

AN ABSTRACT OF THE THESIS OF
Anneke M. (Tucker) Griffith for the degree of Honors Baccalaureate of Science in Bioresource Research presented on March 5, 2012. Title: The Inhibition of α -Amylase and α -Glucosidase by Bioflavonoids.

Abstract Approved:

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Various flavonoid-rich plant extracts have shown efficacy in the treatment of diabetes. Although it is often assumed that dietary flavonoids exert biological antioxidant effects, it is possible that the observed effects work in carbohydrate metabolism. In this study we evaluated the effect of catechins and catechin containing extracts on α -amylase and α -glucosidase activity, enzymes of great importance in carbohydrate metabolism and diabetes mellitus. Grape seed extract, white tea and green tea were tested, as well as their constituent catechins, or flavon-3-ols: catechin, epicatechin, epigallocatechin, epigallocatechin gallate, gallic catechin gallate, and epicatechin gallate. In all tests, acarbose, a pharmacological inhibitor of α -glucosidases was included as a positive control. For each compound, the concentration required for 50% inhibition of enzyme activity was determined to compare the potency. For α -amylase, grape seed extract demonstrated a similar α -amylase inhibitory activity to acarbose and white and green tea extracts showed a weaker inhibition. In α -glucosidase experiments, grape seed, green and white tea extracts inhibited enzymatic activity much more strongly than acarbose. The inhibitory activities of individual catechins are also reported. With this information, it is possible that catechins and certain plant extracts play a role in the prevention or treatment of type II diabetes mellitus.

Key Words: α -amylase, α -glucosidase, diabetes, bioflavonoids, catechins
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Inhibition of α -Amylase and α -Glucosidase by Bioflavonoids

By

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Table of Contents

INTRODUCTION	1
Bioflavonoids of Interest	8
Goal of Investigation	10
MATERIALS & METHODS	11
Materials	11
α -Amylase Activity Assay	11
α -Glucosidase Activity Assay	13
Statistics.....	15
RESULTS	16
α -Amylase Results.....	16
α -Glucosidase Results.....	19
DISCUSSION	22
Further Investigative Plans	27
Summary.....	28
BIBLIOGRAPHY	30

List of Figures

Figure 1: Structures of Acarbose and Miglitol.....	5
Figure 2: Bioflavonoid Classification	7
Figure 3: Structures of Importance in Bioflavonoids	9
Figure 4: Graphical Results of α -Amylase Inhibition.....	17
Figure 5: Graphical Results of α -Glucosidase Inhibition	20
Figure 6: Structural Comparison of Flavan-3-ols.....	25

Dedication

To Betsy and Russel, who have motivated me to pursue a goal larger than any career, reach for the unimaginable, and strive to be perfectly who I am meant to be. Thank you for your insight, creativity, passion and health.

And to the many other incredible people in my life, through growing up, volleyball and FFA: Thank you for being *YOU*, and for being part of *ME*.

The Inhibition of α -Amylase and α -Glucosidase by Bioflavonoids

INTRODUCTION

More than 190 million people worldwide suffer from diabetes (Kawai 2011, Benalla 2010): a disease characterized by elevated fasting blood glucose levels resulting from the body's inability to produce or use insulin. The World Health Organization expects the incidence of diabetes to double by the year 2025. There are two classifications of diabetes mellitus. Type I, characterized by an autoimmune destruction of the insulin-producing beta cells of the pancreas, results in an inability to process glucose from the blood stream. The second classification of this disease is called type II and is characterized by abnormalities in both insulin secretion and action resulting from life-style choices and some genetic influence (O'Reilly 2011). As with most lifestyle-related diseases, diabetes is a chronic and progressive disease (Selea 2011). Type II especially is common among aging populations, and those with unhealthy diets, obesity and sedentary lifestyles (Matsui 2006).

The total expenditures for diabetes in the United States in 2007 is estimated at \$174 billion, which comprised \$116 billion in excess healthcare expenditures and \$58 billion in reduced production of goods and services in the US economy. More specifically, these expenditures included \$27 billion for direct treatments of diabetes and \$58 billion for treatment of diabetes-related complications, both

chronic and acute, and comprised 11% of all healthcare expenses in 2007 (Bolin 2009). The complications include microvascular conditions such as diabetic retinopathy, nephropathy and neuropathy, as well as macrovascular complications such as atherosclerosis, coronary artery disease and cerebrovascular disease. The expenditures for the more common, long term implications of diabetes, such as glaucoma, cataracts, foot health problems and death, totaled \$31 billion (Mattila 2010). A diabetic patient in 2007 incurred healthcare costs that were approximately 2.3 times higher than those of patients without diabetes (Bolin 2009). Diabetes and its complications adversely affect a patient's health-related quality of life, as the symptoms progress towards these micro- and macro-vascular complications (O'Reilly 2011).

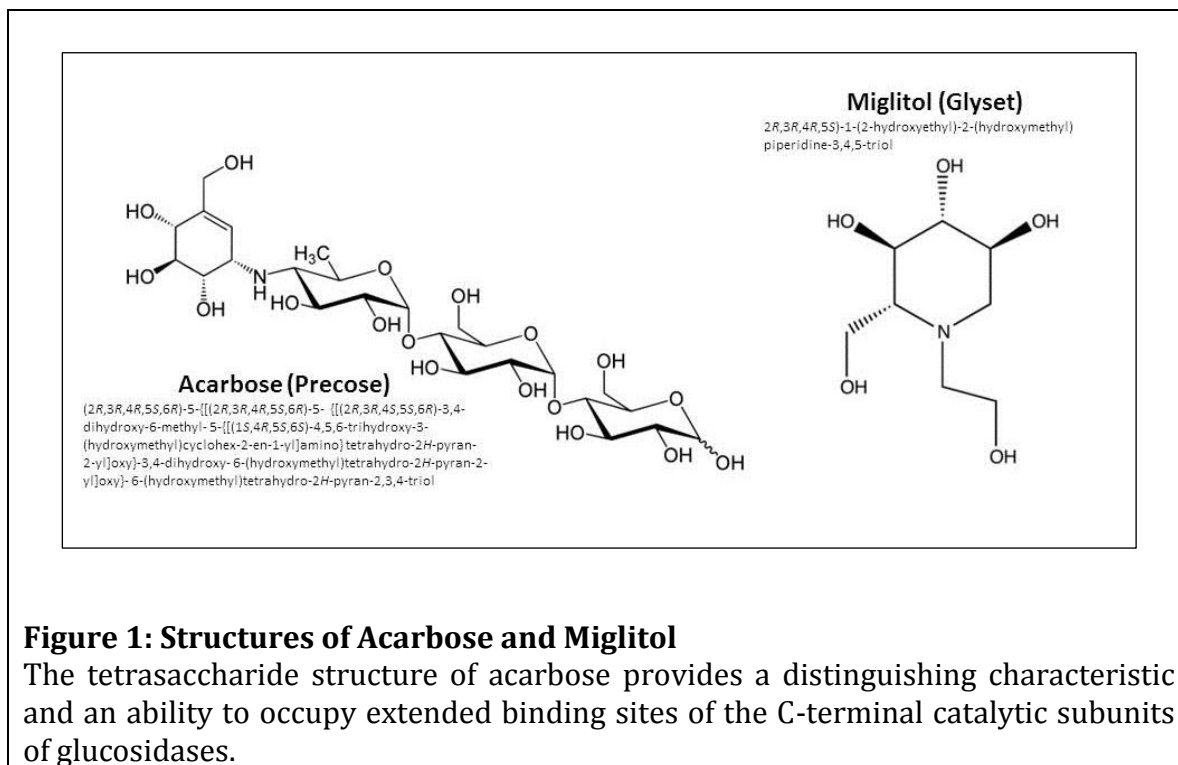
The debilitating symptoms, expensive treatments and complications occurring as a result of diabetes may be prevented or delayed by strict glycemic control (Voulgari 2010). However, it is well established that maintaining blood glucose concentrations of less than 110 mg/dL (Nemoto 2011) in fasting diabetic patients is often quite difficult. Many current research articles and conventional therapies focus on various ways to control hyperglycemia, targeting various organs involved in glucose metabolism as a solution to the symptoms and treatment of diabetes. One option for treatment currently available for glycemic control is sulfonylureas and meglitinides. This modality reduces glycemic levels in the blood through increases in preprandial insulin secretions, but come with serious risks for

