and sucrose as substrates whereas no inhibition was observed when using PNP-G as a substrate (Oki *et al.*, 1999). Like D-xylose and (+)-catechin, caffeic acid was active in the maltose/sucrose model but not in the PNP-G model.

Figure 1 displays the Lineweaver-Burk plots for rutin, delphinidin and ferulic acid. As shown, these compounds displayed competitive inhibitory activity towards intestinal AGH. Figure 2 shows

Table 1: Percent inhibition was calculated for each compound in the endpoint AGH assay. A 10 mM solution for each compound was prepared in methanol or water and tested as described in the Materials and Methods section. Final concentrations for each compound were 100 μM

Compound	Inhibition (%)
Acarbose	50.000±0.66
Cyanidin	12.301±1.85
Malvidin	33.535±7.71
Pelargonidin	36.901±2.54
Delphinidin	23.403±3.67
Quercetin	18.957±0.77
Rutin	75.611±4.11
Cinnamic acid	20.504±3.54
Ferulic acid	15.117±2.06
Gallic acid	37.886±4.88
Tannic acid	76.244±8.29
Caffeic acid	NI

Results are expressed as Mean±SE; NI: No inhibition

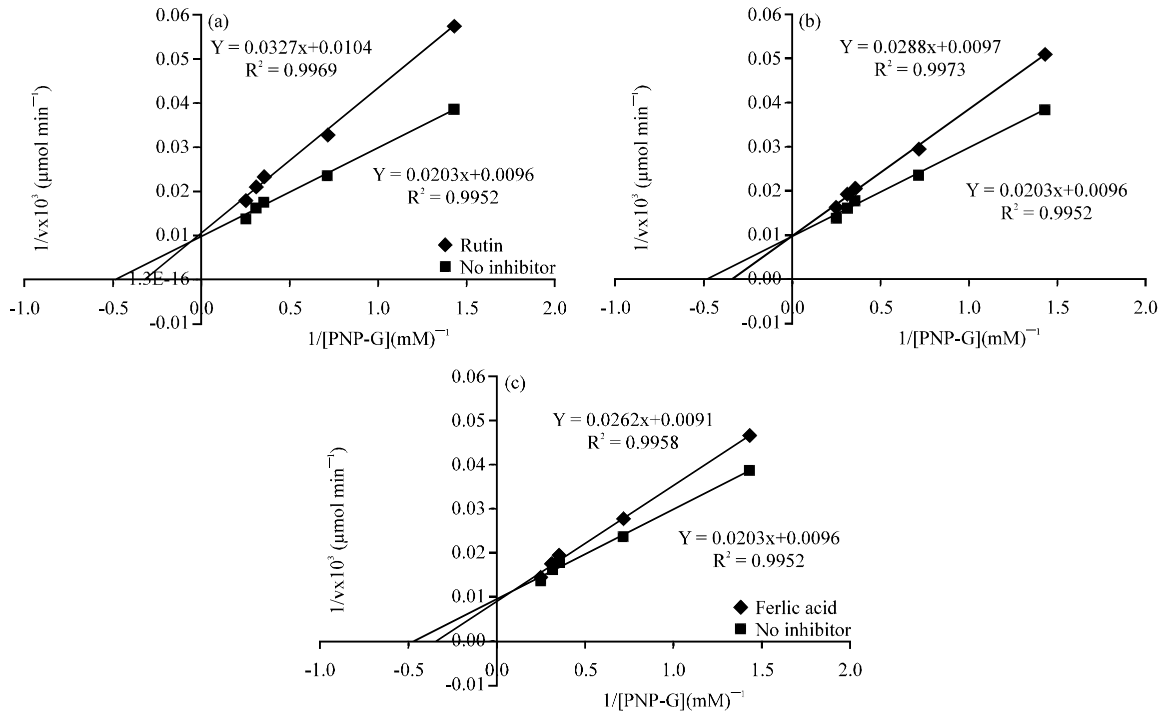

Fig. 1(a-c): Lineweaver-Burk plots for Rutin, Delphinidin and Ferulic acid displayed competitive inhibition kinetic behavior towards intestinal α-glucosidase

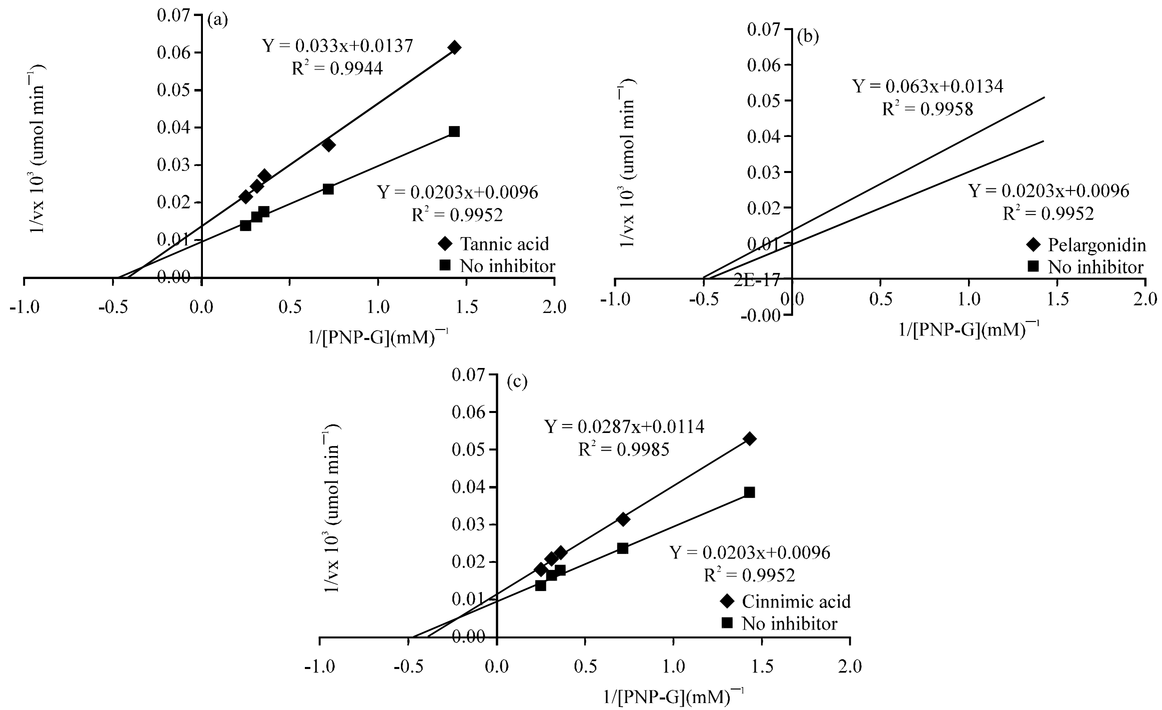

Fig. 2(a-c): Lineweaver-Burk plots for Tannic acid and Pelargonidin displayed noncompetitive inhibitory kinetic behavior towards intestinal α-glucosidase. The Lineweaver-Burk plot for Cinnamic acid displayed mixed inhibitory kinetic behavior towards intestinal α-glucosidase

Table 2: Kinetic data for the compounds tested in this study which displayed significant AGH inhibitory activity in the endpoint AGH assay. Inhibitor concentrations were 100 μM except for delphinidin and malvidin which were 10 μM and tannic acid which was 2 mM

Vmax (μmol min^{-1})	Km (mM)	pVmax	pKm	Inhibition	Type
No Inhibitor	104.84±5.17	2.131±0.13	----	----	-----
trans-Cinnamic acid	87.90±2.95	2.525±0.10	0.022	0.043	Mixed
Gallic acid	103.30±3.32	2.858±0.08	0.809	0.0014	Competitive
Tannic acid	73.65±3.37	2.438±0.17	9.9x10^{-4}	0.181	Noncompetitive
Ferulic acid	119.32±6.89	3.126±0.18	0.131	0.0021	Competitive
Rutin	93.70±5.38	2.881±0.28	0.113	0.041	Competitive
Quercetin	124.33±5.92	4.299±0.30	0.038	1.5x10^{-4}	Mixed/competitive
Cyanidin	95.73±10.28	3.078±0.55	0.951	0.030	Competitive
Pelargonidin	77.52±3.56	2.003±0.114	0.0014	0.261	Noncompetitive
Malvidin	118.44±11.49	7.172±0.96	0.390	0.0020	Competitive
Delphinidin	104.20±4.99	3.011±0.18	0.931	0.0042	Competitive