hypoglycemia compared to other treatments (Bennett 2011, Krentz 2005). Another therapy uses thiazolidinediones to increase the sensitivity of fat and muscle cells to insulin by initiating signals that activate the synthesis of peroxisome proliferator-activated receptor proteins (Taylor 2009). The increased sensitivity helps to lower blood glucose levels, but unfortunately comes with a greater risk for congestive heart failure and bone fractures (Bennett 2011). Metformin is recognized as a first line agent to treat type II diabetes (Bennett 2011), and is part of a class of therapeutical drugs called biguanides. This class of pharmaceuticals initiates a cascade of events that ultimately blocks the release of glucose from the liver and maintains a lower level of fasting blood glucose. Yet again, this treatment is associated with a greater risk for gastrointestinal adverse effects and requires the presence of insulin (Bennett 2011, Krentz 2005).

Another method of treatment for type II diabetes is to limit the amount of glucose that reaches the bloodstream. The digestion of starches to glucose requires multiple reactions, involving two primary enzymes, α -amylase and α -glucosidase. These enzymes, collectively called glucosidases, play essential roles in carbohydrate metabolism (Bolen 2007). α -Amylase is both a component of saliva and secreted from the pancreas as an endohydrolase that cleaves the internal α -(1,4) bonds of starch into shorter, linear and branched dextrin chains. The resultant dextrin mixture is then further hydrolyzed into glucose by α -glucosidase, located on the microvilli brush border of the intestine (Ren 2011). The complementary activities of

these two enzymes allow for digestion of a spectrum of food starches from more than 400 plants that comprise two-thirds of most human diets (Jones 2011).

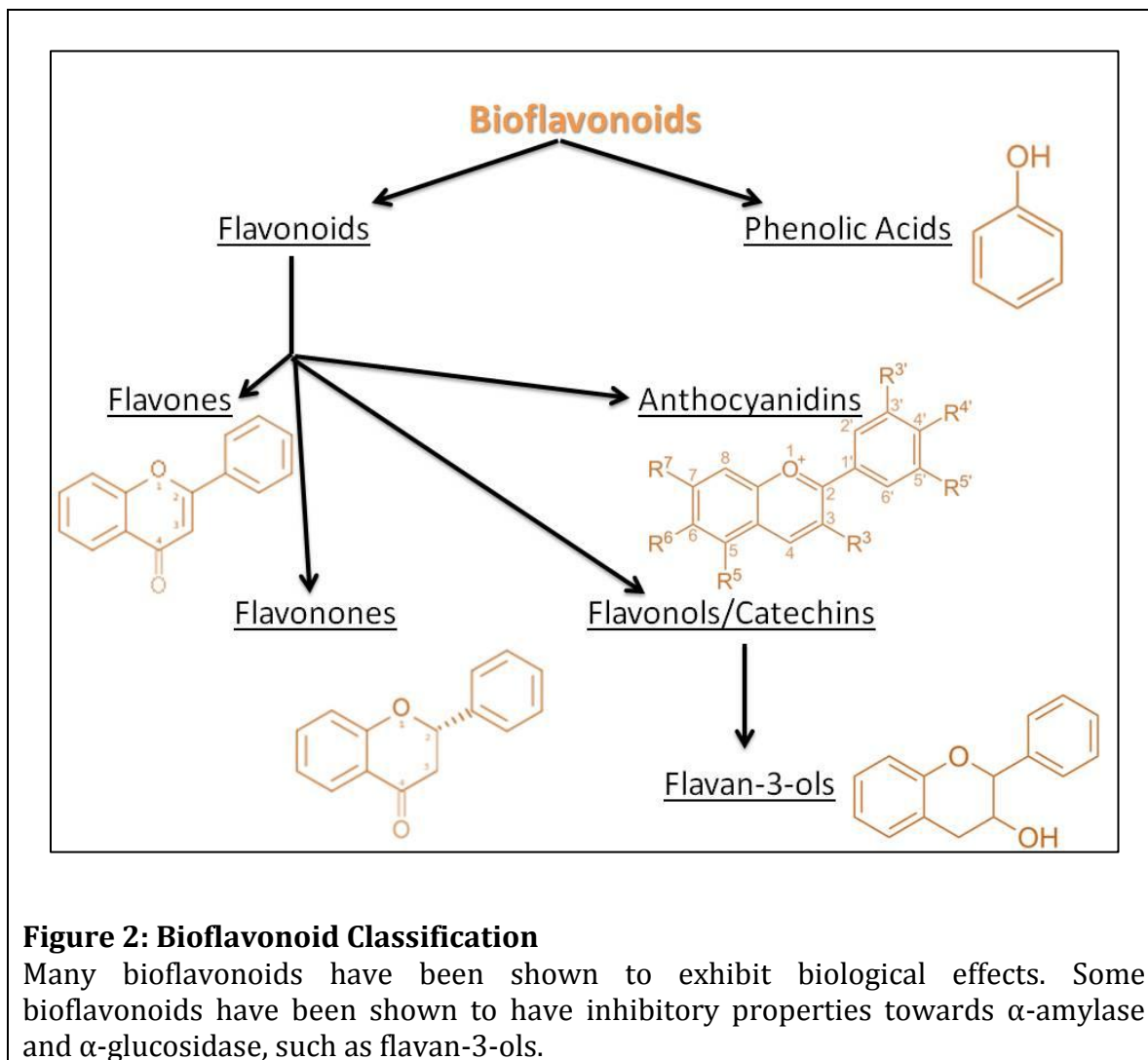
α -Glucosidase inhibitors are drugs that inhibit glucosidases in the intestine and suppress postprandial elevation of plasma glucose by delaying the digestion and absorption of carbohydrates (Nemoto 2011, Krentz 2005). The overall effect of glucosidase inhibition also reduces the occurrence of insulin resistance, thereby preventing further insulin-dependent disorders (Jones 2011). Inhibition of pancreatic α -amylase in particular is a major therapeutic target for type II diabetes (Qin 2011). Currently, there are two pharmaceutical glucosidase inhibitors that have shown value in controlling hyperglycemia: acarbose and miglitol (Figure 1) (Jones 2011). These compounds show a higher inhibitory activity for α -glucosidase compared to α -amylase, and competitively and reversibly inhibit both enzymes (Jones 2011, McDougall 2005). However, acarbose shows a broader affinity for α -glucosidase and α -amylase inhibition because of the ability of the tetrasaccharide analogue to occupy extended binding sites of the C-terminal catalytic subunits as compared to occupying the N-terminal domains of the enzyme (Jones 2011). Also, these prescription medications are frequently used in conjunction with other oral hypoglycemic agents with different mechanisms of action, such as those mentioned previously, or with insulin (Nemoto 2011).



Unfortunately, gastrointestinal side effects such as abdominal pain, flatulence, and diarrhea from undigested starches are among the disadvantages of α -glucosidase inhibiting pharmaceuticals because of the relatively large doses that need to be prescribed (Jones 2011, Bhat M. 2008). Also, a greater risk of life-threatening hypoglycemia from metabolizing too little glucose because of the powerful effect of the drugs at times outweighs the benefits of pharmaceutical intervention (Hirshberg 2003).

Bioflavonoids are a large family of polyphenolic compounds having a common chemical structure (Figure 2) and found in plant based foods. These polyphenols (polyhydroxylated aromatic phytochemicals) are separated into classes

of flavonoids and phenolic acids. The flavonoids can be further divided into structural classes of flavones, flavanones, flavonols, flavan-3-ols (or catechins), and anthocyanidins. Flavonoids are of particular interest because of their reported antioxidant and anti-inflammatory effects (Lotito 2006). In addition, flavan-3-ols specifically have been found to competitively inhibit duodenal oligosaccharide digestion by α -amylase and α -glucosidase (Ryu 2010). As explained above, inhibition of these carbohydrate digestive enzymes may be of use to patients with type II diabetes as well as the growing pre-diabetic population in the U.S. and around the world (Krentz 2005, Kamiyama 2010, Li 2009). Included in the structural family of flavan-3-ols and commonly known as catechins are the flavan-3-ol monomers and their oxidative coupling products, theaflavins. These compounds have a reported inhibitory effect on the glucosidase enzymes, especially those with a gallate addition to their structure (Kamiyama 2010, Ryu 2010). Previous research has demonstrated that orally-applied green tea extract, which contains many monomeric flavan-3-ols, including epigallocatechin gallate and epicatechin gallate, may inhibit glucose absorption from the lumen of the intestine (Park 2009, Johnston 2005). Therefore, it is conceivable that natural, dietary inhibitors of glucosidases that likely pose fewer adverse side effects than pharmaceutical drugs could be useful in the prevention or treatment of type II diabetes and pre-diabetes.



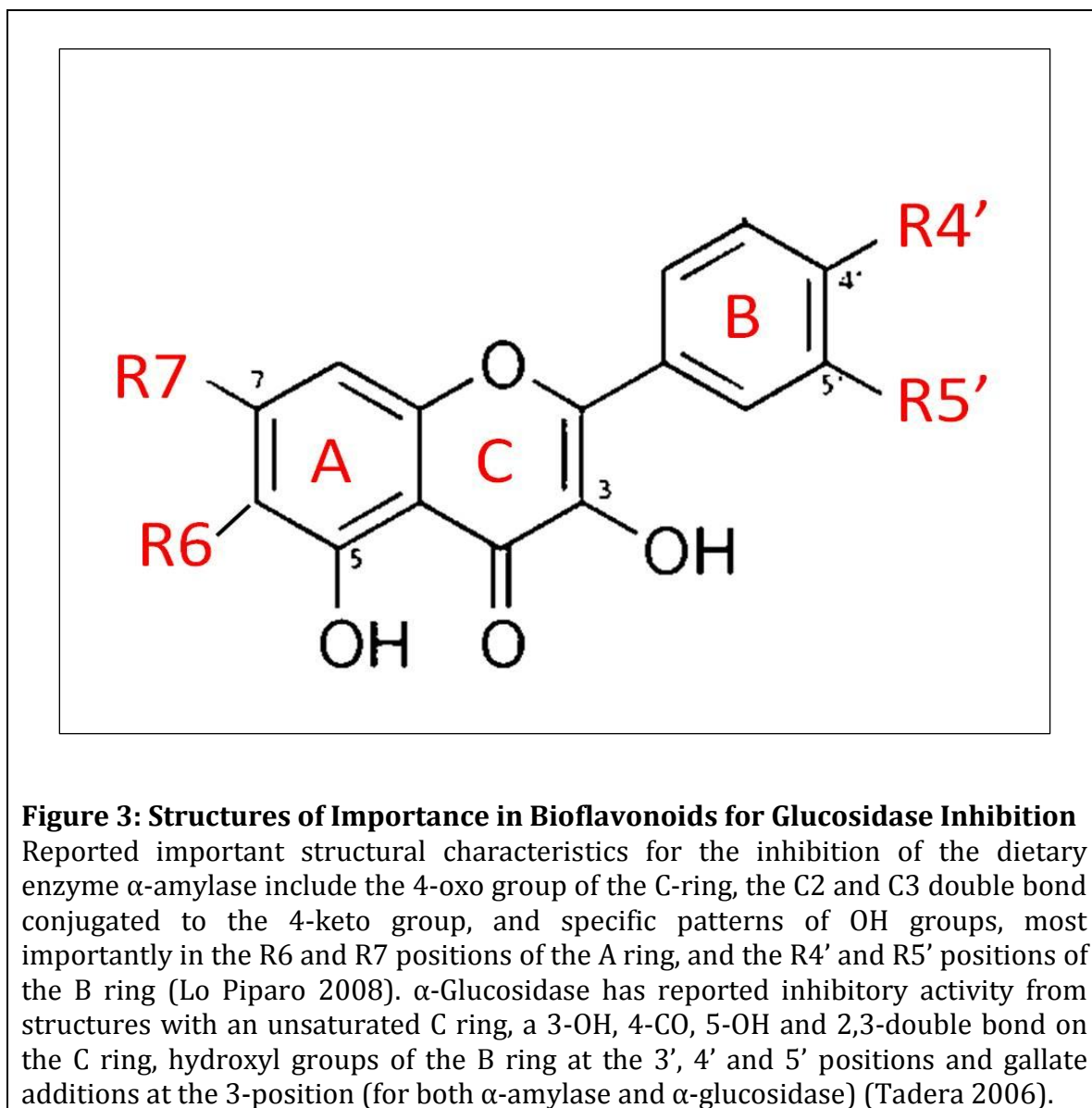
To gain further insight into the potential protective effects of flavan-3-ols against postprandial hyperglycemia, we investigated the inhibitory activity of grape seed, green tea and white tea extracts and their constituent catechins on isolated α -amylase and α -glucosidase enzymes.