Results are expressed as Mean±SE

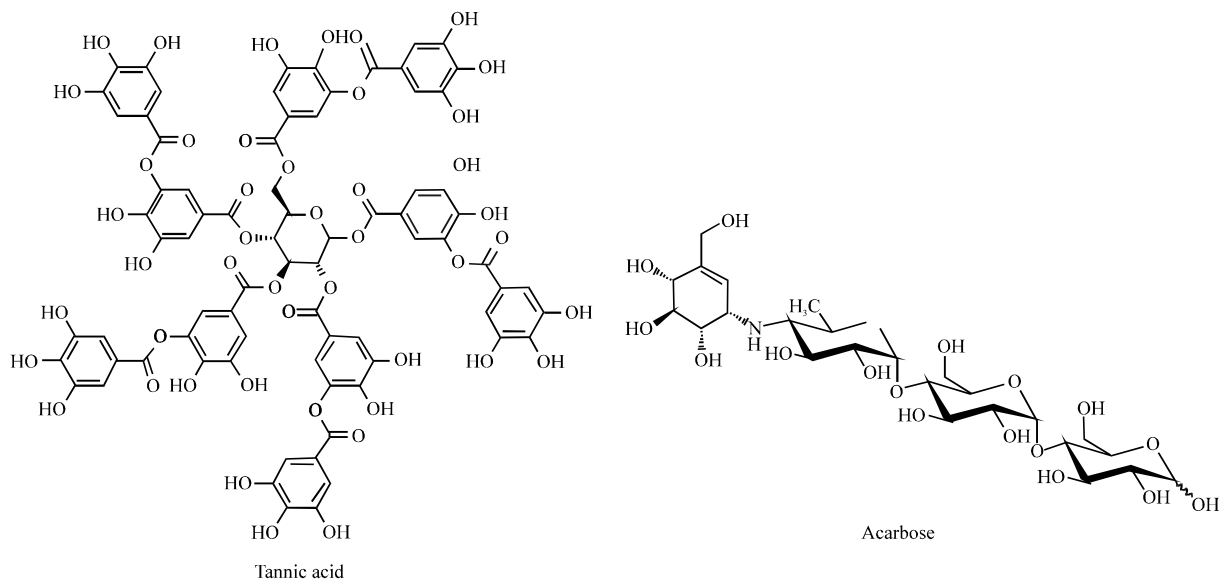

Fig. 3: Chemical structures for the polyphenol tannic acid and the synthetic AGH inhibitor Acarbose

the Lineweaver-Burk plots for tannic acid, pelargonidin and trans-cinnamic acid. Tannic acid and pelargonidin displayed noncompetitive inhibition while trans-cinnamic acid displayed mixed inhibiton towards intestinal AGH. A complete set of kinetic data is shown in Table 2. The following compounds from Table 2. ferulic acid, gallic acid, rutin, cyanidin. malvidin and delphinidin show competitive inhibition. However, tannic acid and pelargonidin demonstrate noncompetitive inhibition. *trans*-Cinnamic acid and quercetin showed mixed inhibition. This can be seen in Fig. 1 and 2.