Bioflavonoids of Interest

Previous research has suggested that particular functional groups of certain flavan-3-ols contribute to their biological importance and their efficacy of inhibition of glucosidases (Gamberucci 2006). Specifically, the structures of interest for the inhibition of α -amylase (Figure 3) are the 4-oxo-flavonoid nucleus and the C2-C3 double bond conjugated to the 4-keto group of the C-ring, and specific patterns of hydroxyl groups, located on the R6 and R7 positions of the A ring and the R4' and R5' positions of the B ring (Lo Piparo 2008). For the inhibition of α -glucosidase, an unsaturated C ring, a 3-OH, 4-CO, 5-OH and 2,3-double bond on the C ring, hydroxyl groups of the B ring at the 3', 4' and 5' positions and gallate additions at the 3-position (for both α -amylase and α -glucosidase) have been shown to increase the inhibitory activity (Tadera 2006).

Teas are a rich source of flavan-3-ol compounds (Ryu 2010). Purple grapes and grape products, including red wine and grape seed extract, also contain many of these polyphenols, and are an abundant source of catechins (Figure 3) and proanthocyanidins (oligomeric chains of flavan-3-ols) in the human diet (Mandic 2008). The teas and extracts tested in this work included grape seed, green tea, and white tea extracts. One individual catechin in particular, epigallocatechin gallate (EGCG), has been strongly linked to the beneficial effects of green tea in both genetic and dietary models of insulin resistance (Sae-tan 2001). This same catechin is also

found to have insulin enhancing activity in vitro (Josic 2010). The catechins that were tested include these structural features of importance: catechin, epicatechin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallicocatechin gallate (GCG), and epicatechin gallate (ECG).



Goal of Investigation

The goal of this study was to determine the efficacy of grape seed, green tea, and white tea extracts and their constituent monomeric flavan-3-ols at inhibiting the starch-substrate digestive enzymes, α -amylase and α -glucosidase, compared to acarbose, a conventional pharmaceutical drug currently in use. If the compounds are inhibitory, this research will lead to testing in animal or human models to determine if this is an alternative or additional treatment for type II diabetes mellitus.

MATERIALS & METHODS

Materials

α -Amylase from human saliva (Type IX-A), α -glucosidase from *Saccharomyces cerevisiae* (Type I), catechin, epicatechin, EGC, ECG, EGCG, GCG, p-nitrophenyl α -D-glucopyranoside (pNPG) and acarbose were obtained from Sigma-Aldrich (St. Louis, MO). Grape seed extract (composed primarily of catechin, epicatechin, epicatechin gallate and proanthocyanidins) (Mandic 2008), green tea extract (composed primarily of epigallocatechin, epigallocatechin gallate, and some flavan-3-ol oligomers) (Kim 2011, Yang 2000), white tea extract (comprised primarily of monomeric flavan-3-ols) (Zhao 2011) and TeaVigo® (a purified form of approximately 94% EGCG) (Wolfram 2005) were supplied by USANA Health Sciences, Inc. (Salt Lake City, UT). EnzChek® ultra amylase assay kit (E33651) was purchased from Invitrogen (Life Technologies, Grand Island, NY) and 96-well microplates were used for the α -amylase assay.

α -Amylase Activity Assay

The method used to measure α -amylase inhibition was similar to that described by Deutschlander (2009) and Lo Piparo et al. (2008) with some

modifications. The test compound was dissolved in 100% dimethylsulfoxide (DMSO) to a concentration of 8 mM and diluted with a phosphate buffer containing 50 mM NH_2PO_4 , 50mM NaCl, 0.5 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, and 0.1% bovine serum, resultant pH 6.0. α -Amylase from human saliva was prepared fresh at 10 mU/ml by dissolving in the phosphate buffer. Starch from the EnzCheck® ultra amylase assay kit was also mixed with buffer to a concentration of 20 $\mu\text{g}/\text{ml}$. This kit contains a starch derivative, called DQ™ starch substrate, labeled with BODIPY® FL dye. When the substrate is degraded by α -amylase, digestion relieves the quenching and yields highly fluorescent fragments. The accompanying increase in fluorescence was monitored, and is proportional to amylase activity (Molecular Probes™ 2006).

Fifty μl of test compound solutions and 25 μl of 10 mU/ml of α -amylase were mixed and incubated for 30 minutes at room temperature. Starch solution was then added at a volume of 25 μl to each well, resulting in a final experimental concentration of 5 $\mu\text{g}/\text{ml}$ for starch, 2.5 mU/ml of enzyme, and 2.5 μM , 5 μM , 12.5 μM , 50 μM , 100 μM , 250 μM and 500 μM of test compound. DMSO percentages ranged from 0.03125%-6.25%. The final volume in all wells was 100 μl . Fluorescence intensity was measured in a fluorometric microplate reader, the SpectraMax GeminiXS, using SoftMax Pro software made by Molecular Devices (Sunnyvale, CA). Enzymatic activity was determined every minute for 60 minutes by measuring the fluorescence of the starch using an excitation wavelength of 485 nm and emission wavelength of 530 nm. Reaction rates were calculated from the slope

of the product formation over time. Enzymatic activity was standardized in each assay in the control wells on the 96-well plate, calculated from the initial linear rate of reaction. Test wells with DMSO and enzyme alone resulted in no effect on enzyme activity, and are therefore not considered in discussion or results. Results were compared to the control well that had only enzyme and starch to represent 100% activity. The inhibitory activity was calculated utilizing a four point regression model to convert the data to the IC_{50} value, defined as the concentration of a compound required to inhibit 50% of the enzymatic activity, by GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). In some cases, 50% inhibition was not attained and therefore the IC_{50} was either approximated or not determined.

α -Glucosidase Activity Assay

To test α -glucosidase inhibition, the methods described by Deutschlander et al. (2009) and Li et al. (2009) were followed. The test compounds were dissolved in DMSO to a concentration of 8 mM. These stock solutions were then diluted with 0.1 M potassium phosphate (K-Ph) buffer containing 1 M K_2HPO_4 and 1 M KH_2PO_4 at pH 6.8. The test compounds were diluted to 5 μ M, 10 μ M, 25 μ M, 100 μ M, 200 μ M, 500 μ M, and 1 mM. The enzyme solution was prepared fresh and contained 0.1 U/ml of α -glucosidase from *Saccharomyces cerevisiae* in K-Ph buffer. The substrate used was pNPG, which releases the product p-nitrophenol after degradation by α -glucosidase. The released product yields a yellow color when mixed with a stopping reagent.

This yellow color was measured spectrophotometrically (Deutschlander 2009). The substrate was prepared at a concentration of 3 mM in K-Ph buffer, and the reaction stop solution contained 1 M Na₂CO₃ in H₂O at pH 11.2.

A pre-incubation of the enzyme and the test compounds in solution for 30 minutes at 37°C began the experiment. Then, 20 µl of α-glucosidase solution, 5 µl test compound and 72 µl K-Ph buffer were added in each well for a total volume of 97 µl and incubated for another 15 minutes at 37°C. Subsequently, 3 µl of pNPG was added to each well to reach a total volume of 100 µl, and the solution was incubated for another 15 minutes at 37°C. The experimental concentrations were 20 mU/ml enzyme, 90 µM pNPG, and 0.25 µM, 0.5 µM, 1.25 µM, 5 µM, 10 µM, 25 µM, or 50 µM of test compound. DMSO percentages ranged from .003125%-0.625%. The reaction was stopped by the addition of 100 µL of the reaction stop solution for a total experimental volume of 200 µl. The absorbance was measured spectrophotometrically at 412 nm using a SpectraMax spectrophotometer with SoftMax Pro software (Molecular Devices). The inhibitory activity was calculated in the same manner as in the α-amylase experiment.

Statistics

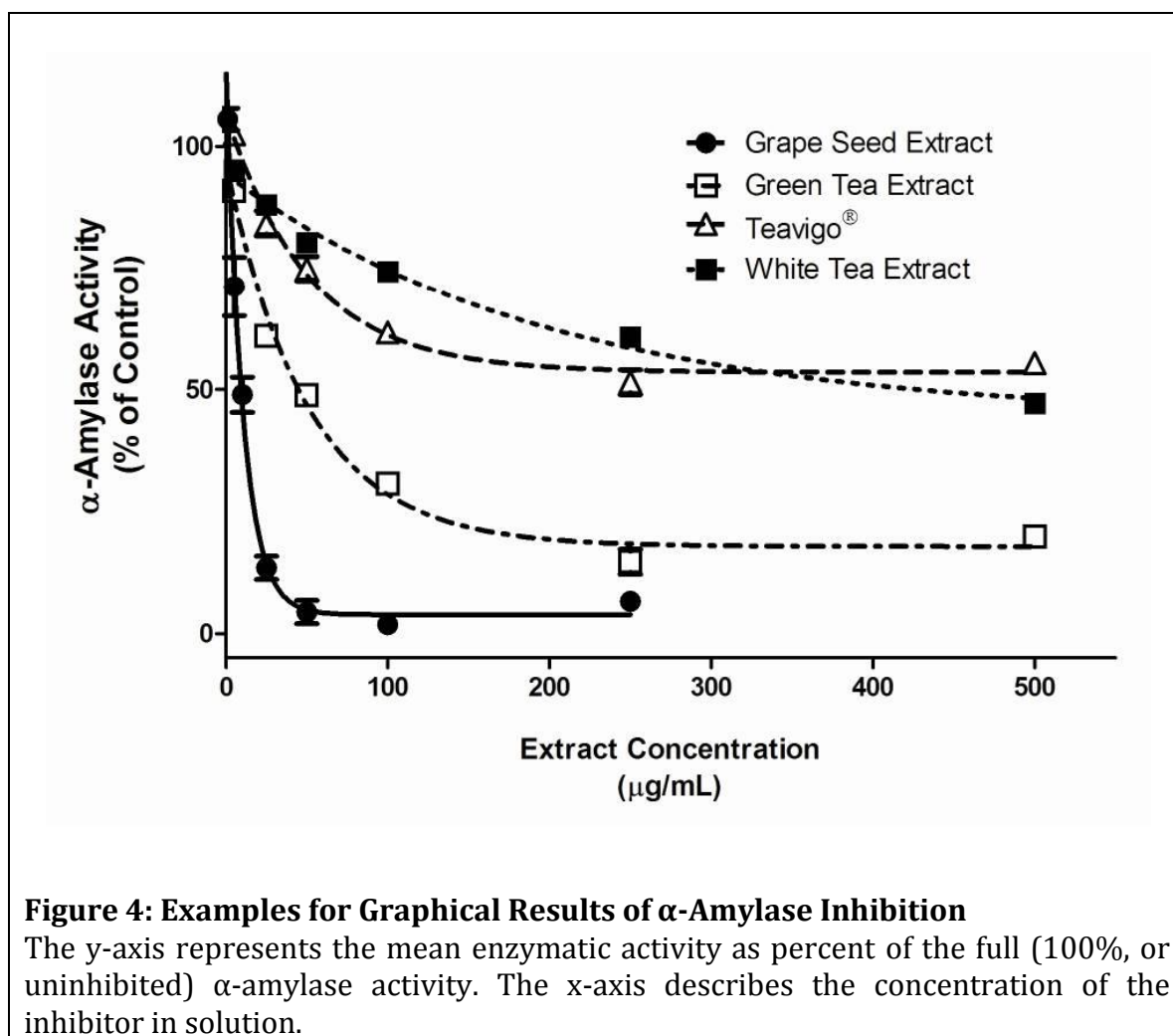
Differences in calculated IC₅₀ values were analyzed by unpaired t-tests and one-way ANOVA employing Tukey-Kramer post-hoc analysis to compare data sets (GraphPad Prism). Differences between means were considered significant if $p < 0.05$, as denoted in applicable tables, figures, and the text.

RESULTS

α -Amylase Results

To determine the inhibitory effects of flavan-3-ols and tea or grape seed extracts on α -amylase activity, we used a special fluorescently labeled starch as a substrate for degradation by the enzyme. We compared these tests to “normal” α -amylase activity, which was standardized using only the labeled substrate and enzyme. Also, these flavan-3-ols and extracts were compared to the pharmaceutical drug currently used as an inhibitor of α -amylase, acarbose. This information was analyzed using the IC_{50} values, or the concentration required for 50% inhibition of enzymatic activity for each compound to determine potency. In our tests, acarbose exhibited an IC_{50} value of 6.90 ± 0.81 $\mu\text{g/ml}$. Of all the flavonoid compounds tested, grape seed and tea extracts were found to be more effective inhibitors of α -amylase than individual flavonoids (Table 1, Figure 4). The results from grape seed extract resulted in an IC_{50} of 8.74 ± 0.81 $\mu\text{g/ml}$ and green tea extract showed an IC_{50} of 34.9 ± 0.90 $\mu\text{g/ml}$. These two extracts were the most successful inhibitors of all catechins and extracts tested. On the other hand, white tea extract resulted in an IC_{50} value of 378 ± 134 $\mu\text{g/ml}$ at the highest concentration tested (1 mg/ml). It is interesting that white tea, which is the least processed of all teas and, hence, contains mostly monomeric flavan-3-ols, was less effective than green tea and grape seed extract. In addition to monomeric flavan-3-ols, green tea and grape seed extract also contain

flavan-3-ol dimers and oligomers, called theaflavins and proanthocyanidins, respectively, which have been linked to α -glucosidase and α -amylase inhibition (Gamberucci 2006).