The structures of all compounds used in this study are shown in Fig. 3-5. Examination of these structures will show that three compounds malvidin, rutin and ferulic acid have either glycosidic or ether linkages. Examination of the data in Table 1 and 2 show that each of these compounds

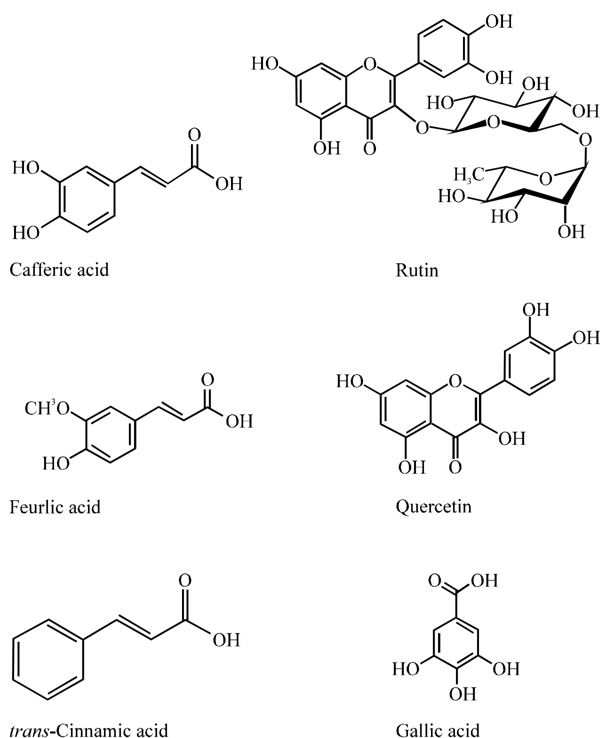

Fig. 4: Chemical structures of non-anthocyanidin polyphenols used in this study

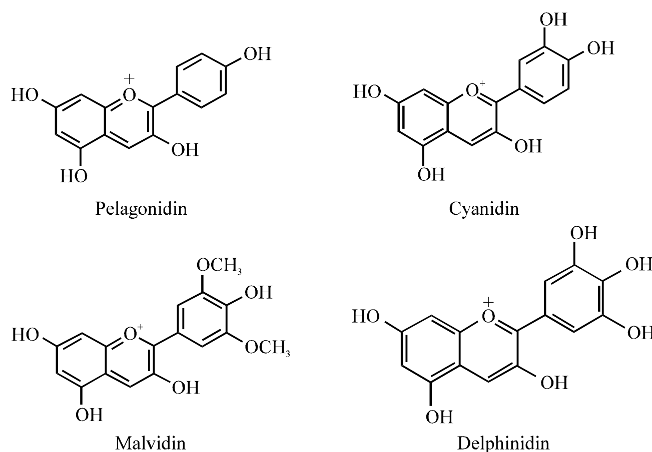

Fig. 5: Structures of anthocyanidins tested in this study

displayed significant inhibitory activity and competitive inhibitory behavior. The three compounds gallic acid, cyanidin and delphinidin also displayed competitive inhibition despite the fact they contain no ether or glycosidic linkages. The anthocyanidin pelargonidin exhibits noncompetitive inhibition. The structures of pelargonidin, delphinidin and cyanidin differ only in the positioning and number of hydroxyl groups. However, malvidin contains two ether linkages or methoxy groups ($-OCH_3$) and displays competitive inhibition as expected. Because of the similarity of the structures of pelargonidin, delphinidin and cyanidin one might suspect that they bind to the enzyme at the

same site. This would lead to the possibility that cyanidin and delphinidin are displaying allosteric competitive inhibition rather than true competitive inhibition. This would have to be determined by further experimentation.

The fact that many natural products show AGH inhibitory activity is very significant and could lead to the development of new antidiabetic drugs or a dietary approach to type-2 diabetes (Kolawole *et al.*, 2011; Priya *et al.*, 2012; Khan *et al.*, 2013; Saha *et al.*, 2011). Further studies need to be done to determine the scope of each of the methodologies used and to determine the molecular mechanism of AGH inhibition by each of the compounds. Once natural compounds which have high AGH inhibitory activity are identified, varieties of crops could be developed which contain high contents of these natural inhibitors. We believe that use of rat intestinal AGH produces more relevant data than AGH obtained from yeast. Due to differences in activity sometimes seen with the PNP-G method and maltose/sucrose method we would recommend testing compounds using both methods.

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