Individual flavan-3-ols were less inhibitory than extracts. GCG was the most potent, with an IC_{50} of approximately 17 $\mu\text{g/ml}$. The inhibition by epicatechin could

not be determined ($> 1\text{ mg/ml}$), while the IC_{50} of its diastereoisomer, catechin, was $160 \pm 67\ \mu\text{g/ml}$ (Table 1). Other individual bioflavonoids containing a 3-gallate moiety, such as epigallocatechin gallate and epicatechin gallate, also resulted in limited inhibition even at the highest concentration tested ($1\ \text{mg/mL}$). No inhibition was found for epigallocatechin. Similarly, TeaVigo, which contains $\geq 94\%$ EGCG, inhibited α -amylase activity with an IC_{50} of only $44.2 \pm 6.10\ \mu\text{g/ml}$ (Table 1).

Table 1: α-Amylase Results		
Table of triplicate and averaged results, described as an IC_{50} value.		
Test Compound/Extract	n Value	IC_{50} Value (mean \pm SEM)
Acarbose	7	$6.90 \pm 0.81^a\ \mu\text{g/ml}$
Catechin	3	$160 \pm 67^{b,c,d}\ \mu\text{g/ml}$
Epicatechin	3	value not determined
Epicatechin Gallate	2	$\sim 27\ \mu\text{g/ml}$
Epigallocatechin	3	value not determined
Epigallocatechin Gallate	2	$\sim 24\ \mu\text{g/ml}$
GCG	2	$\sim 17\ \mu\text{g/ml}$
Grape Seed Extract	3	$8.74 \pm 0.81^{a,b}\ \mu\text{g/ml}$
Green Tea Extract	4	$34.9 \pm 0.90^c\ \mu\text{g/ml}$
TeaVigo®	4	$44.2 \pm 6.10^c\ \mu\text{g/ml}$
White Tea Extract	4	$378 \pm 134^d\ \mu\text{g/ml}$
Different letter superscripts denote significant differences in IC_{50} values as determined by unpaired t-test ($P < 0.05$ versus acarbose). Values presented are mean \pm standard error or approximate values if weak inhibition was observed.		

α -Glucosidase Results

To determine if the results we obtained from α -amylase were applicable to other glucosidase enzymes, we conducted similar experiments with α -glucosidase. Using pNPG as the substrate, we were able to measure the yellow color of the enzyme's degradation product, p-nitrophenol. We compared uninhibited activity to the inhibitory effects of tea and grape seed extracts and individual flavan-3-ols, as well as acarbose. Acarbose exhibited an IC_{50} value of $91.0 \pm 10.8 \mu\text{g/ml}$. In addition, grape seed and green tea extracts, which were potent inhibitors of α -amylase (Table 1), proved even more inhibitory towards α -glucosidase (Figure 5). Green tea, grape seed and white tea extracts showed IC_{50} values of 0.49 ± 0.05 , 1.15 ± 0.16 , and $2.52 \pm 0.42 \mu\text{g/ml}$, respectively. However, only the results from green tea were statistically significant. As expected of the catechins, only those with gallate side chains (ECG, GCG, and EGCG) were potent inhibitors, with IC_{50} values of 18.1 ± 5.20 , 1.35 ± 0.12 , and $0.25 \pm 0.01 \mu\text{g/ml}$, respectively (Figure 5 and Table 2), however, the difference between ECG and the other two gallated catechins was not statistically significant. Furthermore, the strong inhibition by EGCG was almost identical to that of TeaVigo® ($IC_{50} = 0.28 \pm 0.03 \mu\text{g/ml}$), which was statistically indistinguishable. The more basic structure of catechin resulted in an IC_{50} of approximately $31 \mu\text{g/ml}$, and no inhibition was found for epicatechin (Table 2). Overall, α -glucosidase was more strongly inhibited by flavan-3-ol compounds compared to α -amylase, in agreement with previous research (Jones 2011) and showed that all extracts and several

catechins, except for catechin, epicatechin and epigallocatechin, were more potent and significant inhibitors of the enzyme than acarbose (Table 2, Figure 5).

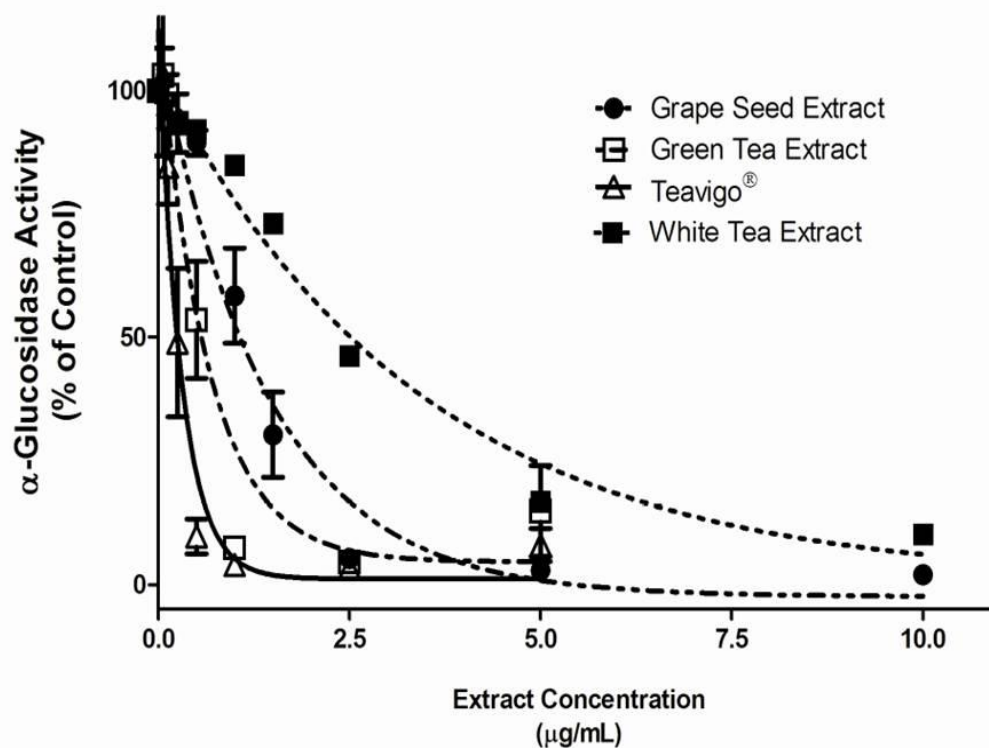


Figure 5: Examples for Graphical Results of α -Glucosidase Inhibition

The y-axis represents the mean enzymatic activity as percent of the full (100%, or uninhibited) α -glucosidase activity. The x-axis describes the mean concentration of the inhibitor in solution.

Table 2: α-Glucosidase Results		
Results for α -glucosidase assays described as IC ₅₀ values.		
Test Compound/Extract	n Value	IC ₅₀ Value (mean \pm SEM)
Acarbose	7	91.0 \pm 10.8 ^a μ g/ml
Catechin	2	~31 μ g/ml
Epicatechin	2	>290 μ g/ml
Epicatechin Gallate	3	18.1 \pm 5.20 ^f μ g/ml
Epigallocatechin	2	no value determined
Epigallocatechin Gallate	3	0.25 \pm 0.01 ^d μ g/ml
Gallocatechin Gallate	3	1.35 \pm 0.12 ^{b,c} μ g/ml
Grape Seed Extract	3	1.15 \pm 0.16 ^b μ g/ml
Green Tea Extract	3	0.49 \pm 0.05 ^c μ g/ml
TeaVigo [®]	3	0.28 \pm 0.03 ^d μ g/ml
White Tea Extract	3	2.52 \pm 0.42 ^e μ g/ml
Different letter superscripts denote significant differences in IC ₅₀ values as determined by unpaired t-test (P<0.05 versus acarbose). Values presented are mean \pm standard error or approximate values if weak inhibition was observed.		

DISCUSSION

In this study, we compared the *in vitro* inhibition of glucosidases by monomeric flavan-3-ols (catechins), two different tea extracts, grape seed extract and a currently used pharmaceutical drug. The two target enzymes are those primarily responsible for the digestion of starches in the human diet, α -amylase and α -glucosidase. The potency of the inhibition was determined experimentally, and the data were expressed using an IC_{50} value where obtainable. These results show that several catechins, and grape seed and tea extracts are potent inhibitors of α -amylase and α -glucosidase activity, although some have a greater affinity towards one enzyme. These results also support the previous research that suggests that gallate moieties are of importance in glucosidase inhibition (Gamberucci 2006). Several of these inhibitors are equally if not more potent than acarbose *in vitro*.

To measure α -glucosidase and α -amylase enzyme activity, we used methods reported by Deutschlander (2009) and Lo Piparo (2008). In the case of the α -glucosidase experiment, this method was chosen because of the ease of adaptation for use with various glycohydrolase enzymes (Collins 1997). In previous α -amylase studies, a porcine variant of the enzyme was used because of its availability. However, it has been shown that it is important to use human α -amylase, because there is a 14% difference in the amino acid composition of α -amylase in the two species (Lo Piparo 2008). Interestingly, Deutschlander found different results when

testing acarbose as a positive control (Deutschlander 2009). Their concentration for a 50% inhibition of the α -glucosidase activity was 4.75 $\mu\text{g}/\text{ml}$, compared to our result of $91.0 \pm 10.8 \mu\text{g}/\text{ml}$. We used a different buffer, which may explain the differences in results between the two studies. Also, Lo Piparo found acarbose to inhibit α -amylase with an IC_{50} value of 0.643 $\mu\text{g}/\text{ml}$ (Lo Piparo 2008), compared to $6.90 \pm 0.81 \mu\text{g}/\text{ml}$ in our experiments. However, we used a DQTM starch from an enzyme assay kit, instead of the potato starch used by Lo Piparo. Hence, in our assays, acarbose was about 20- and 10-fold less potent in inhibiting α -glucosidase and α -amylase, respectively, than reported by other investigators (Deutschlander 2009, LoPiparo 2008) using different incubation and assay conditions.

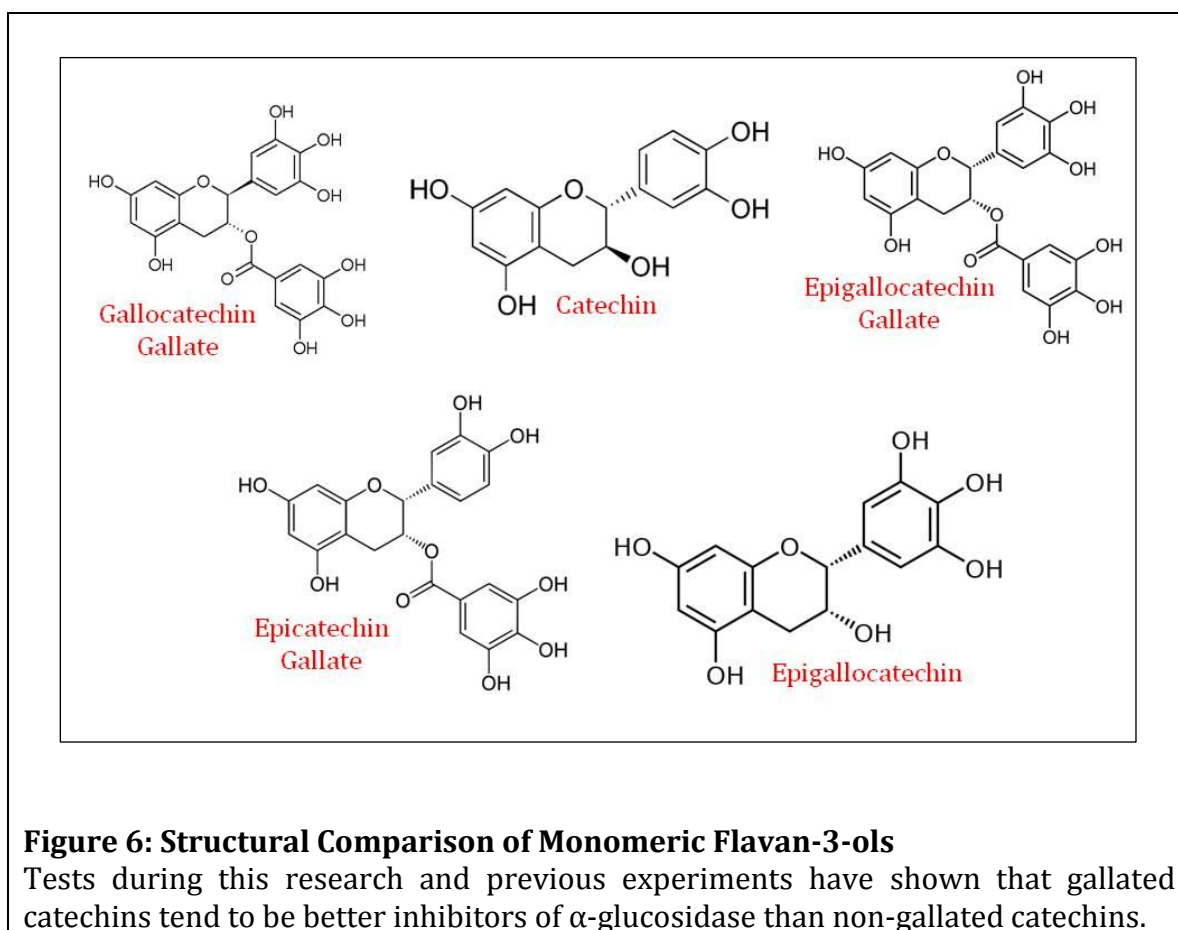
Our data suggest a greater affinity for α -amylase of the oligomeric compounds in tea extracts and grape seed extract than the monomeric bioflavonoids. Both grape seed and green tea extracts contain proanthocyanidins, oligomeric forms of epicatechin and catechin (Mandic 2008), which may play a role in the inhibition of α -amylase. Fermentation in teas leads to the formation of theaflavins and proanthocyanidins (Zhao 2011). White tea and green tea are made from the same leaves, but the method of enzymatic oxidation of polyphenols during fermentation, in addition to growing season and geographical region, contribute to the differences in chemical composition (Zhao 2011). The less fermented white tea extract, which contains a relatively large amount of monomeric flavan-3-ols, did not inhibit α -amylase activity as potently as green tea extract. Likewise, the individual flavan-3-

ols tested did not result in strong inhibition. It is, therefore, possible that the larger structures of the oligomers contribute to the inhibitory effect on α -amylase activity of green tea and grape seed extracts.

In contrast to α -amylase, α -glucosidase was potently inhibited by several monomeric flavan-3-ols. It has been found previously that those flavan-3-ol compounds with a gallate side chain (Figure 6) have a particularly high affinity for α -glucosidase (Gamberucci 2006). Thus, we expected that the only compounds that would not effectively inhibit α -glucosidase would be catechin and epicatechin, two of the three flavan-3-ols lacking a gallate moiety that were tested. The third flavan-3-ol that was ineffective was epigallocatechin, which actually contains a gallate moiety. However, it is not linked to the 3-position of the C-ring but instead represents a tri-hydroxylated B-ring (Figure 6). Further kinetic testing of the mode of inhibition for the enzyme would provide more insight into the differences that did not allow this compound to interact with the enzyme. The most successful inhibition followed the rationale provided by Gamberucci (2006), in that white and green tea extract, TeaVigo[®], and the monomer EGCG were strong inhibitors. These results, compared to acarbose, show promising ability as candidates for a supplement or replacement of pharmaceutical drugs.

The above results indicate that α -glucosidase is effectively inhibited by monomeric flavan-3-ols, whereas proanthocyanidins appear to be important for inhibition of α -amylase. Catalytic domain differences between the two enzymes and

different binding specificities of the various monomeric and polymeric flavan-3-ols (Gamberucci 2006) could explain our results. Therefore, it would be interesting to perform additional kinetic testing of specific catechins and proanthocyanidins to get better insights into their mode of enzyme inhibition.



Interestingly, acarbose also showed different affinities for the two enzymes.

Comparison of the IC_{50} values for both enzymes by acarbose showed a greater

affinity for α -amylase than α -glucosidase. The IC_{50} value for α -glucosidase is much higher than the IC_{50} for α -amylase, which is surprising because acarbose is prescribed specifically for α -glucosidase inhibition of disaccharide digestion (Krentz 2005). This may be caused by the large saccharide chain of acarbose (Figure 1), which has different binding affinities for α -amylase than α -glucosidase. This is consistent with the observations that oligomeric flavan-3-ols appear to inhibit α -amylase more effectively than α -glucosidase. Potentially, it is the larger inhibitors that can occupy different or more domains on the enzyme that exert a greater inhibition of α -amylase, and a stronger response from the monomers may suggest a stronger inhibition towards α -glucosidase.

It is possible that whatever compound or extract is selected as an inhibitor would be used in conjunction with current therapies, not as a replacement for pharmaceutical drug intervention. There was some consistent inhibition of both enzymes by grape seed extract and green tea extract, but the different IC_{50} values may indicate that this would not be a complete treatment method. A combination of grape seed extract extract and TeaVigo® or EGCG would provide the most potent inhibition of both enzymes. Our results could be used to lower the cost of treatment and lessen side effects of pharmaceutical drug intervention, if found to be as potent in human or animal models. These types of experiments will be necessary to determine if any of these compounds could be sufficient treatments, on any scale, for type II diabetes mellitus and pre-diabetes.

Further Investigative Plans

Enzymatic inhibition by flavanoids is a complex process that presents inconsistencies between studies with isolated enzymes in the lab and *in vivo* in human studies. For instance, green tea was found by Kamiyama and colleagues to exhibit *in vitro* inhibition of α -glucosidase, with inhibition concentration values as low as 16 μ M (Kamiyama 2010). However, Josic and colleagues found that green tea and catechins exerted no inhibition or lowering of postprandial glucose after consuming a basic meal of starch and protein in human subjects (Josic 2010). These differing results present the challenge for this research, which seeks to find an inhibitor that is potent and effective not only *in vitro* but also *in vivo*.

As demonstrated by the previous research, several factors need to be taken into consideration prior to application in medicine. First, the solubility of these compounds in humans may pose a challenge: It has been shown that several flavonoids have low solubility in water and at acidic pH, as low as 1.5. The acidity (pH) of gastric juices in the lumen of the stomach ranges from 1.35-3.5. However, flavonoid solubility increases 4-fold at pH 8 (Chebil 2007). Yet, a 4-fold increase in solubility cannot be expected in the lumen of the intestine, which is slightly more acidic than pH 8, and so the exact intestinal solubility of the bioflavonoid should be determined for sufficient experimental dosing. Because of the challenges presented with solubility, the vehicle of delivery to the intestinal brush border and pancreatic

secretions should also be considered in the test drug design. With the promising results found with the proanthocyanidins and oligomeric compounds in the α -amylase experiment, it is possible that more fermented teas, such as black tea, could have inhibitory effects as well. However, because we do not know the exact composition of the dimers and oligomers present in the fermented teas that we tested, it will be important to investigate the composition further. Also, because these inhibitors are natural compounds, they have not been specifically designed for the inhibition of α -amylase or α -glucosidase, like pharmaceutical drugs. Eating a mixed meal of fats, proteins and carbohydrates could potentially compromise the inhibition with unanticipated reactions. Another consideration is the timing of the treatment, such as instructions to the patient to take the compound before, during or after consuming a meal. Human subjects could be used to investigate this target window for the frequency of dosage.

Summary

The catechins and extracts tested in this investigation showed promising inhibitory characteristics on α -amylase and α -glucosidase activity, which may be of future medical significance. Green tea and grape seed extract might be particularly useful for limiting post-prandial glycemia and insulinemia because of their strong inhibition of both α -amylase and α -glucosidase. In addition, the amount of EGCG and

TeaVigo® required to inhibit 50% of α -glucosidase activity was 400 times less than the amount of acarbose required for 50% inhibition. It is possible that using these compounds to target α -glucosidase and α -amylase inhibition would be an effective method of controlling post-prandial blood glucose levels and effective adjuncts to oral hypoglycemic drugs in the treatment of type II diabetes mellitus.

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